Hybrid Nanotrimers for Dual $T_1$ and $T_2$-Weighted Magnetic Resonance Imaging

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ABSTRACT Development of multifunctional nanoparticle-based probes for dual $T_1$- and $T_2$-weighted magnetic resonance imaging (MRI) could allow us to image and diagnose the tumors or other abnormalities in an exceptionally accurate and reliable manner. In this study, by fusing distinct nanocrystals via solid-state interfaces, we built hybrid heteronanostructures to combine both $T_1$ and $T_2$-weighted contrast agents together for MRI with high accuracy and reliability. The resultant hybrid heterotrimers showed high stability in physiological conditions and could induce both simultaneous positive and negative contrast enhancements in MR images. Small animal positron emission tomography imaging study revealed that the hybrid heterostructures displayed favorable biodistribution and were suitable for in vivo imaging. Their potential as dual contrast agents for $T_1$ and $T_2$-weighted MRI was further demonstrated by in vitro and in vivo imaging and relaxivity measurements.

KEYWORDS: hybrid · nanotrimers · dumbbell structures · $T_1/T_2$-weighted magnetic resonance imaging · positron emission tomography

As one of the most powerful noninvasive diagnostic techniques, magnetic resonance imaging (MRI) is widely applied for clinical whole body imaging and can provide anatomical and functional information about soft tissues and organs with excellent spatial resolution. The signal intensity in MRI, corresponding to the magnetic relaxation rate of water protons, could be enhanced by appropriate exogenous contrast agents. There are two principal relaxation processes: longitudinal ($T_1$ recovery, spin–lattice) or transverse ($T_2$ decay, spin–spin) relaxation. $T_1$ recovery generates positive contrast, but $T_2$ decay reduces the imaging intensity, resulting in negative contrast. The introduction of exogenous contrast agents could accelerate the relaxation process of either $T_1$ or $T_2$, thereby enhancing the imaging contrast. Common $T_1$ contrast agents are paramagnetic complexes containing gadolinium ($\text{Gd}^{3+}$), manganese ($\text{Mn}^{2+}$), or iron ($\text{Fe}^{3+}$) ions. Those relatively small molecules usually have a short circulation time, due to their fast renal excretion. They leave the vascular system within a few minutes, making it relatively hard to have a high resolution image acquisition. Moreover, the use of functionalized paramagnetic complexes as targeting contrast agents is limited because of the arduous and lengthy modification process and poor detection sensitivity. On the other hand, the superparamagnetic iron oxide nanoparticles (SPIOs) as $T_2$ MR contrast agents are very useful for biomedical applications as they are not subject to strong magnetic interactions in dispersion and are readily stabilized under physiological conditions. As $T_2$-weighted contrast reagents, SPIOs can be magnetically saturated in the normal range of magnetic field strengths used in MRI scanners, thereby establishing a substantial locally perturbed dipolar field that leads to a remarkably shortening of proton relaxation of the surrounding solid tissue area. However, due to the negative contrast effect and susceptibility artifacts, distinguishing the region of signals induced by SPIOs from low-level background MR...
signals arising from adjacent tissues such as bone or vasculature is a major challenge, especially when the signal-to-noise ratio is low.

Recently, $T_1/T_2$ contrast agents have been developed and actively studied for dual $T_1$ and $T_2$ mode MRI, because they have the ability to validate reconstruction and visualization of the data in an accurate and reliable manner, and to acquire complementary and self-confirmed information to permit meaningful interpretation.\(^\text{10}\) Several approaches have been applied to develop $T_1/T_2$ contrast agents. In the first approach, some nanomaterials could intrinsically show both $T_1$ and $T_2$ contrast effects in the dual mode MRI but also suffer certain limitations. For instance, ultrasmall SPIOs (~3 nm size) display great potential as $T_1$ contrast agents, but their $T_2$ contrast effect is weak.\(^\text{11}\) FeCo alloy nanoparticles (NPs) exhibit both high $T_1$ and $T_2$ contrast effects and the mechanism underlying this phenomenon is still unclear.\(^\text{12}\) As paramagnetic materials, several lanthanide (Ln) oxide NPs (Ln = Gd, Dy, and Ho) and manganese oxide NPs have been extensively studied as potential candidates for either $T_1$ or $T_2$ MRI contrast agent or both.\(^\text{5,13–19}\) Compared to either regular $T_1$ or $T_2$ contrast agents, the MRI contrast effects of Ln oxide NPs are relatively weak at the same scanning condition. Moreover, their acute and chronic toxicity remain unclarified. In the second approach, in order to combine both $T_1$ and $T_2$ contrast effect together, Park and co-workers used Gd-labeled magnetite (Fe$_3$O$_4$) NPs as contrast agents for dual $T_1$ and $T_2$ weighted MRI.\(^\text{20}\) Later, Cheon and co-workers found that the magnetic coupling induced by $T_2/T_1$-core/shell structures can result in undesirable quenching of both $T_1$ and $T_2$ signals in MRI images.\(^\text{10}\) They then optimized the thickness of silica shell to modulate the degree of $T_1$ and $T_2$ coupling, thereby leading to a significant increase in $T_1$ contrast effect, but the $T_2$ contrast effect still lost one-third of original value because the thick silica shell dramatically reduces the magnetic field to the surrounding water molecules.\(^\text{10}\) On the basis of the similar IONP/silica core/shell structures, Yang and co-workers recently designed the dual-contrast $T_1$ and $T_2$-weighted MR contrast agents modified with an arginine–glycine–aspartic acid (RGD) peptide for selectively targeting αvβ3 over-expressed tumor in vitro and in vivo.\(^\text{21}\) However, coupling of different chemical functionalities on a single component NP surface is often a strenuous and low yield synthetic process, and the presence of contrast agents and targeting molecules as well as other functional reporters on the same NP surface may interfere with the targeting capability and contrast effects. In the third approach, Gao and co-workers demonstrated that embedding Gd oxide crystals into iron oxide nanoparticles could synergistically enhance both $T_1$ and $T_2$ contrast effects in MR imaging, probably due to the fact that the Gd spins of Gd$_2$O$_3$ buried in iron oxide NPs are parallel to the local magnetic field induced by those superparamagnetic iron oxide NPs under an external magnetic field.\(^\text{22}\) This strategy is attractive for some reasons such as facile and reproducible synthetic methods, though challenges still remain if ones try to incorporate targeting moieties and biocompatible coating on the single external surface of those NPs.

The interference inevitably occurs when both $T_1$ and $T_2$ contrast agents are integrated into a single composite, especially when they are in close proximity. Although the conventional core/shell structures could efficiently reduce their magnetic coupling by introducing a separating layer between them (Figure 1a, left panel), the $T_2$ contrast effect is compromised as the thick shell decreases the magnetic field to the surrounding water molecules. In this study, we first developed a synthetic strategy for engineering the architecture of heterogeneous nanoparticles, which could help us to further understand the magnetic coupling at the nanoscale. After gaining insight from the synthesis strategies, we chose dumbbell-like NPs or so-called ‘Janus’ NPs, which have two different components within one single structure, as solid-state analogues of bifunctional organic molecules to construct hybrid nanotrimers (HNTs) (Figure 1a, the right panel), in which iron oxide and gold nanocrystals are connected with a platinum nanocube. In our previous study, dumbbell-like heteronanostructures with two different nanomaterials (Au and iron oxide: Au–IO) within one hybrid structure also showed capability of combining two different moieties together for dual modal imaging.\(^\text{23}\) In this study, the surface of Au component in Au–IO NPs were then modified with Gd to test its use for $T_1/T_2$ MRI. However, induced $T_1$ contrast effect could be adversely affected by the attenuation of local magnetic field because there is a strong magnetic coupling between iron oxide and Gd species in the structure of the dumbbell-like Au–IO NPs. To reduce the undesired magnetic coupling effect, we designed a strategy to build such HNTs to combine both $T_1$ and $T_2$ contrast agents together by fusing IONP, Pt, and gold nanocrystals via solid-state interfaces (Figure 1b). The size of gold nanocrystals could be further enlarged by controlling the seed-mediated and epitaxial growth processes during the synthesis. Meanwhile, we optimized the sizes of Pt cubes to adjust the distance of IONP and gold nanocrystals in order to manipulate the magnetic coupling effect. The resultant heterotrimer with large gold crystals show real dumbbell morphology, which are named as dumbbell-hybrid nanotrimers (DB-HNTs). Ones with a large center-to-center distance between gold and iron oxide are called as extra large dumbbell-hybrid nanotrimers (XDB-HNTs). The iron oxide components were modified with polyethylene glycol (PEG) chains to make the whole NPs water-soluble and biocompatible. The surface of gold were covalently immobilized with Gd chelators (dithiol derivative 1,4,7,10-tetraazacyclododecan-1,4,7,10-tetraacetic acid
(DOTA) chelator, or cystamine-DOTA2, Supporting Information Figure S1). The resultant hybrid heterostructures could induce both simultaneous positive and negative contrast enhancement in MR images. The \textit{in vivo} biodistribution of hybrid heterostructures favorable for molecular imaging was confirmed using small animal PET. The potential of Gd–DB-HNTs as dual contrast agents for \( T_1 \) and \( T_2 \) weighted MRI was demonstrated by conducting \textit{in vitro} relaxivity measurements and \textit{in vivo} imaging.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of Dumbbell Hybrid Heterostructures.** The syntheses of complex hybrid heterostructures sequentially involved a set of the seed-mediated nucleation and epitaxial growth processes. According to the previously published procedures with a slight modification,\textsuperscript{23–25} the monodisperse Au–IONP heterodimers were synthesized by thermal decomposition of iron precursor...
Table 1. Summary of Size Measurement by TEM and DLS and Zeta Potentials of Various Heteronanoparticles

| sample       | TEM analysis (nm) | D_{Au-Pt-C0} (nm) | DLS (nm) | zeta potential (mV) |
|--------------|-------------------|--------------------|----------|---------------------|
| Au–IONP      | 10.2 ± 0.6        | 5.1 ± 0.4          | 19.5 ± 0.6| –13.4 ± 0.8         |
| HNTs         | 11.6 ± 0.2        | 7.0 ± 0.4          | 21.2 ± 0.5| –15.1 ± 0.9         |
| DB-HNTs      | 11.7 ± 0.4        | 9.1 ± 0.6          | 24.6 ± 0.7| –16.0 ± 1.3         |
| XDB-HNTs     | 12.5 ± 1.2        | 9.8 ± 0.6          | 26.8 ± 0.9| –16.4 ± 1.2         |

* A minimum of 200 discrete particles was measured from each of at least two widely separated regions of the sample. ** D: the center-to-center distance between iron oxide and gold nanocrystal.

... and gold nanocrystals. When 5 nm Pt nanocrystals to adjust the distance of iron oxide components and gold nanocrystals. When 5 nm Pt nanocubes were used as seeds (Supporting Information Figure S2), we could end up with getting extra large dumbbell HNTs (XDB-HNTs) (Figure 1f,j). As shown in Table 1, the center-to-center distance between iron oxide and gold nanocrystal (D_{Au-Pt-C0}) was significantly increased when large gold nanocrystals were grown on the Pt seeds. As expected, the large 5 nm Pt nanocubes created the longest distance (12.9 ± 0.7 nm) between iron oxide and gold within XDB-HNTs. Although much bigger HNTs with similar dumbbell shapes could be obtained when the 7 nm cubic Pt particles were used as seeds, two or more gold crystals could grow on Pt cubes to form complex heterostructures (Supporting Information Figure S6), which could adversely influence the quality of the products. In short, all four heterostructures (Au–IONP, HNTs, DB-HNTs, and XDB-HNTs) share a structural similarity, a dumbbell shape, in which either Au component is embedded into IONP, or Au and IONP are connected via a robust Pt spacer.

High-resolution TEM (HRTEM) images of HNTs indicate that all three components are well-crystallized and they have coherent interfaces (Figure 2). The d-spacing between adjacent (111) planes in IONP component is 4.84 Å, which is characteristic of magnetite. The lattice fringes of Pt and Au components are very similar (2.20 and 2.32 Å, respectively) and they are related to (111) planes of Pt or Au in the fcc phase. Moreover, the d-spacing of Pt is approximately half of that of the (111) planes of Fe_{3}O_{4}. Thus, the formation and stability of Pt–IONP and Pt–Au interfaces are largely attributed to their lattice matching. Energy-dispersive X-ray spectroscopy (EDS) was further applied to determine the compositions of various HNT nanostructures (Figure 2b and Supporting Information Figures S4 and S5). It is apparent that the HNTs are composed of Fe_{3}O_{4}, Pt and Au crystals. The forward and inverse Fast Fourier transforms (FFT) were applied to corresponding HR-TEM images to distinguish iron oxide, cubic Pt, and Au nanocrystals within various HNTs. The inset in diffractogram pattern (Figure 2c) of HNTs showed the splits of the (111) peaks into two spots: one for Pt and the other for Au crystal, due to the slight difference in planar distances of the Pt and Au crystals. Inverse FFT reconstructions of IO, Pt, or Au NPs...
using their own reflections provided the real-spatial distributions of IO, Pt, and Au in HNTs (Figure 2d–f). It was further confirmed by the overlay image of all reconstructed IO, Pt and Au inverse FFT images (Figure 2g). Likewise, the lattice images of DB-HNTs and XDB-HNTs coupled with the forward FFT images revealed the crystal structures and orientations of Au and IONP around Pt cube (Figure 2h,i). All of components showed well-defined crystal structures and coherent interfaces. Importantly, they all have dumbbell shapes; Au and IONP component are tightly connected to each other via a cubic Pt crystal.

As seen in Supporting Information Figure S7, all of dumbbell HNTs are superparamagnetic at room temperature and they all show similar loops with saturation moment of 26 emu/g of Fe3O4. The slow increase in magnetic moment from hysteresis loops with the field up to 5T is due to the interface communication between the nanoscale Pt, Au, and Fe3O4 crystals, which leads to the change of magnetization behaviors of Fe3O4 nanoparticles within all types of HNTs.24

**Surface Coating and Gd Chelating of Hybrid Heteronanostructures.** A surface modification step is necessary to provide the NPs water-soluble, biocompatible and functionalizable. According to our previously published procedure,23 all four hybrid heterostructures were coated with bio-compatible PEG chains in order
by increasing the size of spherical gold component, both Gd–DB-HNTs and Gd–XDB-HNTs have much higher Gd/Fe molar ratios (0.083 or 0.079) than Gd–HNTs (0.037).

The hydrodynamic sizes and zeta potentials of four types of heterostructures were determined by dynamic light scattering (DLS) analysis. The average hydrodynamic diameters of Au–IONPs, HNTs, DB-HNTs, and XDB-HNTs were 19.5 ± 0.6, 21.2 ± 0.5, 24.6 ± 0.7, and 26.8 ± 0.9 nm, respectively (Table 1 and Supporting Information Figure S10). A significant increase in size from 19 to 27 nm was attributed to the epitaxial growth of gold components on Pt crystals. After the gold components were coated with negatively charged DOTA ligand, zeta potential measurements clearly showed that all four types of heterostructures had negative surface potentials (Table 1). Subsequent complexation with Gd ions gave rise to a remarkable increase in surface zeta potentials of all four NPs (Supporting Information Figure S12), due to the neutralization of the negative surface charge caused by the coordination of cationic Gd ions with carboxylate groups of DOTA ligands.

**Magnetic Resonance (MR) Phantom Test.** To evaluate the feasibility of using various Gd incorporated HNTs as dual T₁- and T₂-weighted contrast agents, we measured their relaxivities in aqueous medium on a 7.0 T MR scanner. Figure 3 shows the T₁-weighted MR images of Gd–Au–IONPs, Gd–HNTs, Gd–DB-HNTs, and Gd–XDB-HNTs at different concentrations based on iron contents. Both Gd–DB-HNTs and Gd–XDB-HNTs displayed a signal enhancement on the T₁-weighted images with a concentration-dependent manner. It is noticed that their contrast effects are more significant than that of Gd–HNTs at the same condition, but there is almost no T₂ contrast effect observed for Gd–Au–IONPs. Although the larger Au surface area could result in the more Gd loading, the distance between Au and IONP components played an important role in regulating the magnetic coupling effect so that both Gd–DB-HNTs and Gd–XDB-HNTs provided the most significant contrast effect among these heterostructures. To quantitatively determine the MR contrast effect, the relaxivity coefficient (r₁) was calculated from T₁ mapping images acquired by using an inversion recovery sequence. On the basis of the total concentrations of both Fe and Gd, the r₁...
values of Gd–Au–IONPs, Gd–HNTs, Gd–DB-HNTs, and Gd–XDB-HNTs are 1.65, 1.74, 3.88, and 4.12 mM [Fe\(^{+3}\)] \(^{-1}\)s, respectively (Table 3). It is clear that Gd–DB-HNTs have 141% higher \(r_1\) value compared to the commercial contrast agent, DOTArem (gadoterate meglumine, Guerbet, Aulnaysous-Bois, France, \(r_1 = 2.75\) mM \([Fe^{+3}\]) \(^{-1}\)s), due largely to the slower tumbling rate of Gd-complex on the NPs than as free molecules.\(^2\)

For Gd–HNTs, however, the \(T_1\) MR contrast effect was quenched to some extent because the coupling between electron spins of the Gd complex and nuclear spins of water was perturbed by the additional magnetic field generated by nearby iron oxide components.\(^10\) Such quenching effect could be remarkably reduced by enlarging the distance between \(T_1\) and \(T_2\) contrast agents (or \(D_{Au\rightarrow IO}\)). In this case, with an increase in the size of gold components, the percentage of Gd-complex immobilized on gold surface away from the iron oxide components gradually increased, thereby resulting in reduction of the quenching effect. In a similar manner, the interaction between the \(T_1\) and \(T_2\) contrast agents could be efficiently separated or attenuated using large Pt nanocubes. With the larger center-to-center distance between iron oxide and gold component, Gd–XDB-HNTs provided higher \(r_1\) value than Gd–DB-HNTs. Both Gd–DB-HNTs and Gd–XDB-HNTs showed obvious \(T_1\) contrast effect among four heterostructures. On the other hand, the iron oxide components in the heterostructures can serve as \(T_2\) contrast enhancement agents. Since all four heterostructures have iron oxide components with similar sizes and shapes, their \(T_2\) weighted MR images at a same iron concentration gradient (in the range from 0 to 0.5 mM) displayed similar contrast effects (Figure 4b). Accordingly, the relaxivity coefficients (\(r_2\)) of Gd–Au–IONPs, Gd–HNTs, Gd–DB-HNTs, and Gd–XDB-HNTs were calculated from \(T_2\) mapping images (Table 3). Because the sizes of iron oxide components are roughly close, they all have similar relaxivities, which are close to that of a commercial available \(T_2\) contrast agent (Feridex, 158.3 mM \([Fe^{+3}\]) \(^{-1}\)s).

It suggests that all heterostructures with iron oxides can be efficiently used as \(T_2\) contrast agents. Moreover, there are no significant changes in relaxivity of Gd-chelated NPs compared to that of unchelated NPs, indicating that the \(T_2\) relaxation process induced by magnetic iron oxide cores could not be adversely influenced by the Gd-coating on the nearby Au components. Interestingly, there is a slight decrease in relaxivity coefficient with a decrease in the size of iron oxide component (Tables 1 and 3), probably due to both thermal agitation and the surface spin canting of small iron oxide particles. Finally, the ratios of \(r_2\) to \(r_1\) are calculated in order to estimate the efficiency of both \(T_1\) and \(T_2\) contrast effects of all the heterostructures. For the purpose of comparison, we determined each relaxivity of contrast agent with each element content. For example, the calculation of the \(r_1\) relaxivity of each particle is based on different concentrations of Gd. They are listed as \(r_1^I\) values in

**Table 3.** \(r_1\) and \(r_2\) of Various Dumbbell Heterostructures and DOTArem

| heterostructures     | \(r_1\) (mM [Fe\(^{+3}\)] \(^{-1}\)s) | \(r_1^I\) (mM [Gd] \(^{-1}\)s) | \(r_2\) (mM [Fe] \(^{-1}\)s) | \(r_2/r_1^I\) | \(r_2/r_1\) |
|----------------------|-------------------------------|-----------------------------|----------------------------|----------------|-------------|
| Gd–Au–IONPs          | 1.65                          | 43.6                        | 123                       | 2.8            | 75          |
| Gd–HNTs              | 1.74                          | 18.6                        | 131                       | 7.0            | 75          |
| Gd–DB-HNTs           | 3.88                          | 30.4                        | 128                       | 4.2            | 33          |
| DB-HNTs              | 2.34                          | -                           | 125                       | -              | -           |
| Gd–XDB-HNTs          | 4.13                          | 32.1                        | 136                       | 4.2            | 33          |
| DOTAm                | -                             | 2.75                        | 2.02                      | 0.73           | -           |
Table 3. Even though the $r_2/r_1$ ratio of Gd/C0 DB-HNTs was 33, their $r_2/\rho_1$ ratio was only 4.2, indicating that they could be both efficient $T_1$ and $T_2$ contrast agents. Although Gd/C0 XDB-HNTs have slightly higher $r_1$ values, they do not have better $r_2/r_1$ ratio than Gd/C0 DB-HNTs because of their larger size of iron oxide component.

Stability and Cytotoxicity. To validate the stability of functionalized heterostructures, we measured the hydrodynamic size distribution of functionalized DB-HNTs over the time course of a 48 h incubation in mouse serum using DLS. The average sizes of DB-HNTs remained same over the course of 48 h incubation, indicating that the DB-HNTs are stable in a physiological condition (Supporting Information Figure S11a). We further validated the binding stability of Gd ions to the functionalized DB-HNTs by analyzing the trace amount of each element in the eluent/supernatant of every aliquot from stability test ICP-MS, and found the free Gd$^{3+}$ was nearly undetectable (Supporting Information Figure S13). The absence of any free Gd ions in the eluent confirmed the strong binding of Gd ions to DOTA-conjugated DB-HNTs.

In vitro cytotoxicity of Gd/C0 DB-HNTs was evaluated by a standard tetrazolium dye (MTT) based colorimetric assay of viability of a fibroblast cell line (NIH-3T3). As seen in Supporting Information Figure S11b, there was no obvious decrease in cell viability after the cells were
incubated with Gd–DB-HNTs at various concentrations in the range of 1–200 μg Fe/mL for 24 and 48 h. The cell viability still remained above 90% for 24 h or 85% for 48 h even at the concentration of 200 μg Fe/mL, indicating that Gd–DB-HNTs are biocompatible and minimally cytotoxic in the given concentration range.

**PET Imaging and Quantification.** The in vivo behavior of DB-HNTs was then evaluated by positron emission tomography (PET). Instead of being chelated with Gd ions for MRI study, the DOTA molecules on the DB-HNTs were efficiently labeled with PET radionuclide ⁶⁴Cu at a specific activity of 222 MBq/nmol particles (or 4 μCi/μg Fe) with a radiolabeling yield of 60–70%. Figure 4a showed the representative coronal and transverse PET and computed tomography (CT) images of HT-29 tumor-bearing mice at 4 and 24 h after tail-vein injection of 100 μCi of ⁶⁴Cu-DB-HNTs. The HT-29 tumor was clearly visualized with high contrast relative to the background after 4 and 24 h post-injection of radiolabeled NPs. On the basis of the quantitative analysis of region of interest (ROI), PET images revealed that the tumor accumulation of radiolabeled NPs at 4 h post-injection was 2.7 ± 0.8 percentage injected dose per gram (%ID/g) and increased to 4.0 ± 1.0%ID/g after 24 h post-injection. After accurate PET/CT image alignment, the nonspecific uptakes of NPs by major organs delineated and localized by CT images were detected and quantified in the PET images (Figure 4b). The liver and spleen uptake were prominent after 4 h post-injection (18.7 ± 1.1, 11.5 ± 0.8%ID/g), indicating a majority of injected NPs are taken up by the MPS. A significant decrease (5.1%ID/g) in liver uptake after 24 h post-injection highlighted that NPs were able to be eliminated through hepatic excretion. More importantly, the kidney uptake of injected NPs was 3.5 ± 0.5%ID/g at 4 h post-injection and slightly increased to 4.1 ± 0.5%ID/g after 24 h post-injection, indicating that the renal excretion was also involved in the elimination process of NPs from mice. Even though XDB-HNTs have slightly bigger sizes than DB-HNTs, they show very similar in vivo behavior as DB-HNTs (Figure 4c,d). The favorable in vivo distribution, tumor accumulation and clearance through both hepatobiliary and renal system indicate that the DB-HNTs is a novel type of highly promising NPs for in vivo applications.

**Dual T₁ and T₂-Weighted MRI.** To validate the ability of Gd–DB-HNTs as dual T₁/T₂ mode MRI agents in living subjects, we performed the in vivo MRI of mice bearing HT-29 tumors injected via the tail vein with Gd–DB-HNTs (200 μL, 8 mg Fe/kg mouse body weight). When administered in the bloodstream, the Gd–DB-HNTs favor extravasations through leaky vasculature due to their size and surface properties, followed by deep penetration and retention at the tumor sites, which is a phenomenon known as the Enhanced Permeability and Retention (EPR) effect. Figure 4e shows the transverse T₁- and T₂-weighted images of tumor before injection and at 4 h post-injection. Compared with the pre-injection images, T₁-weighted tumor images showed obvious positive signal enhancement, while the tumor signal from T₂-weighted images was getting much darker. Since the tumor appeared hyperintense relative to muscle on T₂-weighted images before tail-vein injection of Gd–DB-HNTs, it consequently became easily identifiable on the post-injection images because of the obvious hypointenses within the tumors. Such a significant change in contrast was attributed to the accumulation of the injected Gd–DB-HNTs in the tumor via the EPR effect. On the other hand, the hyperintense signal from the tumor sites on T₁-weighted image after intravenous injection of particles further validated that Gd–DB-HNTs were accumulated within the tumor after 4 h. As seen in Figure 4e, the variation in the signal intensities on T₁-weighted and T₂-weighted images most likely reflected the inhomogeneities of tumors, such as tumor vessel, cell density, necrotic core, or susceptibility effects between tissues. Although calculated T₁ or T₂ maps could clearly identify those substantial changes in T₁ or T₂ values in the ROIs compared to the surrounding tissues, the quantification of the concentration of accumulated Gd–DB-HNTs within the tumors from T₁ or T₂ measurement is inexact, possibly due to the lack of correlation between the number of the particles and either T₁ or T₂ value, especially when monitoring tumor uptake overtime. To obtain the reproducible and dependable results for signal intensity measurement, we manually drew ROIs covering the whole tumor volume on both T₁- and T₂-weighted images. According to the quantification analysis of the MR signal in the tumors, there was a sharp increase (36%) in T₁-weighted signal intensity after 4 h post-injection, while the T₂-weighted signal intensity was decreased to 72% of initial scans (Figure 4f). These results indicated that Gd–DB-HNTs provided both positive T₁ and negative T₂ contrast enhancement for MR tumor imaging. It is worth noting that, when the T₁ and T₂ contrast intensities are comparable, the dual-contrast effect probes could provide optimal contrasts on both T₁- and T₂-weighted images because of their synergistic combination of the two relaxation effects.

**CONCLUSIONS**

By fusing Au, Pt, and IONP nanocrystals together via solid-state interfaces, and selectively modifying distinct surfaces, we built hybrid nanotrimmers to combine both T₁ and T₂-weighted contrast agents together in a nanocomposite with desirable shape and size for dual MRI. We further manipulated the spatial arrangement of each component in order to control the distance between T₁

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and $T_2$ species to reduce the magnetic coupling and synergistically enhance both $T_1/T_2$ contrast effects. The novel nanostructures having both positive and negative contrast effects can be potentially used as molecular probes for tumor diagnostic and therapeutic applications with excellent accuracy and reliability.

**METHODS**

**Materials.** Hydrogen tetrachloroaurate(III) (HAuCl₄) and platinum(II) 2,4-pentanedionate (Pt(acac)₂) were ordered from Strem Chemicals, Inc. N-Hydroxy succinimide (NHS), N-hydroxy sulfo succinimide (sulfo-NHS), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Pierce Biotechnology. Iron pentacarbonyl (Fe(CO)₅), oleic acid (technical grade 90%), oleylamine (technical grade 90%), 1-octadecene (technical grade 90%), cystamine, and methoxy-PEG glycol (mPEG 5000) were ordered from Sigma-Aldrich. The metal chelator, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (N-hydroxy succinimide ester), DOTA-NHS-ester, was obtained from Macrocyclics. Unless otherwise mentioned, all other chemicals were purchased from Sigma/Aldrich. All solvents and chemicals were used as received. The radioisotope ⁶⁴Cu was provided by the Department of Medical Physics, University of Wisconsin at Madison (Madison, WI). Dotarem (GD-DOTA) was purchased from Guerbet, Inc.

**Characterizations.** Mass spectra of synthetic polymers were recorded by a time-of-flight (TOF) mass spectrometer (AB SCIEX TOF/TOF 5800, Applied Biosystems) equipped with a matrix-assisted laser desorption ionization (MALDI) source. A CRC-15R PET dose calibrator (Capintec Inc., Ramsey, NJ) was used for all radioactivity measurements. Graphical image analysis was performed using ImageJ. The elemental analyses were performed using inductively coupled plasma mass spectrometer (ICP-MS). The NPs were suspended in freshly prepared aqua regia (trace metal grade 70% nitric acid HNO₃/36% hydrochloric acid HCl (Fisher Scientific), 1:3 v/v) and heated until completely dissolved, and then diluted up to 8 mL with double-distilled water. The NPs were precipitated by adding cold ethyl ether and purified by chromatography to afford product mPEG-5000-COOH as a white powder.

**Synthesis of Cystamine – DOTA₂.** The cystamine dihydrochloride (1.5 mg, 6.5 μmol) was dissolved in 0.2 mL of DMF containing 5 μL of N,N-diisopropylamine. Ten milligrams of DOTA – NHS–ester in 50 μL of DMF was added to the above solution. The resultant mixture was stirred for 4 h at room temperature and the completion of the reaction was monitored by Kaiser test and mass spectra. The final product was identified by MALDI-TOF (Supporting Information Figure S9).

**Synthesis of Methoxy polyethylene Glycol 5000 Acid (mPEG-5000-COOH).** To a solution of methoxy polyethylene glycol 5000 (5 g, 1 mmol) in 30 mL of dry THF was slowly added sodium hydride (60%, 0.2 g, 5 mmol) at room temperature. The solution was stirred for 1 h at room temperature, and then ethyl bromoacetate (1.11 mL, 10 mmol) was added dropwise. The mixture was stirred at 50 °C for 24 h. The solution was filtered and concentrated in vacuum. The residue was hydrolyzed with 20 mL of 1 M NaOH/methanol (1:1, v/v) for 6 h at room temperature. The resultant solution was acidified with 1 M HCl to pH 1.0, and the solvents were then removed under reduced pressure. The residue was dissolved in 10 mL of DCM and dried out in the presence of anhydrous sodium sulfate. The crude product was precipitated by adding cold ethyl ether and purified by chromatography to afford product mPEG-5000-COOH as a white powder.

**Surface Modification of Various HNTs.** Two milligrams of EDAC and 1.5 mg of NHS were added to the solution of the mPEG-5000-COOH (50 mg) in 2 mL of chloroform. The mixture was stirred at room temperature for 30 min, and 2 mg of dopamine in 1 mL of DMF with 2 mg of anhydrous sodium carbonate was then added. The resultant mixture was continuously stirred for 2 h at room temperature. Ten milligrams of dumbbell HNTs (HNTs, DB-HNTs, or XDB-HNTs) in 1 mL of chloroform was added to the above mixture with vigorous stirring. The resulting mixture was stirred at room temperature for 24 h. The PEGylated nanoparticles were precipitated by adding 15 mL of hexane, and collected by centrifugation. After a wash with hexane, the PEGylated HNTs were redissolved in 2 mL of chloroform. Ten milligrams of as-synthesized cystamine – DOTA₂ in 1 mL of DMF was added to the solution. The resultant mixture was vigorously stirred for 24 h at room temperature. The modified HNTs were precipitated by adding 15 mL of hexane, and collected by centrifugation. The washing process was repeated twice. The NPs were dried under the nitrogen flow and redissolved in water. The NPs were purified by dialysis tubing (MWCO = 12 kDa). After a pass through 0.22 μm filter, the resultant NPs were concentrated using a spin filter (MWCO = 30 kDa). The concentrations of Cu, Pt, and Fe were determined by ICP – MS analysis. The same procedure was used to modify Au – IONP and HNTs.

**TEM Analysis.** The TEM images were recorded with a FEI Tecnai G2 F20 X-TWIN transmission electron microscope operating at 200 kV. The HNTs and DB-HNTs were deposited and dried on copper grids covered with a Formvar/carbon support film, followed by plasma cleaning. The washing process was repeated twice and the final products were stored in hexanes (5 mL) in the presence of 0.02 mL of oleylamine.

**Synthesis of Cystamine – DOTA₂.** The cystamine dihydrochloride (1.5 mg, 6.5 μmol) was dissolved in 0.2 mL of DMF containing 5 μL of N,N-diisopropylamine. Ten milligrams of DOTA – NHS–ester in 50 μL of DMF was added to the above solution. The resultant mixture was stirred for 4 h at room temperature and the completion of the reaction was monitored by Kaiser test and mass spectra. The final product was identified by MALDI-TOF (Supporting Information Figure S9).

**Synthesis of Methoxy polyethylene Glycol 5000 Acid (mPEG-5000-COOH).** To a solution of methoxy polyethylene glycol 5000 (5 g, 1 mmol) in 30 mL of dry THF was slowly added sodium hydride (60%, 0.2 g, 5 mmol) at room temperature. The solution was stirred for 1 h at room temperature, and then ethyl bromoacetate (1.11 mL, 10 mmol) was added dropwise. The mixture was stirred at 50 °C for 24 h. The solution was filtered and concentrated in vacuum. The residue was hydrolyzed with 20 mL of 1 M NaOH/methanol (1:1, v/v) for 6 h at room temperature. The resultant solution was acidified with 1 M HCl to pH 1.0, and the solvents were then removed under reduced pressure. The residue was dissolved in 10 mL of DCM and dried out in the presence of anhydrous sodium sulfate. The crude product was precipitated by adding cold ethyl ether and purified by chromatography to afford product mPEG-5000-COOH as a white powder.

**Surface Modification of Various HNTs.** Two milligrams of EDAC and 1.5 mg of NHS were added to the solution of the mPEG-5000-COOH (50 mg) in 2 mL of chloroform. The mixture was stirred at room temperature for 30 min, and 2 mg of dopamine in 1 mL of DMF with 2 mg of anhydrous sodium carbonate was then added. The resultant mixture was continuously stirred for 2 h at room temperature. Ten milligrams of dumbbell HNTs (HNTs, DB-HNTs, or XDB-HNTs) in 1 mL of chloroform was added to the above mixture with vigorous stirring. The resulting mixture was stirred at room temperature for 24 h. The PEGylated nanoparticles were precipitated by adding 15 mL of hexane, and collected by centrifugation. After a wash with hexane, the PEGylated HNTs were redissolved in 2 mL of chloroform. Ten milligrams of as-synthesized cystamine – DOTA₂ in 1 mL of DMF was added to the solution. The resultant mixture was vigorously stirred for 24 h at room temperature. The modified HNTs were precipitated by adding 15 mL of hexane, and collected by centrifugation. The washing process was repeated twice. The NPs were dried under the nitrogen flow and redissolved in water. The NPs were purified by dialysis tubing (MWCO = 12 kDa). After a pass through 0.22 μm filter, the resultant NPs were concentrated using a spin filter (MWCO = 30 kDa). The concentrations of Au, Pt, and Fe were determined by ICP – MS analysis. The same procedure was used to modify Au – IONP and HNTs.

**TEM Analysis.** The TEM images were recorded with a FEI Tecnai G2 F20 X-TWIN transmission electron microscope operating at 200 kV. The HNTs and DB-HNTs were deposited and dried on copper grids covered with a Formvar/carbon support film, followed by plasma cleaning. The washing process was repeated twice. The NPs were dried under the nitrogen flow and redissolved in water. The NPs were purified by dialysis tubing (MWCO = 12 kDa). After a pass through 0.22 μm filter, the resultant NPs were concentrated using a spin filter (MWCO = 30 kDa). The concentrations of Au, Pt, and Fe were determined by ICP – MS analysis. The same procedure was used to modify Au – IONP and HNTs.

**Stability Test.** The colloidal stability of DB-HNTs in aqueous solution was studied by dynamic light scattering (Malvern Zeta Sizer Nano S-90 DLS instrument). The refractive indexes of gold
and platinum were adapted from the Malvern Reference manual (MANO396 Issue 1.0). The refractive index of 0.30 was adapted for gold, while the refractive index of platinum was 4.5. The stability of functionalized DB-HNTs was validated by measuring the dynamic light scattering (DLS) of functionalized DB-HNTs in serum using DLS. The DB-HNTs were added to mouse serum in phosphate buffered saline (PBS) at a concentration of 4 nM and then incubated at 37 °C for 24 h. The aliquots were analyzed at 0, 4, 8, 24, and 48 h post-incubation by DLS. Each aliquot was filtered using a spin filter (MWCO = 30 kDa), and further washed with water 3 times. The eluent was collected and analyzed by ICP-MS for trace amount of Fe, Pt, Au and Gd.

**Cytotoxicity Assay.** Cytotoxicity of Gd–DB-HNTs was evaluated by using a cell viability assay (MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich, St. Louis, MO)). Briefly, NIH-3T3 cells were plated into a 96-well plate at a density of 6 × 10^3 cells/well in DMEM with 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂, and 24 h later, the cells were incubated with Gd–DB-HNTs with different concentrations carried out according to the manufacturer's instruction. After treatment, MTT compound (5 mg/mL in PBS) was diluted 1:100 with DMEM medium into each well, incubated at 37 °C overnight for the chelating reaction. The unbound Gd ions were removed by using a PD-10 column. The eluent was concentrated using a spin filter (MWCO = 30 kDa), and further washed with water 3 times. The concentrations of Au, Pt, Gd, and Fe were determined by using an ICP-MS system.

**Relaxivity Measurement.** A series of concentrations of four types of Gd-incorporated NPs (0, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 mM Fe) corresponding Gd concentrations are determined by measuring the molar ratio of Gd to Fe. All experiments were performed in triplicate. Differences in the distribution of Gd were considered statistically significant when p < 0.05.

**In Vivo Small-Animal PET/CT of Tumor Mice.** 64Cu-DOTA-HNTs or 64Cu-DOTA-NPs (100 μCi in 150 μL PBS) were injected i.v. into the HT-29 tumor-bearing mice at a dose of 5 mg Fe/kg of mouse weight. In vivo small-animal PET/CT imaging was performed 60–90 min post-injection using a Siemens-Inveon PET/CT (Siemens Medical Solution) according to our previous publication.51,52 PET scans were obtained at 4 and 24 h post-injection (pl). Mice were anesthetized with isoflurane (5% for induction and 2% for maintenance in 100% O₂). With the help of a laser beam attached to the scanner, mice were placed in the prone position and near the center of the field of view (FOV) of the scanner and 5 min static scans were obtained. All the small animal PET images were reconstructed and reviewed by the Inveon Research Workplace (IRW). Region of interests (ROIs) for coronal and transaxial slices were drawn over the tumor on decay-corrected whole-body coronal images. Values in 3 to 10 adjacent slices (depending on the size of the tissue or organ) were averaged to obtain a reproducible value of radioactivity concentration. The maximum counts per pixel per minute were determined from the ROIs and were converted to counts per milliliter per minute using a calibration constant. ROIs were then converted to counts per gram per minute based on the assumption of a tissue density of 1 g/mL, and image ROI-derived percentage of the injected radioactive dose per gram of tissue (%ID/g) values were determined by dividing counts per gram per minute by injected dose. No attenuation correction was performed.

**MRI.** Mice bearing HT-29 tumors were anesthetized with 2% isoflurane in oxygen and placed with prone position. MRI was performed using the same instrument, protocols, and conditions as in the phantom MRI study. DOTA-DB-HNTs were injected via tail vein into the HT-29 tumor-bearing mice at a...
dose of 10 mg Fe/kg of mouse weight (n = 3). T2-weighted fast spin–echo MR images were acquired on a 7-T small animal MRI system (GE Healthcare) under the following parameters: repetition time (TE) = 3000 ms, TR = 40 ms, echo train length = 8, FOV = 4 × 4 cm², section thickness = 1 mm, flip angle = 90°. MR images were acquired in both transverse and coronal direction post-injection and at 4 and 48 h after injection. Transversal and coronal MR images were acquired and the signal intensities were measured in defined ROIs using OsiriX imaging software (OsiriX version 3.2; Apple Computer). The NIH standard was used for tumor imaging processing. The ROI analysis was performed according to the previous publications.23

Statistical Method. Measurement data are expressed as the mean ± SD. Statistical analyses of the data were performed using Prism (GraphPad Software). A p-value of less than 0.05 was considered significant.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: TEM images of Pt–IONPs, HRTEM images of HNTs, mass spectra of mPEG-5000-COOH, hydrodynamic sizes of functional Au–IONPs, HNTs, and DB-HNTs and XDB-HNTs, zeta potentials of Gd incorporated DB-HNTs, and stability test of Gd–DB-HNTs. This material is available free of charge via the Internet at http://pubs.acs.org.

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