Mixed lanthanide oxide nanoparticles as dual imaging agent in biomedicine

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There is no doubt that the molecular imaging is an extremely important technique in diagnosing diseases. Dual imaging is emerging as a step forward in molecular imaging technique because it can provide us with more information useful for diagnosing diseases than single imaging. Therefore, diverse dual imaging modalities should be developed. Molecular imaging generally relies on imaging agents. Mixed lanthanide oxide nanoparticles could be valuable materials for dual magnetic resonance imaging (MRI)-fluorescent imaging (FI) because they have both excellent and diverse magnetic and fluorescent properties useful for dual MRI-FI, depending on lanthanide ions used. Since they are mixed nanoparticles, they are compact, robust, and stable, which is extremely useful for biomedical applications. They can be also easily synthesized with facile composition control. In this study, we explored three systems of ultrasmall mixed lanthanide (Dy/Eu, Ho/Eu, and Ho/Tb) oxide nanoparticles to demonstrate their usefulness as dual T2 MRI–FI agents.

There is a continuing research interest in mixed lanthanide oxide nanoparticles because they have both magnetic and fluorescent properties useful for dual imaging in biomedicine. By using different lanthanide ions in synthesis, a variety of mixed lanthanide oxide nanoparticles can be synthesized. These mixed nanoparticles generally have an advantage over core-shell11, hetero-junction12, and dye-coated nanoparticles13 because of their compactness, robustness, stability, and easy synthesis with composition control. In addition, several inherent magnetic and fluorescent properties of mixed lanthanide oxide nanoparticles are valuable in molecular imaging. First, their magnetic and fluorescent properties are not much affected by surface coating due to compactness of 4f-orbitals close to nucleus14. Second, their magnetic properties do not change much with particle diameter for the same reason14. Third, their fluorescent intensities enhance with decreasing particle diameter due to reduced excitation migration to quenching sites that are proportional to particle diameter15,16. Therefore, ultrasmall mixed lanthanide oxide nanoparticles will be extremely useful for dual magnetic resonance imaging (MRI)–fluorescent imaging (FI), as demonstrated in this study.

There is no doubt that dual imaging will play a vital role in diagnosing diseases in the near future. First, dual imaging can generally provide more information useful for diagnosing diseases than the single imaging does17,18. Injection doses can be reduced because a single dose of a dual imaging agent will cover both doses of single imaging agents. Regarding MRI-FI, MRI generally has a high spatial resolution of ~1 mm while FI, a very high sensitivity that is limited by that of an optical device19,20. Therefore, a dual MRI-FI modality will allow us to detect and diagnose diseases very sensitively and in a high spatial resolution21.

This study deals with T2 MRI-FI agents. While FI entirely relies on chemical agents, MRI is greatly improved using contrast agents through contrast enhancements. Here, it is worth to mention that a good MRI contrast agent should satisfy that it should be non-toxic, completely excreted from a body through renal system after some time after intravenous injection, should have a large r1 value and r2/r1 ratio close to one in case of a T1 MRI contrast agent, and a large r2 value in case of a T2 MRI contrast agent to show high contrast MR images at a standard injection dose. T2 MRI contrast agents can be made by nanoparticles but not by molecules because large magnetic moments at room temperature are needed in accelerating transverse water proton relaxation that is induced by fluctuation of local magnetic field generated by nanoparticles22-24. Although lanthanide oxide nanoparticles have only decent magnetic moments at room temperature, they are useful at high MR fields25,26. Their magnetic moments are decent even at ultrasmall particle diameters, extremely useful for in vivo applications...
moments while Eu and Tb strongly fluoresce in visible region, on lanthanide elements. Their fluorescent properties are similar to quantum dots (QDs) except for their weaker fluorescent intensities. That is, they have narrow band widths useful for multiplex imaging and high photostabilities like QDs, which are superior to dyes because dyes have wide band widths and poor photostabilities (i.e., subject to photobleaching and photodecomposition after multiple uses). Lower fluorescent intensities of lanthanide oxide nanoparticles than those of QDs and dyes can be partly overcome through the imaging depth increase using up-conversion compositions.

As summarized in Table 1, Dy, Ho, and Tb have large magnetic moments while Eu and Tb strongly fluoresce in visible region. Although not listed in Table 1, Gd and Er also have large magnetic moments comparable to those of the above three elements. Therefore, ultrasmall mixed lanthanide oxide nanoparticles composed of these elements will be useful for both MRI and FI. Their basic applications to biomedical imagings are schematically represented in Fig. 1. In this study, we explored three systems of ultrasmall mixed lanthanide (Dy/Eu, Ho/Eu, and Ho/Tb) oxide nanoparticles to demonstrate their usefulness as T2 MRI-FI agents by investigating their in vivo T2 MR images and in vitro fluorescent confocal images.

**Results**

Composition, particle diameter, and hydrodynamic diameter. Compositions in DyxEuO3, HoxEuO3, and Ho1-xTbO3 nanoparticles were estimated to be (x, y) = (1.5 ± 0.1, 0.5 ± 0.1), (1.6 ± 0.1, 0.4 ± 0.1), and (1.1 ± 0.1, 0.9 ± 0.1), respectively. Pictures of well-dispersed aqueous sample solutions were inserted in Fig. 2B. HVEM images are also provided for Ho1-xTbO3 nanoparticles with an improved lattice resolution. More HRTEM and HVEM images are provided in Supplementary data.

Particle diameters were estimated using two electron microscopes (EMs) with different acceleration voltages. D-glucuronic acid coated nanoparticles are hydrophilic because they are coated with hydrophilic D-glucuronic acids and thus were not monodispersed but mostly aggregated in EM images because they were dispersed on hydrophobic carbon film on copper grid. This is opposite to hydrophobic nanoparticles synthesized in organic solvents that can be monodispersed on carbon film. From high resolution transmission electron microscopy (HRTEM) and high voltage electron microscope (HVEM) images in Fig. 2A, the average core particle diameters (davgs) of D-glucuronic acid coated ultrasmall Dy1.5Eu0.5O3, Ho1.6Eu0.4O3, and Ho1.1Tb0.9O3 nanoparticles were estimated to be 2.3 ± 0.1, 2.1 ± 0.1, and 2.5 ± 0.1 nm, respectively, using log-normal function fits to observed particle diameter distributions as shown in Fig. 2B. HVEM images are also provided for Ho1.1Tb0.9O3 nanoparticles with an improved lattice resolution. More HRTEM and HVEM images are provided in Supplementary data.

Low resolution TEM and elemental map images are shown in Fig. 2C. Since nanoparticles are so ultrasmall that an elemental map image of each individual nanoparticle could not be obtained. Therefore, we provided overall elemental map images of nanoparticles. As expected, identical elemental map images for all elements in each sample were observed, implying that nanoparticles are mixed (or alloy) Ln2O3 nanocrystals but not physical mixtures of Ln2O3 nanoparticles. The doping-induced particle size tuning effect as observed in up-conversion NaYF4:Yb/Er nanocrystals may not be observed in the present mixed nanoparticles. This is because of similar ionic radii and similar reaction properties between lanthanide ions.

From dynamic light scattering (DLS) patterns in Fig. 2D, the average hydrodynamic diameters (davgs) of D-glucuronic acid coated ultrasmall Dy1.5Eu0.5O3, Ho1.6Eu0.4O3, and Ho1.1Tb0.9O3 nanoparticles were estimated to be 6.7 ± 0.1, 6.4 ± 0.1, and 7.6 ± 0.1 nm, respectively. Pictures of well-dispersed aqueous sample solutions are also inserted in Fig. 2D.

| Ln3+ | Ground state configuration | Fluorescent color | Theoretical magnetic moment (μB) | Observed magnetic moment (μB) | Applicable imaging | Ref. |
|------|----------------------------|-------------------|---------------------------------|-------------------------------|-------------------|-----|
| Eu3+ | 7F0 (4f7)                  | Red               | 0                               | 3.3–3.5                       | FL                | 14,28|
| Tb3+ | 7F0 (4f6)                  | Green             | 9.72                            | 9.5–9.8                        | T2 MRI, FL        | 14,28|
| Dy3+ | 6H1/2 (4f6)                | Green             | 10.65                           | 10.4–10.6                      | T2 MRI            | 14,28|
| Ho3+ | 5I8 (4f10)                 | Red               | 10.6                            | 10.4–10.7                      | T2 MRI            | 14,28|

**Figure 1 | A schematic diagram showing possible applications of ultrasmall mixed lanthanide oxide nanoparticles in the area of MRI and FI.**
We also investigated crystal structures of powder samples of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles by measuring X-ray diffraction (XRD) patterns (Supplementary data). As-prepared powder samples showed very broad patterns as observed in unmixed nanoparticles whereas powder samples after thermal treatment with a thermogravimetric analyzer (TGA) showed sharp peaks, all corresponding to a highly crystallized cubic Ln$_2$O$_3$ due to particle size growth during TGA treatment.

Surface coating. Nanoparticles should be coated with water-soluble and biocompatible ligands for biomedical applications. D-glucuronic acid was used in this study. The surface coating was investigated by recording Fourier transform-infrared (FT-IR) absorption spectra of powder samples of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles in KBr as shown in Fig. 3A. A FT-IR absorption spectrum of a free D-glucuronic acid was also recorded as a reference as shown in Fig. 3A. Stretching
frequencies characteristic of C–H at 2910 cm$^{-1}$, C=O at 1610 cm$^{-1}$, and C–O at 1070 cm$^{-1}$ of D-glucuronic acid in powder samples confirmed the surface coating. Furthermore, the C=O stretch was red-shifted by $\sim 100$ cm$^{-1}$ from that ($= 1710$ cm$^{-1}$) of a free D-glucuronic acid, confirming that its –COOH group was bonded to a nanoparticle. This red shift has been observed in various metal oxide nanoparticles coated with ligands with –COOH group$^{39-43}$, supporting our results. TGA curves were also recorded to estimate the amount of surface coating with D-glucuronic acid as shown in Fig. 3B. The surface coating weight percentages were estimated to be 46.0% for Dy$_{1.5}$Eu$_{0.5}$O$_3$, 46.0% for Ho$_{1.6}$Eu$_{0.4}$O$_3$, and 59.0% for Ho$_{1.1}$Tb$_{0.9}$O$_3$ nanoparticles from the mass drop in the corresponding TGA curves. 5.0% water desorption between room temperature and $\sim 110$ °C was taken into account in all of these values. The remaining corresponded to net masses of ultrasmall Dy$_{1.5}$Eu$_{0.5}$O$_3$, Ho$_{1.6}$Eu$_{0.4}$O$_3$, and Ho$_{1.1}$Tb$_{0.9}$O$_3$ nanoparticles in powder samples. The surface coating weight percentages were converted into average grafting densities corresponding to the average number of D-glucuronic acids coated per unit surface area of a nanoparticle$^{44}$. They were estimated to be 9.4, 8.3, and 17.3 nm$^{-2}$ for D-glucuronic acid coated ultrasmall Dy$_{1.5}$Eu$_{0.5}$O$_3$, Ho$_{1.6}$Eu$_{0.4}$O$_3$, and Ho$_{1.1}$Tb$_{0.9}$O$_3$ nanoparticles, respectively. All of these values were larger than 1.0, showing sufficient surface coating of nanoparticles with D-glucuronic acid$^{44}$.

Magnetic properties. Magnetic properties of ultrasmall mixed lanthanide oxide nanoparticles were investigated by recording both magnetization (M) versus applied field (H) (i.e., M–H) curves at $T = 5$ tesla and $T = 5$ and 300 K (Fig. 4A) and zero-field-cooled (ZFC) M–T curves at $H = 0$ or 100 Oe of three powder samples of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles. All of these values were larger than 1.0, showing sufficient surface coating of nanoparticles with D-glucuronic acid$^{44}$.
oersted (Oe) (Fig. 4B). Here, magnetizations in Fig. 4 corresponded to net magnetizations of ultrasmall mixed lanthanide oxide nanoparticles in powder samples because they were mass-corrected with net masses of nanoparticles in powder samples as described in experimental section. Both coercivities and remanences in M–H curves were zero (i.e., no hysteresis). This lack of hysteresis and no magnetic transition down to T = 5 K in all M–T curves showed that all ultrasmall mixed lanthanide oxide nanoparticles were paramagnetic down to 5 K, consistent with previous reports.\textsuperscript{43–48} Therefore, magnetizations in all samples arise from electron magnetic moments of Dy\textsuperscript{3+} (‘‘H\textsubscript{15/2}’’), Ho\textsuperscript{3+} (‘‘I\textsubscript{8}’’), and Tb\textsuperscript{3+} (‘‘F\textsubscript{6}’’) (see Table 1). Net magnetizations of ultrasmall mixed lanthanide oxide nanoparticles estimated from M–H curves at T = 5 and 300 K were plotted in Fig. 4C. As shown in Fig. 4C, net magnetizations at T = 300 K are decent due to unsaturated magnetizations at room temperature, whereas those at T = 5 K are large due to nearly saturated magnetizations. Magnetizations at room temperature are important for water proton relaxation because the water proton relaxivity (r) is proportional to the square of magnetization\textsuperscript{22–24} and thus, should be as large as possible.

**Relaxivities and map images.** Both inverse longitudinal (1/T\textsubscript{1}) and transverse (1/T\textsubscript{2}) relaxation times measured at 1.5 tesla and 22 °C were plotted as a function of Ln (Ln = Dy or Ho) concentration in Fig. 5A and combined Ln\textsubscript{1}&Ln\textsubscript{2} (Ln\textsubscript{1} = Dy or Ho, Ln\textsubscript{2} = Eu or Tb) concentration in Fig. 5B. Longitudinal (r\textsubscript{1}) and transverse (r\textsubscript{2}) water proton relaxivities were estimated from the corresponding slopes (i.e., from the equation 1/T\textsubscript{i} = r\textsubscript{i} C + I (i = 1 or 2) in which C is the concentration and I, the intercept) and plotted in Fig. 5C. As shown in Fig. 5C, r\textsubscript{1} values were negligible in all samples (i.e., less than 1.0 s\textsuperscript{–1}mM\textsuperscript{–1}) because of orbital contribution to magnetic moments in Dy\textsuperscript{3+}, Ho\textsuperscript{3+}, and Tb\textsuperscript{3+}. Note that only a pure electron spin magnetic moment as in Gd\textsuperscript{3+} can strongly induce a longitudinal water proton relaxation\textsuperscript{22,23}. In fact, gadolinium oxide and gadolinium-based nanoparticles have shown very large r\textsubscript{1} values\textsuperscript{49–52}. However, r\textsubscript{2} values, depending on total magnetic moment of contrast agents\textsuperscript{44},

![Figure 5](www.nature.com/scientificreports)

Figure 5 | Plots of 1/T\textsubscript{1} and 1/T\textsubscript{2} as a function of (A) Dy (or Ho) and (B) combined Dy&Eu (or Ho&Eu or Ho&Tb) concentrations for aqueous sample solutions of D-glucuronic acid coated ultrasmall (I) Dy\textsubscript{1.5}Eu\textsubscript{0.5}O\textsubscript{3}, (II) Ho\textsubscript{1.6}Eu\textsubscript{0.4}O\textsubscript{3}, and (III) Ho\textsubscript{1.1}Tb\textsubscript{0.9}O\textsubscript{3} nanoparticles. (C) Plot of r\textsubscript{1} and r\textsubscript{2} values of three sample solutions estimated from single (‘‘s’’) and combined (‘‘c’’) concentration plots in (A) and (B), respectively. (D) R\textsubscript{1} and R\textsubscript{2} map images of three aqueous sample solutions (Ln = Dy in (I), and Ho in both (II) and (III)). (E) Plot of r\textsubscript{1} and r\textsubscript{2} values of various nanoparticles estimated at 1.5 tesla and 22 °C: D-glucuronic coated Gd\textsubscript{3}O\textsubscript{3} nanoparticle (d\textsubscript{avg} = 1.0 nm)\textsuperscript{50}, PEG-diacid coated Fe\textsubscript{3}O\textsubscript{4} nanoparticle (d\textsubscript{avg} = 1.7 nm)\textsuperscript{58}, D-glucuronic acid coated MnO nanoparticle (d\textsubscript{avg} = 2.5 nm)\textsuperscript{59}, and three kinds of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles studied here.
were decent in all samples because of decent magnetizations of nanoparticles at room temperature. As expected, $r_1$ and $r_2$ values were comparable to those of unmixed lanthanide oxide nanoparticles\(^3\,^{35}\,^{36}\). Both $r_1$ and $r_2$ values became reduced when they were estimated from combined concentration plots as shown in Fig. 5C. This simply come from concentration effect. $r_2$ values of combined concentration plots were reduced by $\sim 15\, s^{-1}\, mM^{-1}$ for L$_{n_2}$ = Tb and $\sim 30\, s^{-1}\, mM^{-1}$ for L$_{n_2}$ = Eu from the corresponding single concentration plots because Tb$^{3+}$ has a slightly smaller magnetic moment than Ho$^{3+}$ whereas Eu$^{3+}$, a lot smaller magnetic moment than both Dy$^{3+}$ and Ho$^{3+}$ (see Table 1).

As expected from negligible $r_1$ and decent $r_2$ values, negligible dose-dependent contrast enhancements in $R_1$ map images and appreciable dose-dependent contrast enhancements in $R_2$ map images were observed in all samples as shown in Fig. 5D. These results suggest that the present ultrasmall mixed lanthanide oxide nanoparticles are potential $T_2$ MRI contrast agents at high MR fields because their $r_2$ values and therefore their contrast enhancements in $R_2$ map images will further increase with MR field because $r_2$ is proportional to the square of MR field as mentioned before\(^22\,^{24}\).

It will be valuable to compare $r_1$ and $r_2$ values of the present nanoparticles with those of other nanoparticles at similar particle diameters. As shown in Fig. 5E, gadolinium oxide nanoparticle showed the largest $r_1$ value because of a large electron spin magnetic moment ($S = 7/2$) of Gd$^{3+}$ as mentioned before\(^6\). Both Fe$_3$O$_4$ and MnO nanoparticles showed the next largest $r_1$ values because both Fe$^{3+}$ and Mn$^{2+}$ have $S = 5/2\,^{69}\). The present nanoparticles, however, showed negligible $r_1$ values because Dy$^{3+}$, Ho$^{3+}$, and Tb$^{3+}$ have both electron orbital (L) and spin (S) magnetic moments as mentioned before, but the largest $r_2$ values due to their decent magnetic moments at room temperature. Therefore, as $T_2$ MRI contrast agents, the present nanoparticles are superior to other nanoparticles at ultrasmall particle diameters. This is important because ultrasmall nanoparticles are useful for in vivo applications because they can be excreted through renal system\(^7\).

**Fluorescent properties.** Fluorescent properties of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles in ethanol were investigated by recording photoluminescence (PL) spectra as shown in Fig. 6. PL spectra of precursor ions in ethanol were also recorded as references as shown in Fig. 6. We observed constant emission intensities and identical PL spectra for repeated measurements, supporting good photostabilities of these nanoparticles.

As shown in Table 1 and Fig. 6, the main fluorescent lanthanide ions in sample solutions were Eu$^{3+}$ and Tb$^{3+}$. The other ions negligibly contributed to PL spectra. Therefore, it was expected that PL spectra of sample solutions would resemble those of Eu$^{3+}$ or Tb$^{3+}$ precursor ions. However, PL spectra of sample solutions were quite different from those of these ions in two respects. First, PL spectra of sample solutions were generally broad. Second, new peaks (assigned as $^{44}\,^{48}$) appeared with position and/or intensity quite different from those of precursor ions. Inserted at the top are photographs of the corresponding sample solutions after UV irradiation ($\lambda_{ex} = 254\, nm$ for samples (I) and (II), and 365 nm for sample (III), solvent = ethanol). The emission assignments in precursor ions are following: Eu$^{3+}$ ($a$: $\mathrm{D}_{0} \rightarrow \mathrm{F}_{1}$ (593 nm), $b$: $\mathrm{D}_{0} \rightarrow \mathrm{F}_{2}$ (618 nm), $c$: $\mathrm{D}_{0} \rightarrow \mathrm{F}_{3}$ (654 nm)); Dy$^{3+}$ ($d$: $\mathrm{F}_{22} \rightarrow \mathrm{H}_{15/2}$ (480 nm)); Tb$^{3+}$ ($e$: $\mathrm{D}_{4} \rightarrow \mathrm{F}_{6}$ (491 nm), $f$: $\mathrm{D}_{4} \rightarrow \mathrm{F}_{2}$ (545 nm), $g$: $\mathrm{D}_{4} \rightarrow \mathrm{F}_{4}$ (384 nm)), which are consistent with reported values\(^6\).

Figure 6 | PL spectra of sample solutions of D-glucuronic acid coated ultrasmall (I) Dy$_{1.5}$Eu$_{0.5}$O$_3$, (II) Ho$_{1.6}$Eu$_{0.4}$O$_3$, and (III) Ho$_{1.1}$Tb$_{0.9}$O$_3$ nanoparticles and four precursor ions ($\lambda_{ex} = 220\, nm$, solvent = ethanol). The $a$$^{44}$- $g$$^{48}$ indicates new peaks in nanoparticles with position and/or intensity quite different from those of precursor ions. Inserted at the top are photographs of the corresponding sample solutions after UV irradiation ($\lambda_{ex} = 254\, nm$ for samples (I) and (II), and 365 nm for sample (III), solvent = ethanol).

Quantum yields (QYs) of these nanoparticles in ethanol solution were estimated by using fluorescein as a standard ($\Phi = 0.95$)\(^\ast\) at $\lambda_{ex} = 220\, nm$. The $\Phi$ was 0.57 for ultrasmall Dy$_{1.5}$Eu$_{0.5}$O$_3$ nanoparticles, 0.43 for Ho$_{1.6}$Eu$_{0.4}$O$_3$ nanoparticles, and 0.48 for Ho$_{1.1}$Tb$_{0.9}$O$_3$ nanoparticles. These values are roughly consistent with those reported on lanthanide oxide nanoparticles by others\(^44\,^{50}\), but slightly higher likely due to their ultrasmall particle diameters because it was found that fluorescent intensity of lanthanide oxide nanoparticles become enhanced with decreasing particle diameter\(^14\).

**In vitro cytotoxicity results.** The toxicities of three aqueous solution samples of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles were investigated by measuring cytotoxicity up to 400 (or 500) $\mu$m combined concentrations using both DU145 and NCTC1469 cells as shown in Fig. 7. They were non-toxic and thus, used for in vivo MR experiments.
In vivo 3 tesla T2 MR images. To measure in vivo 3 tesla T2 MR images, aqueous sample solutions of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles were injected into a mouse tail vein. As shown in Fig. 8, decent negative contrast enhancements in both liver and kidneys were observed one hour after intravenous injection. Therefore, D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles clearly functioned as T2 MRI contrast agents. Stronger negative contrast enhancements will be observed at higher MR fields because $r_2$ is proportional to the square of applied MR field as mentioned before22–24, implying that these nanoparticles are potential T2 MRI contrast agents at high MR fields. Furthermore, antibodies, peptides or targeting molecules will increase the targeting ability of nanoparticles and as a result, provide the improved contrasts in MR images. More 3 tesla T2 MR images in a mouse are provided in Supplementary data.

In vitro fluorescent confocal cellular images. To measure in vitro fluorescent confocal cellular images, aqueous sample solutions were treated to DU145 cells. Control DU145 cells to which no sample solution was treated were also prepared in the same condition. As shown at the top images in Figs. 9A to C, cell nuclei tinted pale blue at $\lambda_{ex} = 405$ nm because they were stained with 4',6 diamidino-2-phenylindole (DAPI). As shown at the bottom image in Fig. 9A, no fluorescence was observed in control cells at $\lambda_{ex} = 488$ nm. However, red fluorescence with treatment with Dy1.5Eu0.5O3 nanoparticles (the bottom image in Fig. 9B) and green fluorescence with treatment with Ho1.1Tb0.9O3 nanoparticles (the bottom image in Fig. 9C) were observed at $\lambda_{ex} = 488$ nm. These results demonstrated that ultrasmall mixed lanthanide oxide nanoparticles functioned sensitively as FI agents. More fluorescent confocal cellular images are provided in Supplementary data.

Discussion

In this study we demonstrated that ultrasmall mixed lanthanide oxide nanoparticles had excellent dual imaging properties. We used three systems of ultrasmall mixed lanthanide (Dy/Eu, Ho/Eu, and Ho/Tb) oxide nanoparticles to demonstrate this. The three main findings are following: (1) ultrasmall mixed lanthanide oxide nanoparticles are compact, robust, and stable, which is extremely useful for biomedical applications. They can be also easily synthesized with composition control. (2) They showed decent magnetizations at room temperature and as a result, appreciable $r_2$ values. Decent negative contrast enhancements in 3 tesla T2 MR images in a mouse were observed, clearly demonstrating their capability as T2 MRI contrast agents. Since $r_2$ is proportional to the square of MR field, these nanoparticles will be extremely useful as T2 MRI contrast agents at high MR fields. (3) They strongly showed fluorescent confocal images in DU145 cells, clearly demonstrating their capability as FI agents. Therefore, ultrasmall mixed lanthanide oxide nanoparticles are potential dual T2 MRI-FI agents that will be extremely useful for biomedical applications. It is worth to mention that although not studied here, we expect that ultrasmall mixed Dy/Tb

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Figure 7 | In vitro cytotoxicity results of aqueous sample solutions of D-glucuronic acid coated ultrasmall (A) Dy1.5Eu0.5O3, (B) Ho1.6Eu0.4O3, and (C) Ho1.1Tb0.9O3 nanoparticles using DU145 and NCTC1469 cells.

Figure 8 | 3 tesla T2 MR images in a mouse: (A) axial views of liver and (B) coronal views of kidneys as indicated with arrows, respectively, before and one hour after injection of an aqueous sample solution of D-glucuronic acid coated ultrasmall Ho1.6Eu0.4O3 nanoparticles into a mouse tail vein. MR images became darker after injection due to negative contrast enhancements by nanoparticles.
**Methods**

**Chemicals.** Dy(NO)₃·xH₂O (99.9%), Ho(NO₃)₃·5H₂O (99.9%), Eu(NO₃)₃·5H₂O (99.9%), Tb(NO₃)₃·5H₂O (99.9%), NaOH (> 99.9%), 50% H₂O₂ aqueous solution, triethylene glycol (99%), CH₃OH (99.9%), and D-glucuronic acid (99.99%) were purchased from Sigma-Aldrich and used as received. C₂H₅OH (99%) was purchased from Duksan (S. Korea) and used as received in washing nanoparticles. Triply distilled water to prepare aqueous sample solutions and the remaining triply distilled water. The sample solution concentration used to measure hydrodynamic diameters was ~0.1 mM Ln (Ln = Dy, Ho, Eu or Tb). An XRD spectrometer (Philips, XPERT PRO MRD) with an unfiltered CuKα radiation (λ = 1.54184 Å) was used to measure the crystal structure of powder samples of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles and TGA curves. TGA curves were recorded between room temperature and 900 °C under a nitrogen atmosphere. For surface coating, 5 mmol of D-glucuronic acid was added to the above nanoparticle solution that was magnetically stirred at 80 °C for 24 hours under atmospheric condition until the precursor salts were completely dissolved in triethylene glycol. The NaOH solution prepared in 5 mL of methanol was slowly added to the above precursor solution using a syringe. The mixture solution was magnetically stirred at 80 °C for 2 hours. Then 7.5 mL of 50% H₂O₂ aqueous solution was slowly added to the reaction solution using a syringe. After addition of H₂O₂, the mixture solution was magnetically stirred for additional 2 hours.

Figure 9 | Fluorescent confocal cellular images with λex = 405 nm (top images) and 488 nm (bottom images): (A) the control DU145 cells and the DU145 cells treated with aqueous sample solutions of D-glucuronic acid coated ultrasmall (B) Dy₁.₅Eu₀.₅O₃ and (C) Ho₁.₁Tb₀.₉O₃ nanoparticles.

Mixed lanthanide oxide nanoparticles are extremely valuable for in vivo applications because they are compact, robust, and stable as mentioned above. This is critical because after intravenous injection they should not be decomposed and their properties should not be changed. As mentioned before, their magnetic properties do not much depend on surface coating and particle diameter due to compactness of 4f-orbitals close to nucleus and their fluorescent intensities rather enhance with decreasing particle diameter due to reduced excitation migration to quenching sites that are proportional to particle diameter implying that they can be made ultrasmall without degrading their magnetic and fluorescent properties.

Note that ultrasmall nanoparticles can be excreted through renal system, which is pre-requisite to in vivo applications. Therefore, ultrasmall mixed lanthanide oxide nanoparticles will be extremely useful for MRI-FI dual agents, as demonstrated in this study. For instance, a dual MRI-FI modality will allow us to detect and diagnose diseases very sensitively through FI and in a high spatial resolution through MRI.

In summary, we synthesized ultrasmall mixed lanthanide (Ln) oxide nanoparticles in which one Ln is used for MR imaging and the other Ln, for fluorescent imaging. We explored three systems (Dy/Eu, Ho/Eu, and Ho/Tb) in which Dy, Ho, and Tb are used for MR imaging and Eu and Tb, for fluorescent imaging. They clearly showed negative contrast in 3 tesla T₂ MR images in a mouse and a very intense fluorescence in confocal images in DU145 cells, demonstrating their usefulness as MRI-FI dual agents.

**Characterizations.** An ICPAES (Thermo Jarrell Ash Co., IRIS/AP) was used to measure the concentration of Ln (Ln = Dy, Ho, Eu, and Tb) in aqueous sample solutions. Ultrasmall particle diameters were estimated using a HRTEM (JEOL, JEM-2100F, 200 kV acceleration voltage) and a HVEM (JEOL JEM-ARM 1300 S, 1.2 MeV acceleration voltage). A copper grid (PELCO No.160, TED PELLA, INC.) covered with an amorphous carbon membrane was placed onto a filter paper and a nanoparticle solution diluted in triply distilled water or ethanol was dropped over the copper grid using a micropipette. A DLS particle size analyzer (UPA-150, Microtrac) was used to measure the hydrodynamic diameters of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles dispersed in triply distilled water. The sample solution concentration used to measure hydrodynamic diameters was ~0.1 mM Ln (Ln = Dy, Ho, Eu or Tb). An XRD spectrometer (Philips, XPERT PRO MRD) with an unfiltered CuKα radiation (λ = 1.54184 Å) was used to measure the crystal structure of powder samples of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles. The scanning step was 0.033° and scan range in 2θ was 15–100°. A FT-IR absorption spectrometer (Mattson Instruments, Inc., Galaxy 7020A) was used to investigate the surface coating in ultrasmall mixed lanthanide oxide nanoparticles. To record FT-IR absorption spectra (400–4000 cm⁻¹), pellets of powder samples in KBr were prepared. A TGA (TA Instruments, SDT-Q 600) was used to estimate the amount of surface coating with D-glucuronic acid in powder samples from the mass drop in TGA curves. TGA curves were recorded between room temperature and 900 °C while air flowed. A superconducting quantum interference device (SQUID) magnetometer (Quantum Design, MPMS-7) was used to measure the magnetic properties of ultrasmall mixed lanthanide oxide nanoparticles. Both M-H curves (~5 ≤ H ≤ 50 mT) and 15 mmol of NaOH in 5 mL of methanol. Then NaOH solution was slowly added to the above precursor solution using a syringe. The reaction temperature was raised to 240 °C and maintained at that temperature for 24 hours with magnetic stirring. The product nanoparticle solution was cooled to 80 °C for surface coating. In case of mixed Ho/Tb oxide nanoparticles, 2.5 mmol of Ho(NO₃)₃·5H₂O and 2.5 mmol of Tb(NO₃)₃·5H₂O were added to 40 mL of triethylene glycol. The mixture solution was magnetically stirred until the precursor salts were completely dissolved in triethylene glycol. The NaOH solution prepared in 5 mL of methanol in a separate flask was slowly added to the above precursor solution using a syringe. After addition of H₂O₂, the reaction mixture was magnetically stirred for additional 2 hours.

For surface coating, 5 mmol of D-glucuronic acid was added to the above nanoparticle solutions that were magnetically stirred at 80 °C for 24 hours under atmospheric condition. The product solutions were cooled to room temperature and transferred to 1 L beakers containing 500 mL of triply distilled water (or ethanol) to wash product nanoparticles. After nanoparticles settled to the beaker bottom, top transparent solutions were decanted and the remaining nanoparticles were washed again with triply distilled water (or ethanol) for three times. When ethanol was used as a washing solvent, nanoparticles were washed again with triply distilled water to remove ethanol. The first half volumes of washed nanoparticles were diluted with triply distilled water to prepare aqueous sample solutions and the remaining half volumes were reduced to powder form by drying them in air for various characterizations.
35 mm cell culture dishes at the density of 2.5 to measure fluorescent images of DU145 cells. The DU145 cells were seeded onto two 50.1 mm, TR
3 tesla T2 MR image measurement to quantify the intracellular adenosine triphosphate (ATP). Both human prostate cancer sample solutions of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles were used to measure the cellular toxicity of aqueous sample solution (0.2–0.3 mM Eu or Tb) and incubated for 48 hours. The nuclei in both dishes were then stained with DAPI and washed again with PBS.

In vivo cytotoxicity measurement. A CellTiter-Glo Luminescence Cell Viability Assay (Promega, WI, USA) was used to measure the cellular toxicity of aqueous sample solutions of D-glucuronic acid coated ultrasmall mixed lanthanide oxide nanoparticles (ATP). Both human prostate cancer cell lines were seeded in two 2.5 cm diam. plates at the density of 2.5 x 10^5 per dish (2 mL, 5% CO_2, 37°C). The animal experiments using mice in this study were approved by the animal research committee of Kyungpook National University (KNU) and carried out in accordance with its the rule. A 3 tesla MRI instrument (GE 1.5 T. Signa Advantage, GE medical system) equipped with the knee coil (EXTREM) was used for excitation of DAPI and nanoparticles were 405 and 488 nm, respectively. At

Relaxivity and map image measurement. A 1.5 tesla MRI instrument (GE 1.5 T Signa Advantage, GE medical system) equipped with the knee coil (EXTREM) was used to measure both T_1 and T_2 relaxation times as well as both R_1 and R_2 map images. A series of aqueous sample solutions with different concentrations (1, 0.25, 0.125, 0.0625, and 0 mM Ln (Ln = Dy or Ho)) were prepared by diluting original sample solutions with a sterile phosphate-buffered saline (PBS) solution. Then, ~2 µL of each test sample solution was treated into the cultured cells, which were further incubated for 48 hours. Cell viability was measured and normalized with respect to the control cell with 0.0 M Ln concentration. The measurement was repeated twice for all sample cells to obtain average cell viabilities.

In vivo 3 tesla T_2 MR image measurement. The animal experiments using mice in this study were approved by the animal research committee of Kyungpook National University (KNU) and carried out in accordance with its the rule. A 3 tesla MRI instrument (SIEMENS 3.0 T MAGNETOM Trio a Tim) was used to measure T_2 spin echo (SE) images of a mouse. An ICR female mouse with weight of 30 g was used for each sample solution. Each mouse was anesthetized by 1.5% isoflurane in oxygen. The typical parameters used for excitation was ~8 watts. The λ_{ex} used for excitation of DAPI and nanoparticles were 405 and 488 nm, respectively. At λ_{em} = 405 nm, DAPI tinted pale blue. The typical magnifications used for imaging were 400 and 1000.
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**Author contributions**

W.X., B.A.B. and C.R.K. carried out syntheses and characterizations of nanophosphors, J.S.B. and Y.C. measured relaxivities and T2 MR images, J.E.B. and K.S.C. measured cytotoxicities and fluorescent confocal images, and Y.C., T.J.K. and G.H.L. led the project.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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