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Gd(III)-Gold Nanoconjugates Provide Remarkable Cell Labeling for High Field Magnetic Resonance Imaging

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Abstract

In vivo cell tracking is vital for understanding migrating cell populations, particularly cancer and immune cells. Magnetic resonance (MR) imaging for long-term tracking of transplanted cells in live organisms requires cells to effectively internalize Gd(III) contrast agents (CAs). Clinical Gd(III)-based CAs require high dosing concentrations and extended incubation times for cellular internalization. To combat this, we have devised a series of Gd(III)-gold nanoconjugates (Gd@AuNPs) with varied chelate structure and nanoparticle-chelate linker length, with the goal of labeling and imaging breast cancer cells. These new Gd@AuNPs demonstrate significantly enhanced labeling compared to previous Gd(III)-gold-DNA nanoconstructs. Variations in Gd(III) loading, surface packing, and cell uptake were observed among four different Gd@AuNP formulations suggesting that linker length and surface charge play an important role in cell labeling. The best performing Gd@AuNPs afforded 23.6 ± 3.6 fmol of Gd(III) per cell at an incubation concentration of $27.5 \mu\text{M}$ —this efficiency of Gd(III) payload delivery (Gd(III)/cell normalized to dose) exceeds that of previous Gd(III)-Au conjugates and most other Gd(III)-nanoparticle formulations. Further, Gd@AuNPs were well-tolerated in vivo in terms of biodistribution and clearance, and supports future cell tracking applications in whole-animal models.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00389. Synthetic scheme of lipoic acid-modified Gd(III) chelates; cell viability and counts after Gd@AuNP treatment; Gd(III) uptake of uncoupled chelates (PDF)

Notes

The authors declare no competing financial interest.

INTRODUCTION

In vivo cell tracking is a burgeoning field of research because it is considered the crucial technology for the advancement of cell-based therapy.^{1–4} Cell therapy has implications for a variety of diseases and disorders such as cancer,^{5–7} cardiovascular diseases,^{8,9} neurodegenerative diseases,^{10,11} and musculoskeletal¹² disorders by providing unique opportunities for tissue regeneration, targeted therapy, and drug delivery. In the context of cancer, cell tracking technology would elucidate the behavior of cancer cells at the molecular level, and further understanding of their metastatic potential. However, the outcomes of cell therapy have been variable as the fate of transplanted cells remains indeterminate.

Magnetic resonance (MR) imaging is ideally suited for noninvasive clinical diagnosis, molecular imaging, and cell tracking.^{2,13–17} MR imaging can visualize live specimen, and benefits from high spatiotemporal resolution and excellent soft-tissue contrast while avoiding radiolabels or ionizing radiation. However, MR imaging suffers from lower sensitivity compared to competing modalities such as fluorescence, bioluminescence, positron emission tomography, or single-photon emission computed tomography, all of which have little or no background. Paramagnetic or superparamagnetic contrast agents are used to enhance the sensitivity of MR imaging. Paramagnetic gadolinium(III) [Gd(III)]-based agents are the most frequently used MR contrast agents.^{1,18,19} These agents are typically chelated Gd(III) which reduce the longitudinal relaxation time of nearby water protons, aided by the high magnetic moment and symmetrical S state of the Gd(III) ion.^{1,18,20,21} Regions which incorporate Gd(III) appear brighter on a T_1 -weighted image.

Most clinical and preclinical magnets now operate at higher field strengths (3T or higher) in order to increase resolution, signal-to-noise ratio, and contrast-to-noise ratio. However, the benefits of high-field MR imaging must outweigh the resulting susceptibility artifacts and altered relaxation kinetics. These factors must be taken into account when designing a new contrast agent for high-field performance. Additionally, clinical Gd(III)-based MRI contrast agents are not effectively internalized by cells.^{22,23}

To address these issues, Gd(III) chelates have been conjugated to nanoparticles, including gold nanoparticles, for improved relaxivity along with increased agent permeability and

retention within cellular compartments.^{18,20,21,24} Our group previously devised cell permeable, Gd(III)-enriched DNA–gold nanoparticle conjugates (DNA-Gd(III)@AuNPs) for cellular T_1 -weighted MR imaging.²¹ These constructs demonstrated high stability, cellular uptake, and surface loading of Gd(III). DNA-Gd(III)@AuNPs were used to successfully track the implantation of neural stem cells into rat brain regions affected by stroke.⁴ However, apart from being a carrier for the Gd(III) chelates and optical dyes, it is likely that the presence of DNA limits the amount of Gd(III) that can be loaded onto the AuNP surface.

To overcome this, we recently synthesized DNA-free Gd(III)-Au nanoconjugates by coupling lipoic acid-modified, amine-functionalized Gd(III) chelates directly to the surface of AuNPs (Lip-Gd@AuNPs).²⁵ Lip-Gd@AuNPs were the first T_1 -weighted CAs to accumulate in the pancreas and provide enhanced image contrast. Although this system was designed for pancreatic localization, the DNA-free strategy is ideal for developing cell tracking agents for T_1 -weighted MRI. Herein, we report the synthesis of a series of DNA-free Gd(III)-Au nanoconjugates (Gd@AuNPs) for cancer cell labeling. Four different Gd(III)-based chelates were modified with lipoic acid generating a terminal dithiolane, which was in turn coupled to thiols on the AuNP surface (Figure 1). By modifying the linkers lengths and coordination chemistry of the Gd(III) chelates, we demonstrate that surface loading and cellular delivery of Gd(III) can be optimized for *highly efficient* labeling of cancer cells.

RESULTS AND DISCUSSION

Synthesis and Characterization of Gadolinium–Gold Nanoconjugates

Synthesis of Gd@AuNPs—The overall synthetic scheme for the Gd@AuNPs is presented in Figure 1 and Figure S1. To investigate the role of surface chemistry on our AuNP system and optimize cellular delivery of Gd(III), we studied four different Gd(III) chelates coupled to AuNPs.

Three amine-functionalized Gd(III)-tetraazacyclododecane-triacetic acid (DO3A) complexes of varying linker lengths (3-, 6-, and 12-carbons), and an amine-functionalized Gd(III)-diethylene triaminepentaacetic acid (DTPA) with a four-carbon linker were synthesized according to previously reported protocols.^{25,26} The DTPA complex is negatively charged, while the DO3A complexes are neutral. The four different chelates were peptide-coupled to lipoic acid-NHS ester in 1:1 DMSO:pH 8.5 carbonate buffer, and purified by reverse-phase high performance liquid chromatography (HPLC) at approximately 60% yield generating **1a–4a**, respectively (Figure S1).

The synthesis of citrate-stabilized AuNPs was carried out by literature procedures.²⁵ The diameter of the AuNPs was 18.0 ± 2.1 nm as determined by analysis of transmission electron microscopy (TEM) images. The hydrodynamic radius of the AuNPs suspended in water was 26.1 ± 0.2 nm with a polydispersity index of 0.08 ± 0.01 as determined by dynamic light scattering (DLS). Four, separate 10 nM AuNP suspensions in in Dubecco's phosphate buffered saline (DPBST) in 0.01% Tween20 were aliquoted. To each of these AuNP suspensions was added **1a**, **2a**, **3a**, or **4a**, dissolved in 1:1 MeOH:water. The AuNP mixtures were agitated overnight at ambient temperature. The crude mixtures were purified

by four rounds of ultracentrifugation, and the final particles were suspended in DPBST as 1 mL stocks at a gold concentration of 250 nM yielding four, distinct, DNA-free Gd(III)@AuNP complexes **1–4** (Figure 1). It is notable that the coupling occurs without the aid of any reducing agents.

Characterization of Gd@AuNPs—Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure Gd(III) and Au content of complexes **1–4** (Table 1). Taking into account the Gd(III) and gold ratios from ICP-MS, the average diameter of AuNPs and the spherical packing of gold atoms, the loading of Gd(III) per AuNP was calculated. Similarly, the coverage density of Gd(III) was determined by assuming a spherical surface area of AuNPs. Hydrodynamic size and polydispersity of AuNPs in solution were measured using DLS, and are not significantly different among complexes **1–4**. It is evident that complex **3** has the greatest surface loading and coverage of Gd(III), followed by complexes **1** and **2** (which have comparable levels) and last by complex **4**, which has lowest surface loading of Gd(III).

This trend suggests the 12-carbon alkyl chain in complex **3a** confers greater packing efficiency on the AuNP surface, while the negative charge of **4a** may prevent the same level of surface coverage. It is likely that complexes **1a–2a** have linker lengths that are suboptimal compared to complex **3a**, but their neutral surface charge confers better packing on AuNP than complex **4**. Overall, these results suggest that DO3A-based Gd(III) chelates are able to pack more efficiently on the AuNP surface compared to DTPA-based Gd(III) chelates, most likely due to charge on the DTPA ligand and steric differences between the two chelates. Specifically, **1a–3a** are neutral while **4a** bears a negative charge, and the latter would be unable to pack as efficiently due to electrostatic repulsion. Notably, each of **1–4** demonstrate greater Gd(III) loading than previously reported DNA-Gd(III)@AuNPs, suggesting that polyvalent DNA bearing smaller Gd(III) chelates do not pack as efficiently.²¹

We investigated the ability of **1–4** to decrease the T_1 relaxation time, referred to as longitudinal relaxivity (r_1). r_1 is the slope of the linear plot of $1/T_1$ versus Gd(III) ion concentration (Table 2). Measurements were made at 1.4 and 7 T. The ionic relaxivity r_1 of **1–4** at 1.4 T is comparable to the value reported for DNA-Gd@AuNPs.²¹ The ionic r_1 values of **1–4** at 1.4 T are higher than those of small-molecule clinically used Gd(III) chelates, whose r_1 values range between 3.3–4.0 mM⁻¹ s⁻¹.²⁷ This can be explained by an increase in the rotational correlation time, τ_r , mediated by conjugation to the AuNP surface. A longer τ_r corresponds to a slower tumbling rate that ultimately elongates the longitudinal relaxation of the Gd(III) ion. At 7 T, we observe a drop in ionic relaxivity for all complexes, which is expected of Gd(III)-based contrast agent behavior at higher field strengths.²⁸

The particle relaxivity is computed by multiplying the number of Gd(III) ions per particle and the ionic relaxivity. This is a useful parameter to compare the efficiencies of AuNPs with different surface chemistries as realized in complexes **1–4**. For example, while the ionic relaxivity for complex **3** is lower than that for complex **2**, the particle relaxivity is greater since complex **3** confers greater packing density of Gd(III) ions.

Notably, complex **1** posed a challenge for further characterization as it demonstrated low colloidal stability in DPBST, suggestive of shorter linker lengths of the Gd(III) chelates conferring less salt stability than complexes **2** and **3**. As such, complex **1** was omitted from further analysis and not utilized for subsequent cellular studies.

Cancer Cell Labeling Using Gd(III)@AuNP Constructs

A majority of clinically utilized Gd(III)-based contrast agents do not accumulate in cells.^{22,23} As a first step in evaluating the efficacy of cell-permeable contrast agents, it is vital to determine their cellular uptake and general biocompatibility in vitro. MDA-MB-231 m-Cherry human breast cancer cells were treated with various Gd(III)-equivalent concentrations of complexes **2**, **3**, or **4** and incubated for 24 h at 37 °C. The treated cells were harvested and counted through a flow cytometric live/dead assay. Cell viability and counts indicated that Gd@AuNPs were well-tolerated across a wide dose range (Figure S2). The cellular Gd(III) concentration per cell was quantified through ICP-MS (Figure 2A). Significant differences in the cell uptake of Gd(III) were observed. Complex **2** demonstrated the greatest overall uptake of Gd(III), and achieved approximately four times greater Gd(III) uptake over complex **3** and 20-fold greater than complex **4**. Cells incubated with the unbound chelates **2a**, **3a**, and **4a** resulted in negligible Gd(III) uptake per cell (Figure S3).

These results suggest that while **3a** conferred the greatest packing density of Gd(III) on the AuNP surface, the longer alkyl linker prevented cellular internalization to the same extent as complex **2**. For complex **4**, it appears that the negative surface charge in addition to the relatively poor surface loading confers the least favorable conditions for cellular penetration in this particular cell line. Notably, for the same dosing concentration, all formulations of Gd@AuNPs are superior in terms of cellular Gd(III) delivery compared to the previously synthesized DNA-Gd(III)@AuNP.²¹

In addition, time-dependent uptake for the cellular delivery of Gd@AuNPs was performed with MDA-MB-231 m-Cherry cells (Figure 2B). Cells were incubated with 50 μ M Gd(III)-equivalent concentration of complex **2**, **3**, or **4** for 1, 2, 4, 8, and 24 h at 37 °C. After each time point, cells were harvested, counted, and the Gd(III) content was quantified. The similar trend of cell uptake of Gd(III) between complexes **2**, **3**, and **4** was observed. However, the uptake of complex **4** did not appear to be time-dependent. Maximum cell uptake of complex **4** was achieved within 1 h. This suggests that complex **4** behaves similarly to clinical Gd(III) chelates where extracellular and intracellular Gd(III) concentrations equilibrate quickly resulting in insignificant cellular uptake of Gd(III). Conversely, the cell uptake of complexes **2** and **3** was time-dependent. A significant increase in cell uptake of **2** and **3** was observed between 8 and 24 h incubation, suggesting that uptake occurs concurrently with cell division. This pattern of time-dependent uptake corroborates that surface charge and linker length significantly contribute to degree of particle internalization by MDA-MB-231 m-Cherry cells.

To visualize AuNP uptake, MDA-MB-231 m-Cherry cells were incubated with 50 μ M Gd(III)-equivalent concentration of complexes **2**, **3**, or **4** for 24 h and subsequently harvested for TEM. The intrinsic contrast afforded by the gold core of Gd@AuNPs enables the

visualization of all three complexes in the intracellular space. In particular, complex **2** appeared in more abundance relative to complexes **3** and **4**, and appeared to undergo phagocytosis in large aggregate form (Figure 3A,D). Furthermore, the phagosomes appear to merge together and sequester the AuNPs in a single, large vesicle (Figure 3A,D). In a similar fashion, this behavior is observed for complexes **3** and **4** (Figure 3B,C,E,F). This further corroborates the differences in cell uptake between complex **2** compared to complexes **3** and **4**. In general, these studies reveal that complexes **2–4** undergo a form of endocytosis (or phagocytosis) and accumulate inside the cell for Gd(III) cell labeling.

MR Imaging of MDA-MB-231 m-Cherry Cell Pellets Labeled with Gd@AuNPs

We investigated the ability of the various Gd@AuNPs to produce contrast enhancement via MR imaging. MDA-MB-231 m-Cherry cells were labeled with complexes **2**, **3**, or **4** for 24 h, after which the cells were harvested and concentrated to a pellet. MR images of the cell pellets were acquired at 7 T. The cells were incubated with complex **2**, **3**, or **4** at a normalized Gd(III) concentration of approximately 15 μM (Figure 4).

As observed in Figure 4, cells incubated with complex **2** demonstrated the shortest T_1 relaxation time and greatest positive (bright) contrast, and this was significantly shorter and brighter than demonstrated by cells incubated with complexes **3** and **4** ($p < 0.05$ for one-way ANOVA). Finally, for a relatively low dose of complex **2**, there is marked contrast enhancement compared to unlabeled cells, and when compared to DNA-Gd(III)@AuNP.²¹

Preliminary In Vivo Studies

Complex **2**, the most promising agent from the cellular studies, was selected for preliminary in vivo MR imaging. Mice were IV injected with 4.0 nmol/kg body weight of complex **2** (approximately 9.0 $\mu\text{mol/kg}$ body weight of Gd(III)). Immediately after injection, a series of fast FLASH MR images were acquired on Bruker Biospin 9.4T magnet to visually observe the distribution of the agent over 10 min (Figure 5A,B). In the T_2 weighted image (Figure 5A), the aorta and vena cava are dark and easily seen. The left kidney and stomach are easily identifiable. Figure 5B represents the subtraction of the postcontrast T_1 weighted image from the corresponding precontrast image, termed the area-under-curve (AUC). The AUC image is displayed to demonstrate net contrast enhancement, and the T_2 -weighted image is shown as an anatomical reference. In the AUC image, part of the interior of the kidney is starting to enhance. The AUC image suggests that complex **2** is still in circulation during the FLASH image acquisition, as evidenced by the bright vessels. As expected, clearance is relatively slow. Therefore, the AUC image does not show accumulation in the kidney or liver in the first 10 min. After 3 h, organs were harvested, and quantification of Gd(III) was performed using ICP-MS ($n = 3$) and reported as total μg of Gd(III) per gram of tissue (Figure 5C). There is significant accumulation of complex **2** in clearance organs but negligible accumulation in other vital organs. These results indicate that complex **2** is well-tolerated by mice in the short term and shows promise for future in vivo longitudinal imaging studies in mice.

Conclusions

We have reported the facile synthesis of Gd@ AuNPs with four unique surface chemistries. Specifically, complexes **1–3** were synthesized bearing DO3A-based Gd(III) chelates with alkyl linkers of different lengths, while complex **4** was negatively charged LipGdDTPA@ AuNP as previously reported.²⁵ Complexes **2–3** were stable in DPBST, whereas complex **1** was not, suggesting that the shorter alkyl linkers confer less salt stability than longer linkers. The newly synthesized Gd@ AuNPs demonstrated greater Gd(III) loading, surface coverage, cellular Gd(III) delivery, and MR imaging efficacy than previously reported DNA-Gd(III)@ AuNPs. Interestingly, complex **2** was significantly better at delivering a Gd(III) payload to MDA-MB-231 m-Cherry breast cancer cells compared to complexes **3** and **4**. It is likely that the longer linker of **3a** and the negative charge of **4a** limit the cellular penetration of complexes **3** and **4**, respectively, as evidenced by lower Gd(III) uptake and sparse intracellular localization observed in TEM images. Therefore, while complexes **2** and **4** showed unprecedented performance as T_1 -weighted CAs of the pancreas,²⁵ only complex **2** is optimal for cell labeling.

Future Directions

Gd@ AuNPs, specifically DO3A-based Gd(III) chelates conjugated to AuNPs, show significant promise toward in vivo cell tracking studies. The short-term in vivo pilot study reported herein suggests that the material is biocompatible and suitable for long-term studies in mice bearing tumor xenografts of human cancer cells. Gd@ AuNPs label cells ex vivo with high efficiency and can be used to track transplanted cells in vivo, thereby enabling the success of cell therapy.

MATERIALS AND METHODS

General Synthesis Protocols and Characterization Techniques

Reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). For flash chromatography, standard grade silica gel was purchased from Sorbent Technologies (Norcross, GA, USA). For NMR and mass spectroscopy, a Bruker 500 MHz Avance III NMR spectrometer and a Bruker Amazon X LC-MS Ion Trap Mass Spectrometer (Billerica, MA, USA) were used, respectively. Final purification was accomplished using a Varian Prostar 500 HPLC (Santa Clara, CA, USA) using a Waters 4.6×250 mm $5 \mu\text{m}$ Atlantis C18 column (Milford, MA, USA). Mobile phases consisted of Millipore water, 0.05% trifluoroacetic acid in Millipore water, and acetonitrile. Gd@ AuNP particles were assessed for hydrodynamic size by dynamic light scattering using a Brookhaven ZetaPals particle size analyzer. For structural characterization of Gd@ AuNPs, TEM images were acquired using a JEOL 1230 microscope.

Synthesis of **1a**

Gd-DO3A-C3 amine (0.050 g, 0.091 mmol) and 2 mL of 100 mM carbonate buffer pH 8.5 were added to a 10 mL round-bottom flask. Lip-NHS (0.030 g, 0.099 mmol), synthesized according to published procedure,²⁵ and 3 mL DMSO were added in a separate glass vial. After Lip-NHS was dissolved, the solution was added to the Gd-DO3A-C3 amine solution,

and stirred at room temperature for 12 h. Reaction was monitored by ESI-MS for the disappearance of starting material. Final product was purified by semipreparative HPLC. The mobile phase was composed of Millipore water and HPLC grade acetonitrile (ACN). Initial conditions of 0% ACN were held constant for 5 min, then gradually increased to 32% ACN between 5 and 17 min, followed by a further increase to 40% ACN at 31 min. The product peak eluted between 21 and 23 min, as monitored by UV absorption at 210 nm. The product was collected and lyophilized to a fluffy pale yellow solid. (m/z) observed: 747.1, calculated: 747.01 $[M + H]^+$.

Synthesis of 2a

2a was synthesized as previously reported.²⁵

Synthesis of 3a

3a was synthesized by an analogous procedure to **1a** and **2a**. Specifically, Gd-DO3A-C12 amine (0.047 g, 0.069 mmol) and 2 mL of 100 mM carbonate buffer pH 8.5 were added to a 10 mL round-bottom flask. Lip-NHS (0.025 g, 0.08 mmol), synthesized according to published procedure,²⁵ and 3 mL DMSO were added to a separate glass vial. After Lip-NHS was dissolved, the solution was added to the Gd-DO3A-C12 amine solution, and stirred at room temperature for 12 h. Reaction was monitored by ESI-MS for the disappearance of starting material. Final product was purified by semipreparative HPLC. The mobile phase was composed of Millipore water and HPLC grade acetonitrile (ACN). Initial conditions of 0% ACN were held constant for 5 min, then increased to 5% ACN between 5–10 min, and held constant at 5% ACN until 20 min. ACN was then gradually increased to 100% through 25 min and held constant at 100% ACN until 40 min. The product peak eluted between 32 and 35 min, as monitored by UV absorption at 210 nm. The product is collected and lyophilized to a fluffy pale yellow solid. (m/z) observed: 873.23, calculated: 873.01 $[M + H]^+$.

Synthesis of 4a

4a was synthesized as previously reported.²⁵

Low-Field Relaxivity (r_1)

Stock solutions of complexes **1–4** (approximately 500 μ M Gd(III)) were serially diluted four times for five total samples in 150 μ L volumes. Solutions were heated to 37 °C and 150 μ L of each concentration was used for measurement of T_1 relaxation time via a Bruker minispec mq60 NMR spectrometer (60 MHz). An inversion recovery pulse sequence, with a repetition time of 15 s, 10 data points, and 4 averages, was used to acquire data. The remaining solutions were used to prepare samples for ICP analysis of Gd(III) concentration. The slope of the linear fit of the relaxation rate ($1/T_1$, s^{-1}) plotted against the Gd(III) concentration (mM) yields the relaxivity of the agent ($mM^{-1} s^{-1}$).

High-Field Relaxivity (7 T)

A Bruker Pharmscan 7 T (Billerica, MA, USA) imaging scanner fitted with shielded gradient coils at 25 °C was used. Samples of complexes **1–4** were prepared by serial

dilution, and Gd(III) concentration was measured by ICP-MS. Solutions were placed in glass capillary tubes of approximate diameter 1 mm, and the tubes containing the samples were taped around a larger tube containing water. For acquisition of T_1 relaxation times, a rapid-acquisition rapid-echo (RARE-VTR) pulse sequence was used. The following parameter values were utilized: static echo time = 11 ms, variable repetition time = 150, 250, 500, 750, 1000, 2000, 4000, 6000, 8000, and 10000 ms, field of view = 25×25 mm², matrix size = 256×256 , number of axial slices = 4, slice thickness = 1.0 mm, and averages = 3. Paravision 6.0 software (Bruker, Billerica, MA, USA) was used for T_1 analysis, by mono-exponential curve-fitting of image intensities of selected regions of interest (ROIs) from each axial slice.

Metals Analysis by ICP-MS

A computer-controlled (QTEGA v 2.6) Thermo (Thermo Fisher Scientific, Waltham, MA) iCapQ ICP-MS equipped with an ESI SC-2DX autosampler/autodilution system (Elemental Scientific Inc., Omaha, NE) was utilized for metal quantification. ICP-MS analysis was preceded by acid digestion of Gd@AuNPs samples. Specifically, for Gd analyses 5 μ L of Gd@AuNPs sample was added to 400 μ L of 1:1 concentrated nitric acid:concentrated hydrochloric acid (TraceSelect Nitric acid, >69%; TraceSelect HCl, fuming 37%) and mixed thoroughly. Similarly, for Au analyses, 5 μ L of Gd@AuNPs sample was added to 400 μ L of 1:1 HNO₃:HCl as above, and mixed thoroughly. Sample:acid mixtures were heated at 65 °C for at least 2 h. Then, ultrapure H₂O (18.2 Ω ·m) was added up to 10 mL total sample volume. For cells labeled with Gd@AuNPs, 150 μ L of labeled cells suspended in PBS or media were added to 100 μ L 70% nitric acid and heated at 65 °C for at least 4 h. Following digestion, ultrapure water was added for a final sample volume of 3 mL. Individual Au and Gd elemental standards were prepared at 0, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25.0, 50.0, 100, and 200 ng/mL concentrations with 2% nitric acid (v/v), 2% HCl (v/v), and 5.0 ng/mL internal standards up to a total sample volume of 5 mL. Each sample was acquired using 1 survey run (10 sweeps) and 3 main (peak jumping) runs (100 sweeps). The isotopes selected were ¹⁹⁷Au, ^{156,157}Gd and ¹¹⁵In, ¹⁶⁵Ho, and ²⁰⁹Bi (as internal standards for data interpolation and machine stability).

General Cell Culture

All reagents used for cell culture, such as Dulbecco's modified phosphate buffered saline (DPBS), media, and dissociation reagents, were purchased from Life Technologies (Carlsbad, CA). Only CorningBrand (VWR Scientific, Radnor, PA) cell culture flasks, plates, and sera were used. MDA-MB-231 m-Cherry cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in phenol red-free alpha minimum essential media (α -MEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, and 1% sodium pyruvate. Cells were grown in a humidified incubator operating at 37 °C and 5.0% CO₂. Cells were allowed to incubate for 24 h before all experiments. For harvesting cells, 0.25% TrypLE was added and cells were incubated for 5 min at 37 °C in a 5.0% CO₂ incubator. For sterilization, Gd@AuNPs suspensions in media were filtered with 0.2 μ m sterile filters prior to concentration determination and dosing.

Guava ViaCount Assay for Cell Counting and Viability

A Guava EasyCyte Mini Personal Cell Analyzer (EMD Millipore, Billerica, MA) was utilized to count cells and determine viability by a flow cytometric live/dead assay. 50 μL of cell suspension (from each well of 24-well plate) was mixed with 150 μL of Guava ViaCount reagent for 5 min to facilitate cell staining. Stained cells were counted using the ViaCount software module. For each sample, 1000 sampling counts were acquired and gated manually by the operator for live versus dead cells. The number and percentage of live cells were recorded. Instrument reproducibility was assessed biweekly using GuavaCheck Beads and following the manufacturer's suggested protocol using the Daily Check software module.

Cellular Delivery Studies

Cellular delivery studies were performed with MDA-MB-231 m-Cherry cells. Cells were plated at a cell density of approximately 25 000 cells per well for 24-h uptake in a 24-well plate as counted by a hemocytometer. Stock solutions of complexes **2–4** (500 μM Gd(III)) were prepared. Samples from stock solution were diluted with media to 50 μM Gd(III) or less to prepare incubating solutions of 180 μL per well. Cells were incubated with complexes **2**, **3**, or **4** for 24 h.

Time-dependent uptake for cellular delivery studies were performed with MDA-MB-231 m-Cherry cells. Cells were plated at a cell density of approximately 35 000 cells per well in a 24-well plate as counted by a hemocytometer. Cells were incubated with 50 μM Gd(III)-equivalent concentration of complexes **2**, **3**, or **4** for 1, 2, 4, 8, and 24 h. To harvest, cells were rinsed in-plate three times with 500 μL PBS and trypsinized using 100 μL 0.25% TrypLE. Following trypsin treatment, 150 μL of media was added to each well and mixed by a pipet to ensure that all cells were lifted into suspension. 50 μL of the cell suspension was used for cell counting and 150 μL was used for Gd content analysis via ICP-MS.

Cell Pellet Imaging

Approximately 7.5×10^5 MDA-MB-231 m-Cherry cells were incubated in 25 cm^2 T-flasks with complexes **2**, **3**, or **4** suspended in media or plain media for 24 h, rinsed with DPBS (2 $\times$ 1 mL/flask), and harvested with 500 μL of trypsin. After addition of 500 μL of fresh complete media, cells were transferred to 1.5 mL microcentrifuge tubes and centrifuged at $1000 \times g$ at 4.0 $^\circ\text{C}$ for 5 min. The supernatant was removed; the cell pellets were resuspended in 1 mL of complete media, added to 53/4" flame-sealed Pasteur pipets, and centrifuged at $100 \times g$ at 4.0 $^\circ\text{C}$ for 5 min. The bottom sections of the flame-sealed pipets were then scored with a glass scribe, broken into small capillaries, and taped around a larger tube containing water. The samples were imaged using a RF RES 300 1H 089/023 quadrature transmit receive 23 mm volume coil (Bruker BioSpin, Billerica, MA, USA). A rapid acquisition with refocused echoes (RARE) pulse sequence was used. For T_1 -weighting, the following parameters were used: TR = 500 ms, TE = 10 ms, flip angle = 90° , NEX = 3, FOV = $20 \times 20 \text{ mm}^2$, slice thickness = 1 mm, and matrix size = 256×256 .

In Vivo Studies

All mice were handled and processed according to a protocol approved by Northwestern University Animal Care and Use Committee in accordance with current guidelines from the National Institutes of Health Model Procedure of Animal Care and Use. Male C-57 black mice (wild type) were acquired from Charles River (Wilmington, MA), and were housed under pathogen-free conditions. Mice ($n = 3$) were injected IV 4.0 nmol/kg body weight of complex **2** through IV administration which equates to 9.0 $\mu\text{mol/kg}$ body weight of Gd(III). Mice were imaged by MR on a Bruker Biospin 9.4T magnet (Bruker Biospin, Billerica, MA, U.S.A.) within the first 10 min of injection using a standard FLASH sequence. Mice were kept warm using a heated pad and maintained under 1–2% inhaled isoflurane anesthesia. Respiration was monitored using an SA Instruments MR compatible monitoring system (SA Instruments, Stonybrook, NY, U.S.A.) for appropriate gating. After 3 h, mice were euthanized and organs were analyzed for Gd(III) content by ICP-MS. The heart, lungs, liver, spleen, kidneys, muscle, pancreas, fat, stomach, blood, and urine were placed into preweighed Teflon tubes, weighed, and dissolved in 9:1 ACS reagent grade nitric acid/hydrogen peroxide (10 mL for livers and spleens, 1 mL for kidneys, 500 μL for remaining organs). Organ digestion was accomplished using an EthosEZ microwave digestion system (Milestone, Shelton, CT, U.S.A.). Solutions were heated to 120 °C ramp over 30 min followed by a 30 min hold and a 45 min exhaust cycle. The resultant solutions were weighed and an aliquot was transferred to a preweighed 15 mL conical tube. The final ICP-MS samples were prepared as described in Metals Analysis by ICP-MS.

Statistics

Characterization results of Gd@AuNPs are reported as the average and standard deviation of at least three independently synthesized batches. Hydrodynamic radii, polydispersity indices, and relaxivities of complexes **1–4** were compared by one-way ANOVA. Results from in vitro experiments are expressed as the average of three separate experiments, each performed in triplicate. Three mice were used for in vivo MRI and biodistribution analyses. MR images from a single representative experiment are shown. Bar graphs represent averages while error bars represent standard deviations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Synthetic scheme for facile, DNA-free, Gd@AuNPs with varying linker lengths conjugating Gd(III) chelates to the AuNP surface. Gd–Au surface conjugation is accomplished in the absence of reducing agents.

Figure 2.

(A) MDA-MB-231 m-Cherry cell uptake of Gd(III) after incubation with complex **2**, **3**, or **4**. Complex **2** demonstrates the best overall uptake, and approximately 4-fold greater than complex **3** and 20-fold greater than complex **4**. (B) Time course of MDA-MB-231 m-Cherry cell uptake over a 24 h period after 50 μM incubation with complex **2**, **3**, or **4**. Complexes **2** and **3** demonstrate a significant increase in uptake between 8 and 24 h post-incubation, while complex **4** achieves maximal uptake within 2 h.

Figure 3. TEM images of MDA-MB-231 m-Cherry cells following 24 h incubation with complex **2** (A,D), complex **3** (B,E), or complex **4** (C,F). Complex **2** is present in large (0.5 μm) lysosomes as particle aggregates (A,D). Complexes **3** and **4** are more sparsely distributed within the cell (B,C,E,F).

Figure 4.

MR images of MDA-MB-231 m-Cherry cell pellets treated with Gd@AuNPs acquired at 7 T. Cells labeled with complex **2** demonstrate the greatest contrast enhancement and shortest T_1 relaxation time. Cells labeled with complexes **3** and **4** are indistinguishable from unlabeled cells.

Figure 5.

(A,B) FLASH MR images post-IV injection of complex **2**. (A) T_2 -weighted image 10 min post-IV injection. The great vessels are dark and easily seen. The left kidney and stomach are also easily identifiable. (B) AUC image obtained by subtracting postcontrast image from precontrast image. Part of the interior of the kidney is starting to enhance. As evidenced by the bright great vessels, complex **2** is still in circulation during the FLASH image acquisition. Clearance is relatively slow, as expected. Therefore, the AUC does not show accumulation in the kidney or liver in the first 10 min postinjection. (C) Organ accumulation of complex **2** in C57 mice 3 h after IV injection ($n = 3$). Significant accumulation is seen in the liver and spleen while accumulation in other organs is negligible.

Table 1

Characterization of AuNP Constructs, Specifically Gd(III) Loading, Surface Coverage, Hydrodynamic Size, and Polydispersity Index^a

	loading (Gd/AuNP)	surface coverage (Gd/nm ²)	hydrodynamic radius (nm)	polydispersity index
1	1979 ± 370	1.94 ± 0.36	27.2 ± 1.4	0.16 ± 0.01
2	1715 ± 75	1.69 ± 0.07	28.7 ± 1.5	0.08 ± 0.01
3	2308 ± 291	2.27 ± 0.29	30.7 ± 2.6	0.13 ± 0.01
4	1114 ± 116	1.09 ± 0.11	27.8 ± 1.2	0.13 ± 0.01

^aComplex **4** has significantly decreased loading and surface coverage of Gd(III) compared to complexes **1–3** ($p < 0.05$ for two-tailed t -test). The hydrodynamic radii and polydispersity indices are not significantly different among complexes **1–4**.

Table 2Longitudinal Relaxivity (r_1) of AuNP Constructs, Measured at 1.4 and 7 T^a

	$r_{1,\text{ionic}}$ (mM ⁻¹ s ⁻¹) @ 1.4 T	$r_{1,\text{particle}}$ (mM ⁻¹ s ⁻¹) @ 1.4 T	$r_{1,\text{ionic}}$ (mM ⁻¹ s ⁻¹) @ 7 T	$r_{1,\text{particle}}$ (mM ⁻¹ s ⁻¹) @ 7 T
1 ^b	15.6 ± 1.0	30872 ± 1979	3.4 ± 0.1	6728 ± 198
2	14.6 ± 0.7	25039 ± 1201	4.0 ± 0.1	6860 ± 172
3	12.9 ± 0.7	29773 ± 1616	3.5 ± 0.2	8078 ± 462
4	13.7 ± 0.8	15262 ± 891	4.7 ± 0.2	5236 ± 223

^aThe relaxivities of complexes **1–4** are not significantly different for measurements at both field strengths.^bComplex **1** was not stable in DPBST, so relaxivity of Complex **1** was measured in 25% DPBST in water.