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Gd(III)-nanodiamond conjugates for MRI contrast enhancement

Lisa M. Manus¹, Daniel J. Mastarone¹, Emily A. Waters¹, Xue-Qing Zhang², Elise A. Schultz-Sikma¹, Keith W. MacRenaris¹, Dean Ho², and Thomas J. Meade¹

¹Departments of Chemistry, Biochemistry and Molecular and Cell Biology, Neurobiology and Physiology, and Radiology, Northwestern University, Evanston, Illinois 60208-3113

²Robert R. McCormick School of Engineering and Applied Science, Northwestern University, Evanston, Illinois 60208-3100. Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611

Abstract

A Gd(III)-nanodiamond conjugate [**Gd(III)-ND**] was prepared and characterized, enabling detection of nanodiamonds by MR imaging. The **Gd(III)-ND** particles significantly reduced the T_1 of water protons with a per-Gd(III) relaxivity of $58.82 \pm 1.18 \text{ mM}^{-1}\text{s}^{-1}$ at 1.5 Tesla (60 MHz). This represents a tenfold increase compared to the monomer Gd(III) complex ($r_1 = 5.42 \pm 0.20 \text{ mM}^{-1}\text{s}^{-1}$) and is among the highest per-Gd(III) relaxivities reported.

Multifunctional nanomaterials can be modified with specific biomolecules to increase drug loading capacity and target specific proteins, DNA sequences, and other macromolecular structures.^{1–5} However, one common limitation of nanoparticle applications in clinical settings is the difficulty of tracking nanoparticle localization and movement in vivo. Consequently, development of techniques for facile and effective labeling of nanoparticles with imaging agents would greatly improve the utility of nanoparticle systems.

In recent years, significant interest has been placed on carbon-based nanomaterials such as fullerenes and nanotubes for biological applications (biosensors, drug delivery) due to their physical, chemical, and biological properties.^{6–14} However, the biocompatibility of these compounds remains in question.^{15–17} Diamond-based nanoparticles have gained attention as an alternative carbon nanomaterial due to their excellent biocompatibility, which may be due in part to lower induction of cellular oxidative stress than is observed with other carbon nanomaterials.^{18–21} Exposure to detonation nanodiamonds did not result in a significant loss of cell viability in a variety of cell types (MTT assay and luminescent ATP production).²² Nanodiamond powder-based particles (2–8 nm in diameter) generated by detonation techniques have been shown to disperse in aqueous solutions.^{23–24} Due to the extreme synthetic conditions (diamond detonation followed by treatment with oxidizing acids), the surface of a nanodiamond particle is coated with a variety of functional groups allowing for chemical modification.^{25–26} For example, nanodiamonds have been shown to adsorb proteins and act as chemotherapeutic drug carriers.^{27–30}

Correspondence to: Thomas J. Meade.

SUPPORTING INFORMATION AVAILABLE

Supporting material includes a complete description of the materials and methods used in experimentation. Additionally, an ICP-MS analysis of the washing procedures for the Gd(III)-nanodiamond conjugates is presented. After removal of the reaction supernatant, the particles underwent a cycle of pelleting by centrifugation and resuspension in water. ICP-MS was performed on the reaction supernatant and each of the washes to determine Gd(III) content. By the third wash, Gd(III) content was seen to reach a minimum. Four washes in total were collected before the conjugates were resuspended in a 1 mg/mL solution. An analysis of the dynamic light scattering data is included regarding the hydrodynamic size for nanodiamonds and **Gd(III)-ND**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Although advances are being made in covalent and noncovalent modification of the nanodiamond surface, imaging of nanodiamond particles has largely centered on optical imaging with fluorescence spectroscopy.^{19, 20, 31} Ion beam irradiation has been used to form fluorescent centers with red and green emission visible at the cellular level.^{19, 20} Blue fluorescent nanodiamonds (5 nm in diameter) have been produced by covalent attachment of octadecylamine (ODA) to the carboxylate groups of the nanodiamond surface.³¹ While these fluorescent nanodiamonds provide an alternative to toxic quantum dots, they suffer from the similar limitations in tissue penetration as other optical imaging techniques, restricting their use to primarily histological applications. To our knowledge, there are no published reports of nanodiamonds being visualized by other imaging technologies.

Magnetic resonance imaging (MRI) is a noninvasive technique that can be used to obtain tomographic images of opaque organisms.^{32, 33} In comparison to other imaging techniques such as x-ray or PET, MRI requires no ionizing radiation while producing images with high spatial and temporal resolution, excellent soft tissue contrast, and deep tissue penetration.³² Images are based on the ¹H NMR signal of water with signal intensities proportional to the relaxation rates of the nuclear spins. Different concentrations and microenvironments of water in different tissues result in image contrast.

Contrast agents are used to enhance the local signal intensity in cells and tissues that are magnetically similar but histologically distinct. Gd(III) complexes are used due to their seven unpaired electrons and symmetrical S ground state.³⁴ Such agents consist of the paramagnetic metal species with one or more coordination sites available for water to interact with the unpaired electrons of the Gd(III) ion, resulting in a decreased T_1 (longitudinal relaxation time).^{32, 34} The efficiency of a contrast agent to reduce T_1 of water protons is referred to as the relaxivity, r_1 (mM⁻¹s⁻¹), and defined by Equation 1.^{32, 34}

$$\frac{1}{T_1} = \frac{1}{T_{1, \text{Solvent}}} + r_1 [\text{agent}] \quad (1)$$

We selected covalent modification of the nanodiamond surface for attachment of Gd(III) complexes to produce nanodiamonds detectable by MR imaging. An amine functionalized Gd(III) complex with a six carbon linker was synthesized and characterized to peptide couple to the carboxylic acid groups on the nanodiamond surface (**5**, Scheme 1). Synthesis of the Gd(III) chelate was initiated by reacting 6-amino-1-hexanol with di(*tert*-butyl) dicarbonate to give the Boc protected amine (**2**) in high yield. The alcohol was converted to the bromide (**3**) with bromine in the presence of triphenylphosphine and triethylamine (TEA). Alkylation of the secondary amino group of DO3A tris-*tert*-butyl ester with the protected amine gave (**4**). Global deportation with trifluoroacetic acid (TFA) and triethylsilane and subsequent reaction with Gd(III) chloride at pH 6.5 gave the metallated complex. The crude reaction material was purified by reverse phase high performance liquid chromatography (RP-HPLC) and the collected fractions were freeze dried to give the metallated compound (**5**).

An aqueous stock (10 mg/mL) of nanodiamond particles was generated using previously described methods.³⁰ The Gd(III) complex was peptide coupled to the carboxy-functionalized nanodiamond (1 mg/mL) using a ten-fold excess of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) in a 0.1 M HEPES, 0.1 M NaCl buffer, pH 7.0 (Scheme 2). The Gd(III)-nanodiamond conjugate [**Gd(III)-ND**] was pelleted and the supernatant decanted. Excess **5** was separated from **Gd(III)-ND** through a cycle of resuspensions in water and pelleting of **Gd(III)-ND**. The Gd(III) concentrations of these washes were analyzed by inductively coupled plasma mass spectrometry (ICP-MS), showing that four washes were sufficient to relieve excess Gd(III)

reagent (Supporting Information, Figure S1). Nonspecific interactions between **5** and the ND surface appear minimal (Supporting Information). After the final wash, **Gd(III)-ND** was resuspended in 5 mL of water (1mg ND/mL).

ICP-MS analysis showed $48 \pm 3 \mu\text{M}$ of Gd(III) adhered to nanodiamonds per 1 mg/mL nanodiamond reaction. The majority of **5** remained in the reaction supernatant. Transmission electron microscopy (TEM) images of the nanodiamonds before and after reaction suggest the overall integrity of the individual nanodiamonds is retained but there is greater clustering after conjugation to **5** (Figure 1). Modification of the nanodiamond surface groups was investigated by Fourier transform infrared spectroscopy (FTIR) (Figure 2A). The FTIR spectra of the unmodified nanodiamonds and **5** are shown for comparison (Figure 2B, 2C)

MR imaging of three sets of **Gd(III)-ND** was performed with Gd(III) concentrations ranging from $5 \mu\text{M}$ to $48 \mu\text{M}$ (Figure 3). The intense signal of **Gd(III)-ND** was clearly visualized at the highest concentration of Gd(III) ($48 \mu\text{M}$), with a contrast to noise ratio (CNR) versus water of 142. Even at the lowest concentration of Gd(III) imaged ($5 \mu\text{M}$), **Gd(III)-ND** is measurably brighter than water; the CNR versus water was 25, well above the detection limit of the human eye.

T_1 measurements were obtained on six different concentrations of **Gd(III)-ND** at 37°C in water at 1.5 T. A $48 \mu\text{M}$ Gd(III) sample of **Gd(III)-ND** decreased the average longitudinal relaxation time of environmental water protons 10-fold with respect to unfunctionalized nanodiamonds (Table 1). $1/T_1$ increased linearly with increasing concentrations of **Gd(III)-ND** with a slope corresponding to a relaxivity of $58.82 \pm 1.18 \text{ mM}^{-1}\text{s}^{-1}$ (Table 2). This is significantly higher than clinical Gd(III) contrast agents, Gd-DTPA and Gd-DOTA with relaxivity values of $3.8 \text{ mM}^{-1}\text{s}^{-1}$ and $3.5 \text{ mM}^{-1}\text{s}^{-1}$, respectively.³⁴

Solomon–Bloembergen–Morgan Theory summarizes the parameters that can be manipulated to increase the relaxivity of a given Gd(III) contrast agent. These include q , the number of water molecules coordinated to each Gd(III) center; τ_r , the rotational correlation time; and τ_m , the mean residence lifetime of the coordinated waters.^{35, 36} Small molecule Gd(III) agents have a τ_r on the order of tens of picoseconds; the optimal τ_r to maximize relaxivity at 1.5 T is estimated to be several nanoseconds.^{37, 38}

Optimization of τ_r has been approached from conjugation of Gd(III) complexes to nanoparticles, proteins, and peptides with the additional molecular weight of the appendage slowing the rotation of the agent.^{2, 37, 39–46} For example, MS-325 is a blood pool contrast agent and has been shown to have a 9-fold increase in relaxivity upon binding human serum albumin (HSA).³⁷ We are conjugating our complexes to a nanoparticle significantly higher in molecular weight than a protein resulting in a longer rotational correlation time and a larger increase in relaxivity.

Clustering of the modified nanodiamond particles could be a factor in the observed measurements (Supporting Information, Table S1). Hydrodynamic sizes of the nanodiamonds and **Gd(III)-ND** as determined by dynamic light scattering (DLS) suggest that **Gd(III)-ND** forms small clusters after conjugation of the Gd(III) complexes. Before conjugation, the nanodiamonds are approximately 21 nm with no observable concentration dependence in hydrodynamic size. The hydrodynamic size of **Gd(III)-ND** was seen to decrease ($128 \pm 14 \text{ nm}$, $6 \mu\text{M}$ Gd to $55 \pm 4 \text{ nm}$, $0.2 \mu\text{M}$ Gd) upon dilution and ultrasonication, suggesting fragmentation of the **Gd(III)-ND** clusters. $55\text{--}60 \text{ nm}$ appears to be a lower limit at dilute concentrations for the hydrodynamic size of **Gd(III)-ND**. Aggregation has been observed to contribute to the increased relaxivities of Gd(III)-fullerene contrast agents in aqueous solution.^{47, 48} However, unlike Gd(III)-fullerenes, the nanodiamonds do not sequester the paramagnetic metal allowing direct interaction of the bulk water with the Gd(III) complex.

This permits both inner sphere and outer sphere relaxation mechanisms to contribute to the observed relaxivity.

Previous studies using differential scanning calorimetric analysis (DSC) have shown that strong electrostatic potentials on the nanodiamond facets result in a surface-mediated attraction towards surrounding water molecules, effectively creating a nanophase of water at the nanodiamond-solvent interface.⁴⁹ The potent hydration observed has thus been proposed as a mechanism that drives gelation of the nanodiamonds towards a hydrogel state. Nanodiamond facet electrostatic conditions can be differentially charged depending upon the plane being addressed. For example, [111] facets display negatively charged electrostatic fields while [100] facets display positively charged electrostatic fields. Given these conditions, water molecules orient in clusters with opposing alignments/dipoles that are highly attracted to each other, significantly enhancing the adhesion of a robust and continuous hydration layer surrounding the particle surface.⁴⁹ The combination of direct interaction of the water nanophase with the paramagnetic metal and the size of the nanodiamond clusters (130 nm - 55 nm) may help to explain the high relaxivity of the system.

With diamond-based nanoparticles considered as an attractive alternative carbon nanomaterial due to their high biocompatibility, the effect of **Gd(III)-ND** on cell viability, metabolic activity, and cytotoxicity using multiple mammalian cell lines was tested. Preliminary results using human ovary adenocarcinoma (SKOV-3) cells show **5** and **Gd(III)-ND** conjugates do not significantly decrease cell viability compared to doxorubicin (a known, potent cytotoxic agent, Figure 4). There is, however, a decrease in overall cell viability at 24 h for both **5** and **Gd(III)-ND** at Gd(III) incubation concentrations higher than 1.0 μ M [0.0250 mg/mL **Gd(III)-ND**].

To determine the effect of nanodiamond incubation time on cell metabolic activity and cytotoxicity [glucose 6-phosphate dehydrogenase (G6PD) leakage], NIH/3T3 and HeLa cells were cultured. These cell lines were chosen because they are slightly more robust and have higher metabolic activity and G6PD leakage (compared to SKOV-3 cells), a requirement to conduct these assays. As shown in Figure 5A, overall cell metabolism is comparable to control levels at ≤ 0.250 mg/mL **Gd(III)-ND** in NIH/3T3 cells, but significantly decreases at 0.500 mg/mL **Gd(III)-ND**. There was no significant decrease in the metabolic activity of HeLa cells (compared to control 1) using the same conditions. A test for cytotoxicity revealed **Gd(III)-ND** has no significant effect on cytotoxicity compared to unmodified nanodiamonds (Figure 5B) except in NIH/3T3 cells at 0.500 mg/mL **Gd(III)-ND** after 24 h incubation. **Gd(III)-ND** conjugates exhibit relatively low cytotoxicity with IC₅₀ values higher than 0.500 mg/mL **Gd(III)-ND** (according to three separate assays and three cell lines). These results show the addition of a Gd(III)-based contrast agent to the nanodiamond surface does not have a significant effect on overall cell viability.

Utilizing the inherent surface chemistry of detonation nanodiamonds, an amine-functionalized Gd(III) complex was covalently bound allowing visualization of nanodiamond particles by MR imaging. The relaxivity of the Gd(III) contrast agent increased nearly 10-fold in comparison to the free agent (**5**) upon conjugation to the nanodiamond platform. This enhanced contrast is clearly seen in the MR images of modified and unmodified species.

Current efforts are focused on the biodistribution of **Gd(III)-ND** in vivo, applications of **Gd(III)-ND** at the biomaterials interface, and on studying the field dependence of the **Gd(III)-ND** relaxivity. Rotational correlation time (τ_r) is influenced by the strength of the magnetic field, with the theoretical maximum relaxivity decreasing at higher field strengths.^{38, 50, 51} Further study will include relaxivity measurements at 9.4 T to monitor relaxometric decline. Variation in the linker length of the amino-alkane to the macrocyclic chelate will be explored

to manipulate the extent to which hindrance of the chelate rotation plays a role in the increased relaxivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TEM images of the nanodiamonds before (A) and after conjugation (B) to the Gd(III) contrast agent, **5**. The scale bars are 50 nm.

Figure 2. FTIR spectra of the modification of the nanodiamond surface upon conjugation of the amine functionalized Gd(III) complex: (A) **Gd(III)-ND**, (B) **5**, (C) unmodified nanodiamonds

Figure 3.

MR images of nanodiamond samples. 1: water, 2: 1 mg/mL undecorated ND, 3: undecorated ND + coupling reagents, 4: 48 μM Gd(III), 5: 38 μM Gd(III), 6: 22 μM Gd(III), 7: 10 μM Gd(III), 8: 5 μM Gd(III)

Figure 4.

Cell viability (using an MTT assay) of SKOV-3 human ovary adenocarcinoma cells after 24 h incubation with various concentrations of **Gd(III)-ND** (squares), **5** (circles), and doxorubicin (triangles) as a positive control. Cell viability is represented as the percent absorbance with respect to control cells (those incubated with media alone). All conditions were done in triplicate with error bars representing $\pm$ one standard deviation of the mean. 40 μ M Gd(III) is equivalent to $\sim$ 1.0 mg/mL **Gd(III)-ND**.

Figure 5.

Metabolic activity (A, Vybrant® metabolic activity assay kit) and cytotoxicity (B, Vybrant® cytotoxicity assay kit) of NIH/3T3 and HeLa cells after 24, 4.0, and 1.0 h incubations with various concentrations of Gd(III)-ND. Metabolic activity is represented as the percent fluorescence with respect to control cells (those incubated with media alone). Cytotoxicity is represented as the percent fluorescence with respect to lysed cells incubated with media alone. Control 1 are cells incubated with media alone and control 2 are cells incubated with 0.500 mg/mL NDs [no Gd(III)] for 24 h. All conditions were done in triplicate with error bars representing $\pm$ one standard deviation of the mean. 40 μ M Gd(III) is equivalent to $\sim$ 1.0 mg/mL Gd(III)-ND.

Scheme 1.

Synthesis of an amine-functionalized Gd(III) contrast agent (**5**) for attachment to the nanodiamond surface

Scheme 2.

Conjugation of the Gd(III) contrast agent (**5**) to the nanodiamond surface

Table 1

Average T_1 (longitudinal relaxation time) and corresponding Gd(III) concentrations for water, the free Gd(III) complex **5**, unmodified nanodiamonds, and **Gd(III)-ND**. A sharp decrease in T_1 is observed upon conjugation of **5** to the nanodiamond surface [**Gd(III)-ND**].

Sample	[Gd] (mM)	T_1 (ms)
Water	0.000	3820 ± 10
5	1.751	105.7 ± 0.6
5	0.128	1253 ± 27
ND (1mg/mL)	0.001	3103 ± 114
Gd(III)-ND	0.048	319 ± 11

Table 2Gd(III) ionic relaxivity values for **5** and **Gd(III)-ND** at 1.5 T, 37 °C

Complex	r_1 (mM ⁻¹ s ⁻¹)
5	5.42 ± 0.20
Gd(III)-ND	58.82 ± 1.18