

## PAPER

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3, 5857Paramagnetic ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$   
nanoparticles: characterization of  $r_2$  values  
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Paramagnetic ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles ( $d_{\text{avg}} = \sim 2.1$  nm) grafted with various hydrophilic and biocompatible ligands such as poly(ethylene glycol) diacid ( $M_n = 250$  and 600 amu) and polyacrylic acid ( $M_w = 1800$  amu) were synthesized via a one-pot polyol method. Appreciable transverse ( $r_2$ ) and negligible longitudinal ( $r_1$ ) water proton spin relaxivity values were observed for all nanoparticle samples. The  $r_2$  values increased with increasing nanoparticle magnetic moment and decreased with increasing ligand size. Owing to the aforementioned  $r_1$  and  $r_2$  values, the nanoparticle samples exhibited appreciable negative contrast enhancements in *in vivo*  $T_2$  magnetic resonance (MR) images at a 3.0 T MR field after intravenous injection, demonstrating their potential as efficient  $T_2$  MRI contrast agents.

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## Introduction

Nowadays, magnetic resonance imaging (MRI) is the most commonly used technique in diagnosing diseases.<sup>1–4</sup> MRI contrast agents are commonly intravenously injected to improve the sensitivity and resolution in MR images via contrast enhancements.<sup>5–13</sup> MRI contrast agents are classified into  $T_1$  and  $T_2$  MRI contrast agents.<sup>10,11</sup>  $T_1$  MRI contrast agents significantly reduce longitudinal ( $T_1$ ) water proton spin relaxation times in the tissue, making MR images brighter (positive contrast),<sup>10,11</sup> whereas  $T_2$  MRI contrast agents significantly reduce transverse ( $T_2$ ) water proton spin relaxation times in the tissue, making MR images darker (negative contrast).<sup>10,11</sup> At present, molecular Gd-chelates as  $T_1$  MRI contrast agents have gained wide market applications because of their good contrasts and rapid excretion via the renal system within a few hours after intravenous injection.<sup>14–17</sup> Conversely, most of the clinically approved dextran

and carbohydrate-coated superparamagnetic iron oxide ( $\text{Fe}_3\text{O}_4$ ) nanoparticles (SPIONs) as  $T_2$  MRI contrast agents<sup>18–25</sup> are withdrawn from the market due to their lack of clinical users.<sup>18</sup> They had been developed for liver, spleen, and lymph node imaging<sup>18–25</sup> and are excretable via the hepatobiliary system due to their nanosizes ( $> 3$  nm).<sup>26</sup> They have shown drawbacks, such as side effects (*i.e.*, back pains) and less efficiency than Gd-chelates.<sup>18</sup> Therefore, it is challenging to develop a new class of  $T_2$  MRI contrast agents made of ultra-small nanoparticles ( $< 3$  nm), which are excretable via the renal system<sup>27–29</sup> like molecular agents.

The ability of nanoparticles to induce  $T_1$  and  $T_2$  water proton spin relaxations highly depends on the electron magnetic moments ( $j = \ell + s$ ) of metal ion consisting nanoparticles<sup>5,6</sup> where  $j$  represents the total electron magnetic moment,  $\ell$  represents the orbital component, and  $s$  represents the spin component. According to the inner and outer sphere models,<sup>5,6</sup> nanoparticles can significantly induce both  $T_1$  and  $T_2$  water proton spin relaxations if  $\ell = 0$ , whereas they can exclusively induce only  $T_2$  water proton spin relaxations with negligible induction of  $T_1$  water proton spin relaxations if  $\ell \neq 0$ , corresponding to efficient  $T_2$  MRI contrast agents. Former examples of nanoparticle contrast agents are those consisting of  $\text{Fe}^{3+}$  ( $s = 5/2$ ),  $\text{Mn}^{2+}$  ( $s = 5/2$ ), and  $\text{Gd}^{3+}$  ( $s = 7/2$ ).  $\text{Gd}_2\text{O}_3$  nanoparticles have the highest  $T_1$  induction and their  $T_2/T_1$  induction ratio is closest to one,<sup>30,31</sup> making them the most powerful  $T_1$  MRI contrast agents among the nanoparticle

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contrast agents. Later examples of nanoparticle contrast agents include  $\text{Ln}_2\text{O}_3$  nanoparticles ( $\text{Ln} = \text{Dy}, \text{Ho}, \text{and Tb}$ )<sup>32–37</sup> and  $\text{CoO}$  nanoparticles<sup>38</sup> because of the nonzero  $\ell$  of 4f-electrons in  $\text{Ln}^{3+}$  and 3d-electrons in  $\text{Co}^{2+}$ . The SPIONs can significantly induce  $T_2$  water proton spin relaxations with appreciable induction of  $T_1$  water proton spin relaxations<sup>18–25</sup> because they are composed of the following two types of metal ions:  $\text{Fe}^{2+}$  ( $\ell = 2, s = 2, j = 4$ ) and  $\text{Fe}^{3+}$  ( $j = s = 5/2$ ), corresponding to an intermediate example between the former and latter examples. Notably, magnetic moments of  $\text{Ln}_2\text{O}_3$  nanoparticles are nearly particle size-independent because of the compact 4f-electrons in  $\text{Ln}^{3+}$ , which are nearly unaffected by surface-coating ligands as can be noticed from their small energy splitting ( $\sim 100 \text{ cm}^{-1}$ ) by external factors.<sup>39</sup> In contrast, 3d-transition metal oxide nanoparticles have size-dependent magnetic moments and relaxivities<sup>40,41</sup> because of diffuse 3d-electrons, which are significantly affected by external factors as can be noticed from their large energy splitting by ligands ( $\sim 10\,000 \text{ cm}^{-1}$ ).<sup>42</sup> This implies that ultrasmall  $\text{Ln}_2\text{O}_3$  nanoparticles made of  $\text{Ln}^{3+}$  with high  $j$ -values and with  $\ell \neq 0$  can have appreciable magnetic moments at room temperature close to their bulk values, allowing them to have appreciable transverse ( $r_2$ ) and negligible longitudinal ( $r_1$ ) water proton spin relaxivities. This will make them work as a new class of efficient  $T_2$  MRI contrast agents, which had been recently demonstrated in ultrasmall  $\text{Ln}_2\text{O}_3$  nanoparticles ( $\text{Ln} = \text{Dy}, \text{Ho}, \text{and Tb}$ ), where moderate negative contrasts in *in vivo*  $T_2$  MR images were observed.<sup>32,33,35,37</sup>

In addition to the previously described nanoparticle magnetic moments, the  $T_2$  contrast in MR images is sensitive to physical factors arising from ligands such as ligand size and hydrophilicity<sup>43–46</sup> because these factors can influence the strength of magnetic dipole-dipole interactions between the nanoparticles and water proton spins (because  $r_2 \propto 1/L^3$  in which  $L$  is the distance between the nanoparticles and water proton spins)<sup>5,47</sup> and the amount of water molecules interacting with the nanoparticles, whereas in metal ion-chelates, the  $T_1$  contrast in MR images is sensitive to the hydration number which is determined by the types of chelates.<sup>5–7,48</sup> Considering the aforementioned ligand physical factors and that ligand coating is essential to make the nanoparticles colloidally stable and biocompatible for *in vivo* applications, appropriate ligands should be chosen for nanoparticle coating to obtain high  $r_2$  values.

Here, we synthesized ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles grafted with various hydrophilic and biocompatible ligands, namely, poly(ethylene glycol) diacid (PEGD) ( $M_n = 250$  and  $600$  amu) and polyacrylic acid (PAA) ( $M_w = 1800$  amu), and characterized them using various experimental techniques. We explored their potential as efficient  $T_2$  MRI contrast agents by measuring  $r_1$  and  $r_2$  values and *in vivo*  $T_2$  MR images at a  $3.0 \text{ T}$  MR field.

## Results and discussion

### Particle diameters

Various hydrophilic and biocompatible ligand-coated ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles were synthesized *via* a

one-pot polyol method. The particle diameters were determined by obtaining high-resolution transmission electron microscope (HRTEM) images (Fig. 1a–d). The particle diameter ranged from  $1.0$  to  $3.0 \text{ nm}$  and the average particle diameter ( $d_{\text{avg}}$ ) (Table 1) was estimated to be  $2.1 \text{ nm}$  for both PEGD250- and PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles, and  $2.1$  and  $2.2 \text{ nm}$  for PEGD600- and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles, respectively, from log-normal function fits to the observed particle diameter distributions (Fig. 1e). The ligand-coated nanoparticles were also confirmed by energy-dispersive X-ray spectroscopy (EDS), where elements such as C, O, Ho, and Tm were strongly detected (Fig. 1f–i). The physicochemical properties of the previously studied PAA1800-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles ( $d_{\text{avg}} = 1.7 \text{ nm}$ )<sup>35</sup> were added to Table 1 for comparison.

### Hydrodynamic diameters and zeta potentials

The nanoparticle suspension samples in aqueous media are presented in Fig. 2a. Except for the PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles, all samples exhibited excellent colloidal stability: they did not settle down to the beaker bottom until  $> 1$  year after synthesis, whereas the PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles partially precipitated in a week but were redispersed *via* shaking. This is likely because PEGD250 is considerably short enough to attract a sufficient amount of water molecules to stabilize the nanoparticle colloids whereas PEGD600 is long enough (approximately four times longer than PEGD250) and each PAA1800 possesses abundant  $\text{COO}^-$  groups (approximately  $25 \text{ COO}^-$  groups per monomer) to attract a sufficient amount of water molecules to stabilize the nanoparticle colloids. The average hydrodynamic diameter ( $a_{\text{avg}}$ ) was estimated to be  $8.7$  and  $13.5 \text{ nm}$  for the PEGD250- and PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles, respectively, and  $12.0$  and  $20.6 \text{ nm}$  for the PEGD600- and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles, respectively, from a log-normal function fits to the observed dynamic light scattering (DLS) patterns (Fig. 2b and Table 1). Notably, the  $a_{\text{avg}}$  values increased with increasing ligand size, which is likely attributable to an increase in ligand-coating layer thickness and hydration spheres due to the increase in the amount of water molecules attracted by ligands around the nanoparticles with increasing ligand size. The hydrodynamic diameters measured at different times for PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles (Fig. 2c) showed nearly constant  $a_{\text{avg}}$  values over time, indicating negligible aggregation between nanoparticles with time, as consistent with their observed good colloidal stability.

The zeta potential of the nanoparticle suspension samples in aqueous media was measured to be  $10.4$  and  $14.5 \text{ mV}$  for the PEGD250- and PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles, respectively, and  $12.7$  and  $-20.2 \text{ mV}$  for the PEGD600- and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles, respectively (Fig. 2d and Table 1). The positive zeta potential of the PEGD250- and PEGD600-coated nanoparticles in slightly acidic suspension media ( $\text{pH} = 6.5\text{--}6.7$ ) is due to partially protonated oxygens and carboxyl groups of PEGD, thus providing positive values and consistent with previous observations in PEGD600-coated





**Fig. 1** HRTEM images of (a) PEGD250- and (b) PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles and (c) PEGD600- and (d) PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles. (e) Log-normal function fits to the observed particle diameter distributions to obtain  $d_{\text{avg}}$ . EDS spectra of (f) PEGD250- and (g) PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles and (h) PEGD600- and (i) PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles.

**Table 1** Summary of the observed physicochemical properties of various hydrophilic and biocompatible ligand-coated ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles

Nanoparticle	Surface-coating ligand	$d_{\text{avg}}$ (nm)	$a_{\text{avg}}$ (nm)	$\zeta$ (mV)	pH <sup>a</sup>	Surface-coating amount		
						$P^b$ (wt%)	$\sigma^c$ (nm <sup>-2</sup> )	$N_{\text{NP}}^d$
$\text{Ho}_2\text{O}_3$	PEGD250	2.1	8.7	10.8	~6.5	43.2	5.7	79
$\text{Ho}_2\text{O}_3$	PEGD600	2.1	13.5	14.9	~6.7	51.6	3.5	49
$\text{Ho}_2\text{O}_3^e$	PAA1800	1.7	12.7	-32.9	~9.0	45.5	0.85	7
$\text{Tm}_2\text{O}_3$	PEGD600	2.1	12.0	14.7	~6.7	59.5	4.6	64
$\text{Tm}_2\text{O}_3$	PAA1800	2.2	20.6	-21.4	~9.0	48.4	1.1	16

<sup>a</sup> pH of nanoparticle suspension samples in aqueous media. <sup>b</sup> Average amount of ligands coating a nanoparticle (in wt%). <sup>c</sup> Grafting density, i.e., average number of ligands coating a nanoparticle unit surface area. <sup>d</sup> Average number of ligands coating a nanoparticle. <sup>e</sup> Data from ref. 35.

$\text{Fe}_3\text{O}_4$  nanoparticles,<sup>49</sup> whereas the negative zeta potential of the PAA1800-coated nanoparticles is due to numerous negative

$\text{COO}^-$  groups of PAA1800 in basic suspension sample (pH = ~9.0) and consistent with previous observations for nanoparticles





Fig. 2 (a) Photographs of the nanoparticle suspension samples in aqueous media: (i) PEGD250- and (ii) PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles, (iii) PEGD600- and (iv) PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles. (b) Plots of log-normal function fits to the observed DLS patterns. (c) Plots of  $a_{\text{avg}}$  values as a function of time (h). (d) Plots of zeta potentials. (e) Tyndall effects confirming nanoparticle colloidal dispersions in aqueous media: arrows indicate laser light scattering by the nanoparticle colloids.

grafted with numerous  $\text{COO}^-$  group containing polymers.<sup>35,50,51</sup> The nanoparticle colloidal dispersions in aqueous media were confirmed *via* Tyndall effects, where laser light scattering was observed only for the nanoparticle suspension samples due to collisions between the nanoparticle colloids and laser light whereas it was not observed for triple-distilled water (Fig. 2e).

### Crystal structure

As shown in Fig. 3, X-ray diffraction (XRD) patterns of the as-synthesized nanoparticles showed broad peaks, indicating the amorphous feature of the nanoparticles.<sup>52</sup> However, the XRD patterns after thermogravimetric analysis (TGA) displayed sharp peaks, indicating crystallization of the nanoparticles after TGA due to heating up to 900 °C. All peaks after TGA could be assigned with (*hkl*) Miller indices according to body-centered cubic  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$ .<sup>53,54</sup> The estimated lattice constants after TGA were 10.609 and 10.482 Å for  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles, respectively, which are in good agreement with the reported values of 10.6186 and 10.49 Å,<sup>53,54</sup> respectively.

### Surface-coating results

The surface coating of the nanoparticles was investigated by recording Fourier transform-infrared (FT-IR) absorption spectra of the ligand-coated nanoparticles as well as the ligands for reference. As shown in Fig. 4a and b and Table 2, characteristic IR absorption bands of the ligands such as C–H antisymmetric and symmetric stretching vibrations at 2922–2937 and

2868–2876  $\text{cm}^{-1}$ , respectively,  $\text{COO}^-$  antisymmetric and symmetric stretching vibrations at 1547–1593 and 1398–1433  $\text{cm}^{-1}$ , respectively, and C–O stretching vibrations at 1087–1099  $\text{cm}^{-1}$  were observed in the FT-IR absorption spectra of the PEGD250-, PEGD600-, and PAA1800-coated nanoparticles, confirming the successful ligand coating of the nanoparticles. The splitting of the C=O stretching vibrations of PEGD250 at 1723  $\text{cm}^{-1}$  and PEGD600 at 1721  $\text{cm}^{-1}$  and PAA1800 at 1700  $\text{cm}^{-1}$  into the aforementioned  $\text{COO}^-$  symmetric and antisymmetric stretching vibrations in the samples indicate the bridge bonding of the  $\text{COO}^-$  groups of the ligands to  $\text{Ho}^{3+}$  and  $\text{Tm}^{3+}$  of the nanoparticles.<sup>55,56</sup> This bridge bonding was strong, as confirmed from large red-shifts of the  $\text{COO}^-$  antisymmetric and symmetric stretching frequencies by  $\sim 130$  and  $\sim 300$   $\text{cm}^{-1}$  from the C=O stretching frequencies, respectively (Table 2). This corresponds to hard-acid ( $\text{COO}^-$  groups of the ligands) and hard-base ( $\text{Ho}^{3+}$  and  $\text{Tm}^{3+}$  of the nanoparticles) types of bonding.<sup>57–59</sup> The observed absorption frequencies are consistent with the literature.<sup>56,60,61</sup>

Based on FT-IR absorption spectral results, the surface-coating structures of PEGD250, PEGD600, and PAA1800 on the nanoparticle surfaces are schematically proposed in Fig. 5a–c, respectively. As shown in Fig. 5a, one of the two  $\text{COO}^-$  groups of PEGD250 is likely bonded to  $\text{Ho}^{3+}$  of the ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles because of the short length of PEGD250. PEGD600 is likely bonded to the nanoparticles *via* its one or two  $\text{COO}^-$  groups because of its long and flexible length (Fig. 5b). Each PAA1800 possesses approximately 25  $\text{COO}^-$





Fig. 3 XRD patterns of the nanoparticle powder samples before (*i.e.*, as-prepared) and after TGA: (a) PEGD250- (top) and PEGD600-coated ultra-small  $\text{Ho}_2\text{O}_3$  nanoparticles (bottom) and (b) PAA1800- (top) and PEGD600-coated ultra-small  $\text{Tm}_2\text{O}_3$  nanoparticles (bottom). All peaks after TGA could be assigned with  $(hkl)$  Miller indices of body-centered cubic  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$ .<sup>53,54</sup>

groups and thus can allow multiple bonding interactions among its many  $\text{COO}^-$  groups and  $\text{Tm}^{3+}$  of the nanoparticles (Fig. 5c).

The amount ( $P$ ) of ligand-coating of the nanoparticles in wt% was estimated from the mass loss in the TGA curve after considering an initial mass drop between room temperature and  $\sim 105^\circ\text{C}$  due to water and air desorption (Fig. 6). The residual mass in the TGA curve corresponded to the net mass of the  $\text{Ho}_2\text{O}_3$  or  $\text{Tm}_2\text{O}_3$  nanoparticles without ligands. Grafting density ( $\sigma$ ),<sup>62</sup> corresponding to the average number of ligands coating a nanoparticle unit surface area, was estimated using the bulk density of  $\text{Ho}_2\text{O}_3$  ( $8.41\text{ g cm}^{-3}$ ) or  $\text{Tm}_2\text{O}_3$  ( $8.6\text{ g cm}^{-3}$ ),<sup>63</sup>  $d_{\text{avg}}$  estimated from HRTEM imaging, and the above  $P$  value obtained from the TGA curve. The average number ( $N_{\text{NP}}$ ) of ligands coating the nanoparticle was then estimated by multiplying  $\sigma$  with the nanoparticle surface area ( $=\pi d_{\text{avg}}^2$ ). As provided in Table 1, the  $\sigma$  and  $N_{\text{NP}}$  values decreased with increasing ligand size likely because a larger ligand generally occupied a larger space due to its steric effects.

### Magnetic properties

The magnetic properties of the PEGD250- and PEGD600-coated ultra-small  $\text{Ho}_2\text{O}_3$  nanoparticles, and PEGD600- and PAA1800-coated ultra-small  $\text{Tm}_2\text{O}_3$  nanoparticles were investigated by measuring magnetization ( $M$ ) versus applied field ( $H$ ) (*i.e.*,  $M$ - $H$ ) curves at 300 K using a vibrating sample magnetometer



Fig. 4 FT-IR absorption spectra of (a) PEGD250 and PEGD250-coated ultra-small  $\text{Ho}_2\text{O}_3$  nanoparticles (top), and PEGD600 and PEGD600-coated ultra-small  $\text{Ho}_2\text{O}_3$  nanoparticles (bottom), and (b) PAA1800 and PAA1800-coated ultra-small  $\text{Tm}_2\text{O}_3$  nanoparticles (top), and PEGD600 and PEGD600-coated ultra-small  $\text{Tm}_2\text{O}_3$  nanoparticles (bottom).

Table 2 Observed FT-IR absorption frequencies in  $\text{cm}^{-1}$

	(C-H) <sub>as</sub>	(C-H) <sub>ss</sub>	C=O	(COO <sup>-</sup> ) <sub>as</sub> <sup>a</sup>	(COO <sup>-</sup> ) <sub>ss</sub> <sup>a</sup>	C-O
PEGD250	2926	2885	1723	—	—	1101
PEGD600	2923	2888	1721	—	—	1099
PAA1800	2978	2937	1700	—	—	1101
PEGD250- $\text{Ho}_2\text{O}_3$	2926	2874	—	1578 (145)	1398 (325)	1096
PEGD600- $\text{Ho}_2\text{O}_3$	2922	2876	—	1593 (128)	1418 (303)	1093
PEGD600- $\text{Tm}_2\text{O}_3$	2926	2868	—	1589 (132)	1433 (288)	1987
PAA1800- $\text{Tm}_2\text{O}_3$	2937	2868	—	1547 (153)	1402 (298)	1099

<sup>a</sup> The numbers in parentheses correspond to the red shifts from the C=O stretching frequencies of the ligands.

(VSM) (Fig. 7). The mass-corrected net  $M$  values of the nanoparticles without ligands were used in the plots, which were estimated using their net masses that were extracted from their TGA curves shown in Fig. 6. All nanoparticle samples showed paramagnetism with no hysteresis, zero coercivity, and zero remanence in the  $M$ - $H$  curves, which is similar to that of their corresponding bulk  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$ .<sup>64,65</sup> From the mass-corrected  $M$ - $H$  curves, the net  $M$  values of the ultra-small  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles without ligands at 2.0 T and 300 K were estimated to be 4.64 and 1.73  $\text{emu g}^{-1}$  (Table 3), respectively. The bigger net  $M$  value of the ultra-small  $\text{Ho}_2\text{O}_3$  nanoparticles compared with that of ultra-small  $\text{Tm}_2\text{O}_3$  nanoparticles is due to a higher magnetic moment of  $\text{Ho}^{3+}$  ( $^5\text{I}_5$ , 10.6  $\mu_B$ )





Fig. 5 Proposed ligand-coating structures of (a) PEGD250, (b) PEGD600, and (c) PAA1800 via the bridge bonding between the  $\text{COO}^-$  groups of the ligands and  $\text{Ho}^{3+}$  or  $\text{Tm}^{3+}$  of the nanoparticles.



Fig. 6 TGA curves of the PEGD250- and PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles, and PEGD600- and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles.

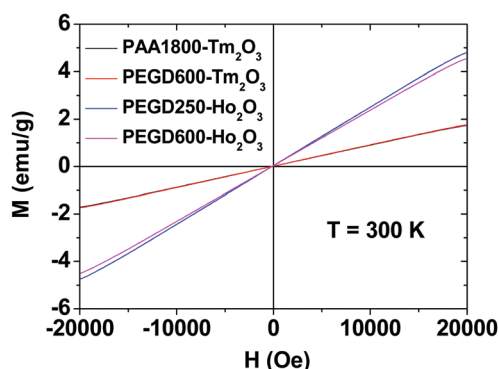


Fig. 7 Mass-corrected  $M$ - $H$  curves of the PEGD250- and PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles, and PEGD600- and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles at 300 K. The net  $M$  values of the ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles without ligands were used in the plots, which were estimated using the net masses of the nanoparticles extracted from the TGA curves.

compared with that of  $\text{Tm}^{3+}$  ( $^3\text{H}_6$ , 7.56  $\mu_B$ ),<sup>66</sup> where  $\mu_B$  is the Bohr magneton.

### $r_1$ and $r_2$ values: nanoparticle magnetic moment and ligand-size effects on $r_2$ values

$r_1$  and  $r_2$  values were estimated from the slopes of  $1/T_1$  and  $1/T_2$  plots as a function of  $\text{Ho}$  or  $\text{Tm}$  concentration, respectively (Fig. 8a and Table 3).  $r_1$  Values were negligible for all nanoparticle samples ( $< 0.2 \text{ s}^{-1} \text{ mM}^{-1}$ ), whereas  $r_2$  values were appreciable with a magnitude that depended on the nanoparticle species and surface-coating ligands. This implies that the ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles can exclusively induce only  $T_2$  water proton spin relaxations with negligible induction of  $T_1$  water proton spin relaxations. This is due to the contribution of 4f-electron orbital motions in  $\text{Ho}^{3+}$  and  $\text{Tm}^{3+}$  of the nanoparticles to the nanoparticle magnetic moments. According to the inner sphere model, only the magnetic moment from electron spin motion can significantly contribute to the  $r_1$  value,<sup>5,6</sup> which is not the case for  $\text{Ho}^{3+}$  and  $\text{Tm}^{3+}$ . However, the  $r_2$  value is proportional to the square of nanoparticle magnetic moment according to the outer sphere model,<sup>5,47</sup> and thus, was appreciable because nanoparticle magnetic moments of the  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles at room temperature were appreciable (Table 3).

Given that  $T_2$  water proton spin relaxation is induced by the magnetic dipole-dipole interactions between the nanoparticles and water proton spins, the  $r_2$  value is proportional to  $M_{\text{NP}}^2/L^3$  in which  $M_{\text{NP}}$  is the nanoparticle magnetic moment (unit: emu/nanoparticle) and  $L$  is the distance between the nanoparticle and water proton spin.<sup>5,47</sup>  $M_{\text{NP}} \propto d_{\text{avg}}^3 M$  for paramagnetic nanoparticles<sup>35</sup> and  $d_{\text{avg}}$  values are nearly the same for all nanoparticle samples for the present study (see Table 1) and thus,  $M_{\text{NP1}} (\text{Ho}_2\text{O}_3 \text{ nanoparticle}) > M_{\text{NP2}} (\text{Tm}_2\text{O}_3 \text{ nanoparticle})$  and  $L_1 (\text{PAA1800}) > L_2 (\text{PEGD600}) > L_3 (\text{PEGD250})$  if  $L$  is assumed to be proportional to the ligand size. This explains the observed increase in  $r_2$  value with increasing  $M$  (Fig. 8b) and a decrease in  $r_2$  value with increasing ligand size (Fig. 8c). Overall,  $M_{\text{NP1}}^2/L_3^3 > M_{\text{NP1}}^2/L_2^3 > M_{\text{NP2}}^2/L_2^3 > M_{\text{NP2}}^2/L_1^3$  explains the observed  $r_2$  values such that  $r_2 (\text{PEGD250-coated Ho}_2\text{O}_3 \text{ nanoparticle}) > r_2 (\text{PEGD600-coated Ho}_2\text{O}_3 \text{ nanoparticle}) > r_2 (\text{PEGD600-coated Tm}_2\text{O}_3 \text{ nanoparticle}) > r_2 (\text{PAA1800-coated Tm}_2\text{O}_3 \text{ nanoparticle})$ . This simple model equation also explains that  $r_2 (\text{PEGD600-coated Ho}_2\text{O}_3 \text{ nanoparticle}) > r_2 (\text{PAA1800-coated Ho}_2\text{O}_3 \text{ nanoparticle; Table 3})^{35} > r_2 (\text{PAA1800-coated Tm}_2\text{O}_3 \text{ nanoparticle})$ .

As shown in the  $R_1$  and  $R_2$  map images (Fig. 8d), dose-dependent contrast enhancements in the  $R_1$  map images were negligible for all nanoparticle samples whereas  $R_2$  map images exhibited appreciable dose-dependent contrast enhancements for all nanoparticle samples, supporting *in vitro* that the ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles may act as efficient  $T_2$  MRI contrast agents.

### *In vitro* cellular cytotoxicity

The cellular cytotoxicity of the PEGD250- and PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles and PEGD600- and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles was investigated by measuring *in vitro* cell viabilities in various types of cell lines,



Table 3 Magnetic properties and water proton spin relaxivities

Nanoparticle	Surface-coating ligand	Magnetic properties at 300 K			Water proton spin relaxivities (22 °C, 3.0 T)	
		Magnetism	Net $M$ (emu g <sup>-1</sup> ) at 2 T		$r_1$ (s <sup>-1</sup> mM <sup>-1</sup> )	$r_2$ (s <sup>-1</sup> mM <sup>-1</sup> )
Ho <sub>2</sub> O <sub>3</sub>	PEGD250	Paramagnetism	4.76	Average = 4.64	0.14	30.39
Ho <sub>2</sub> O <sub>3</sub>	PEGD600	Paramagnetism	4.52		0.17	11.33
Ho <sub>2</sub> O <sub>3</sub> <sup>a</sup>	PAA1800	Paramagnetism	4.1	Average = 1.73	0.13	1.44
Tm <sub>2</sub> O <sub>3</sub>	PEGD600	Paramagnetism	1.74		0.11	5.79
Tm <sub>2</sub> O <sub>3</sub>	PAA1800	Paramagnetism	1.72		0.10	1.03

<sup>a</sup> Data from ref. 35 and net  $M$  was obtained at 1.8 T.

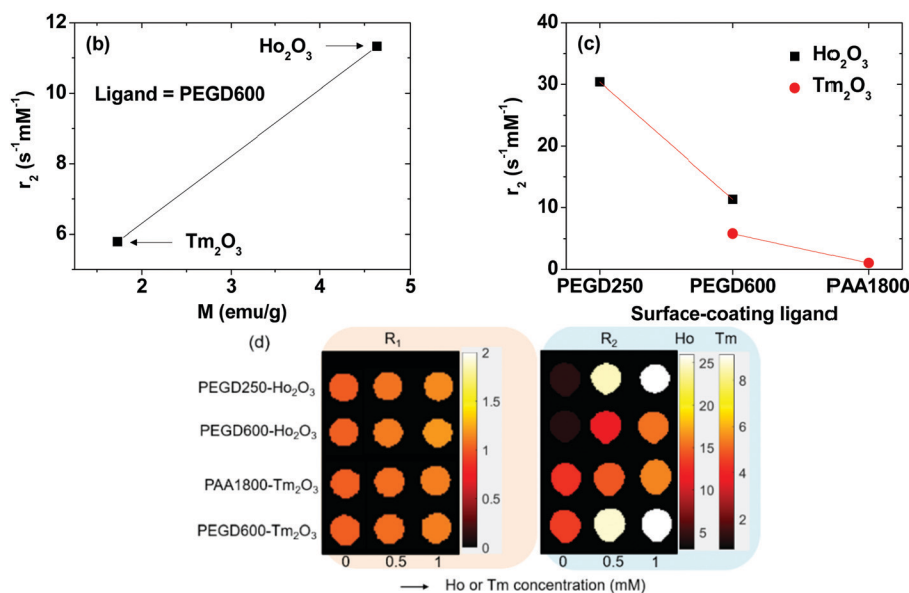


Fig. 8 (a) Plots of  $1/T_1$  and  $1/T_2$  of the nanoparticle suspension samples in aqueous media as a function of Ho or Tm concentration. The slopes correspond to the  $r_1$  and  $r_2$  values, respectively. Plots of the  $r_2$  values as a function of (b) nanoparticle magnetic moment ( $M$ ) (using the average  $M$  in Table 3) and (c) ligand-size (PEGD250 < PEGD600 < PAA1800). (d) Dose-dependent  $R_1$  and  $R_2$  map images of the nanoparticle suspension samples in aqueous media.

such as human prostate cancer (DU145), human embryonic kidney 293 (HEK293), and human liver cancer (HepG2) cell lines 48 h after incubation. As shown in Fig. 9a–c, all samples exhibited considerably low cellular cytotoxicities of up to 500  $\mu$ M Ho and Tm in various cell lines. Dose-dependent cell morphologies were investigated by measuring optical microscope images of control and treated DU145 cells with PEGD250-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> nanoparticles at various Ho concentrations (Fig. 9d). As shown in Fig. 9, the nanoparticles were not localized in the cells but scattered all over the place and heavily covered the cells with the degree of cell coverage which increased with increasing nanoparticle concentration. In addition, the cell morphologies of the treated cells with the nanoparticles were similar to those of the control cells, likely due to the very low cytotoxicities of the nanoparticles.

### Hemolysis assay results

To investigate the hemolytic effects of the nanoparticle samples, the hemolysis assay was performed for all nanoparticle samples and the results are shown in Fig. 10a–c. Photographs of the lysed assay results are shown in Fig. 10a and the estimated lysed

hemoglobin concentrations in mg dL<sup>-1</sup> are plotted in Fig. 10b. The hemolysis rates of the nanoparticle samples are plotted in Fig. 10c. As shown in Fig. 10a–c, only the PEGD600-coated Ho<sub>2</sub>O<sub>3</sub> nanoparticles exhibited slight hemolytic properties for the tested concentration range ( $2.85 \pm 0.48\%$  to  $3.41 \pm 0.16\%$ ). However, the other nanoparticle samples exhibited small hemolysis rates which were less than 2% suitable for *in vivo* applications.

### *In vivo* $T_2$ MR images at a 3.0 T MR field

To demonstrate the effectiveness of the ultrasmall Ho<sub>2</sub>O<sub>3</sub> and Tm<sub>2</sub>O<sub>3</sub> nanoparticles as efficient  $T_2$  MRI contrast agents *in vivo*, the PEGD250-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> nanoparticles and PEGD600-coated ultrasmall Tm<sub>2</sub>O<sub>3</sub> nanoparticles were used for  $T_2$  MR image measurements. These nanoparticles were chosen because they possess higher  $r_2$  values compared with the same kind of nanoparticles grafted with different ligands. *In vivo*  $T_2$  MR images were obtained before (labelled as “pre”) and after intravenous injection of the aqueous nanoparticle suspension samples into mice tails at a 3.0 T MR field. As shown in Fig. 11a and b, negative contrast enhancements







Fig. 9 *In vitro* cell viabilities of (a) PEGD250- and PEGD600-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles in DU145 cell lines, (b) PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles in HepG2 cell lines, and (c) PEGD600- and PAA1800-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles in HEK293 cell lines 48 h after incubation as a function of Ho or Tm concentration. (d) Optical microscope images of control and treated DU145 cells with PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles at various Ho concentrations: the same scale bar applies to all images.

(i.e., darker images) after injection were clearly observed in the liver and kidneys for both nanoparticle samples.

To quantitatively investigate how the negative contrast enhancement changes with time, the signal-to-noise ratios (SNRs) of regions-of-interest (ROIs) in the liver and kidneys (labeled as dotted circles in “pre”  $T_2$  MR images) were plotted as a function of time. As shown in Fig. 11c and d, the negative contrast enhancements initially increased (or SNR-ROI decreased) after injection due to the accumulation of the nanoparticles in the liver and kidneys and then, decreased (or SNR-ROI increased) with time due to the excretion of the nanoparticles from the liver and kidneys because of their ultrasmall particle diameters. Notably, the PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles exhibited higher negative contrast enhancements (maximum =  $\sim 6.0$  in Fig. 11c) compared with those (maximum =  $\sim 4.1$  in Fig. 11d) obtained with the PEGD600-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles because of the higher  $r_2$  value (i.e.,  $30.39 \text{ s}^{-1} \text{ mM}^{-1}$ ) of the former nanoparticles than that of the latter nanoparticles (i.e.,  $5.79 \text{ s}^{-1} \text{ mM}^{-1}$ ). Therefore, it is expected that the other untested nanoparticles will also provide negative contrast enhancements with magnitudes that are proportional to their  $r_2$  values. These results prove that the ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles should act as a new class of efficient  $T_2$  MRI contrast agents.

As shown in Fig. 11c and d, the excretion of the PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles was slightly longer compared with that of the PEGD600-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles. This was likely related to the surface-coating ligands of the PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles,

which resulted in less colloidal stability. Thus, the possible aggregation and interaction of the PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles with biological molecules inside the body of the mice would delay their excretion. It is worth noting that the contrast enhancements will be even higher at higher MR fields because of the unsaturated nanoparticle magnetic moments as can be seen in the M–H curves (Fig. 7) and because the  $r_2$  value is proportional to the square of nanoparticle magnetic moment which will increase with increasing MR field.

#### *In vivo* biodistribution results of the injected nanoparticles

The *in vivo* biodistributions of the PEGD250-coated  $\text{Ho}_2\text{O}_3$  and PEGD600-coated  $\text{Tm}_2\text{O}_3$  nanoparticles which were used for *in vivo* MRI experiments were assessed by measuring the Ho or Tm concentration for the lung, heart, liver, intestine, and kidney using ICP-AES. As shown in Fig. 12, nanoparticles were highly accumulated in the liver with  $26.7 \pm 0.007\%$  and  $30.4 \pm 0.012\%$  for the PEGD250-coated  $\text{Ho}_2\text{O}_3$  and PEGD600-coated  $\text{Tm}_2\text{O}_3$ , respectively. From the results, both nanoparticles can be expected to have a long circulation through the gastrointestinal route promising long-term diagnosis for any liver abnormality. Moreover, the PEGD250-coated  $\text{Ho}_2\text{O}_3$  nanoparticles showed large lung accumulation ( $35.4 \pm 0.002\%$ ), possibly due to their adsorption on red blood cells,<sup>67</sup> which may be related to their observed lower colloidal stability compared with other nanoparticle samples.

#### Histological analysis results

To investigate the *in vivo* toxicity of the PEGD250-coated  $\text{Ho}_2\text{O}_3$  and PEGD600-coated  $\text{Tm}_2\text{O}_3$  nanoparticles which were used for







Fig. 10 Hemolysis assay results: (a) photographs of lysed blood samples, (b) lysed hemoglobin concentration in mg dL<sup>-1</sup>, and (c) hemolysis rate in %.

the *in vivo* MRI experiments, histological changes were assessed for the two major organs, *i.e.*, kidney and liver, which are responsible for excretion and detoxification. As shown in Fig. 13, both nanoparticle samples did not show any morphological changes for the kidney and liver, similar to the untreated mice trends, indicating negligible *in vivo* toxicity.

## Experimental

### Materials

Chemicals including Ho(NO<sub>3</sub>)<sub>3</sub>·5H<sub>2</sub>O (99.9%), Tm(NO<sub>3</sub>)<sub>3</sub>·5H<sub>2</sub>O (99.9%), NaOH (>99.9%), triethylene glycol (TEG, 99%), PEGD (99%, *M<sub>n</sub>* = 250 amu, PEGD250), PEGD (99%, *M<sub>n</sub>* = 600 amu, PEGD600), and PAA (analytical standard grade, *M<sub>w</sub>* = 1800 amu, PAA1800) were purchased from Sigma-Aldrich (Burlington, MA, USA) and used as-received. Ethanol (99.5%) was purchased

from Duksan (Ansan, South Korea) and used as-received for the initial washing of the nanoparticles. Triple-distilled water was used for the final washing of the nanoparticles and preparation of the nanoparticle suspension samples (~20 mM Ho or Tm).

### Synthesis of various ligand-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> and Tm<sub>2</sub>O<sub>3</sub> nanoparticles

The one-pot polyol synthesis of various hydrophilic and biocompatible ligand-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> and Tm<sub>2</sub>O<sub>3</sub> nanoparticles is shown in Fig. 14. In a three-necked round bottom flask, 2.0 mmol of Ho(NO<sub>3</sub>)<sub>3</sub>·5H<sub>2</sub>O or Tm(NO<sub>3</sub>)<sub>3</sub>·5H<sub>2</sub>O and ligand (3.0 mmol of PEGD250 or 2.0 mmol of PEGD600 or 1.0 mmol PAA1800) (Table 4) were dissolved in 20 mL of TEG with magnetic stirring at 60 °C for 2 h under atmospheric conditions. An NaOH solution prepared in TEG by dissolving 7.0 mmol of NaOH in 15 mL of TEG with magnetic stirring at 80 °C was added to the above precursor solution until the solution pH reached 8–10. The reaction solution was homogenized with magnetic stirring at 120 °C for 14 h before cooling to room temperature. To remove unreacted precursors, Na<sup>+</sup>, OH<sup>-</sup>, ligand, and TEG from the product solution, the solution was transferred to a 500 mL beaker and 400 mL of ethanol was added with magnetic stirring for 10 min. The solution was placed in a refrigerator until the nanoparticles settled down to the beaker bottom. The top transparent solution was decanted and the remaining product solution was washed thrice with ethanol using the same process. To remove ethanol from the nanoparticles, the product solution was diluted with 400 mL of triple-distilled water and then rotary evaporated to ~40 mL three times. To further purify the product solution, it was dialyzed against 1.0 L of triple-distilled water using a dialysis tube (MWCO = 1000 amu for the PEGD250- and PEGD 600-coated nanoparticles, and 2000 amu for the PAA1800-coated nanoparticles) for a day with magnetic stirring.

### Physicochemical property characterizations

The particle diameters of various hydrophilic and biocompatible ligand-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> and Tm<sub>2</sub>O<sub>3</sub> nanoparticles were measured using an HRTEM (Titan G2 ChemiSTEM CS Probe, 200 kV; FEI, Hillsboro, Oregon, USA). For measurements, a drop of the diluted nanoparticle suspension sample in ethanol was placed onto a carbon film supported by a 200-mesh copper grid (Pelco No. 160, Ted Pella Inc., Redding, CA, USA) using a micropipette (2–20 µL, Eppendorf, Hamburg, Germany) and allowed to dry in air at room temperature. The copper grid with the nanoparticles was subsequently placed inside the HRTEM vacuum chamber for measurements. An EDS instrument (Quantax Nano, Bruker, Berlin, Germany) installed inside the HRTEM was used to qualitatively identify elements (C, O, Ho, Tm) in the nanoparticle samples. The Ho and Tm concentrations of the aqueous nanoparticle suspension samples were determined using an inductively coupled plasma atomic emission spectrometer (ICP-AES) (IRIS/AP, Thermo Jarrell Ash Co., Waltham, MA, USA). A DLS particle size analyzer (Zetasizer Nano ZS, Malvern, Malvern, UK) was used to measure the hydrodynamic diameters





Fig. 11 *In vivo*  $T_2$  MR images in the liver and kidneys of mice at a 3.0 T MR field before (labeled as “pre”) and after intravenous injection of the aqueous suspension samples of the (a) PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles and (b) PEGD600-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles into mice tails (two mice were used for each sample). Dotted circles in the “pre”  $T_2$  MR images indicate regions-of-interest (ROIs). Plots of signal-to-noise ratios (SNRs) of ROIs in the  $T_2$  MR images before and after intravenous injection of the aqueous suspension samples of the (c) PEGD250-coated ultrasmall  $\text{Ho}_2\text{O}_3$  nanoparticles and (d) PEGD600-coated ultrasmall  $\text{Tm}_2\text{O}_3$  nanoparticles.



Fig. 12 *In vivo* biodistribution results of the PEGD250-coated  $\text{Ho}_2\text{O}_3$  and PEGD600-coated  $\text{Tm}_2\text{O}_3$  nanoparticles 12 h after intravenous injection into mice tails (the number of mice used,  $n = 3$ ).

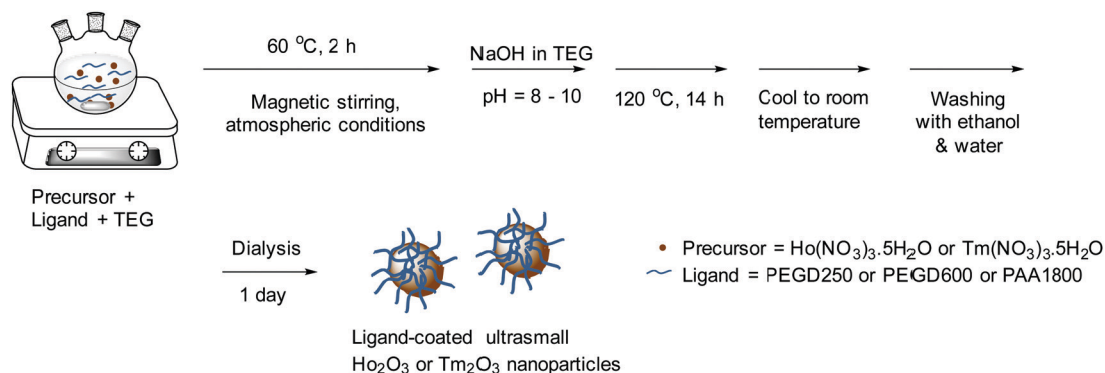


Fig. 13 Optical microscope images of the liver and kidney after H&E staining for the PEGD250-coated  $\text{Ho}_2\text{O}_3$  and PEGD600-coated  $\text{Tm}_2\text{O}_3$  nanoparticles 24 h after intravenous injection into mice tails (the number of mice used,  $n = 3$ ).

and zeta potentials of the nanoparticle suspensions in aqueous media. A multi-purpose XRD instrument (X'PERT PRO MRD, Philips, The Netherlands) with unfiltered  $\text{CuK}\alpha$  ( $\lambda = 0.154184$  nm) radiation was used to characterize the crystal structures of the nanoparticle powder samples. The scanning step and scan range in  $2\theta$  were  $0.033^\circ$  and  $15$ – $100^\circ$ , respectively. The attachment of the hydrophilic ligands to the nanoparticles was probed by recording FT-IR absorption spectra (Galaxy 7020A, Mattson Instrument Inc., Madison, WI, USA) using the powder samples pelletized with KBr. The scan range was

$400$ – $4000$   $\text{cm}^{-1}$ . A TGA instrument (SDT-Q600, TA Instrument, New Castle, DE, USA) was used to estimate the ligand surface-coating amounts by recording TGA curves between room temperature and  $900^\circ\text{C}$  under an air flow. The average amount of surface-coating ligands in wt% was estimated from the mass loss after considering the initial mass drop due to water and air desorption between room temperature and  $\sim 105^\circ\text{C}$ . The net amount of nanoparticles without ligands in the samples was estimated from the remaining mass. A VSM (7407-S, Lake Shore Cryotronics Inc., Westerville, OH, USA) was used to record the





**Fig. 14** One-pot polyol synthesis of various hydrophilic and biocompatible ligand-coated ultrasmall  $\text{Ho}_2\text{O}_3$  and  $\text{Tm}_2\text{O}_3$  nanoparticles (ligand = PEGD250, PEGD600, and PAA1800).

**Table 4** Physical data of various hydrophilic and biocompatible ligands used for the surface coating

Ligand	Molecular weight (amu)	Structure and size ( $n$ )
PEGD250	$M_n = 250$	 $n = \sim 3$
PEGD600	$M_n = 600$	 $n = \sim 11$
PAA1800	$M_w = 1800$	 $n = \sim 25$

M-H curves ( $-2.0 \text{ T} \leq H \leq 2.0 \text{ T}$ ) at 300 K using 20–30 mg powder samples. The net  $M$  values of the nanoparticles without ligands were estimated using the net masses of the nanoparticles extracted from the TGA curves.

#### $r_1$ and $r_2$ relaxivity and $R_1$ and $R_2$ map image measurements

$T_1$  and  $T_2$  water proton spin relaxation times and  $R_1$  and  $R_2$  water proton spin relaxation map images were measured using a 3.0 T MRI scanner (Magnetom Trio Tim, Siemens, Munich, Bayern, Germany). Aqueous dilute nanoparticle suspension samples (1.0, 0.5, 0.25, 0.125, and 0.0625 mM Ho or Tm) were prepared *via* dilution of the original concentrated nanoparticle suspension samples ( $\sim 20$  mM Ho or Tm) with triple-distilled water. These dilute solutions were used to measure  $T_1$  and  $T_2$  relaxation times and  $R_1$  and  $R_2$  map images. Next,  $r_1$  and  $r_2$  water proton spin relaxivities of the nanoparticle suspension samples were estimated from the slopes of  $1/T_1$  and  $1/T_2$  plots *versus* the Ho or Tm concentration, respectively.  $T_1$  relaxation time measurements were conducted using an inversion recovery method. In this method, the inversion time (TI) was varied, and the MR images were acquired at 35 different TI values in the range of 50–1750 ms.  $T_1$  Relaxation times were obtained from nonlinear least-square fits to the measured signal intensities at various TI values. For the measurements of  $T_2$  relaxation times, the Carr–Purcell–Meiboom–Gill pulse sequence was used for

multiple spin-echo measurements, and 34 images were acquired at 34 different echo time (TE) values in the range of 10–1900 ms.  $T_2$  relaxation times were obtained from nonlinear least-square fits to the mean pixel values of the multiple spin-echo measurements at various TE values.

#### *In vitro* cellular cytotoxicity measurements

The *in vitro* cellular cytotoxicity of the nanoparticles was measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA). The intracellular adenosine triphosphate was quantified using a Victor 3 luminometer (PerkinElmer, Waltham, MA, USA). The human prostate cancer (DU145), human embryonic kidney 293 (HEK293), and human liver cancer (HepG2) cell lines were used. The cells were seeded into a separate 24-well cell culture plate and incubated for 24 h ( $5 \times 10^4$  cell density, 500  $\mu\text{L}$  cells per well, 5%  $\text{CO}_2$ , and 37 °C). Four test solutions (10, 50, 100, 200, and 500  $\mu\text{M}$  Ho or Tm) were prepared *via* dilution of the original concentrated nanoparticle suspension samples with a sterile phosphate-buffered saline solution, and 2.0 mL aliquots were used to treat the cells, which were subsequently incubated for 48 h. Cell viabilities were measured thrice to obtain average cell viabilities, which were then normalized with respect to those of untreated control cells (0.0 mM Ho or Tm).

#### Hemolysis assay

Mice blood (balb/c, 19–20 g, male, 6 weeks old) was collected and immediately mixed with heparinized saline (Sigma-Aldrich, catalog no. H3393-50KU, 20 units per ml) to prevent coagulation. Nanoparticle samples (1, 0.5, and 0.25 mM Ho or Tm, 490  $\mu\text{L}$ ) were mixed with the heparinized blood (10  $\mu\text{L}$ ) and incubated at 37 °C for 1 h. 1% TritonX-100 and saline (0.9% NaCl) were used as a positive and negative control, respectively. The incubated blood samples were centrifuged at 10000 rpm for 5 min to remove intact erythrocytes and the supernatants of each sample were obtained. The lysed hemoglobin in the supernatants was quantified with a hemoglobin assay kit (Sigma-Aldrich, catalog no. MAK115) according to the manufacturer's instructions. The hemolysis rate was estimated as follows:

$$\text{Hemolysis rate (\%)} = \frac{[H_{\text{NPs}} - H_{1\% \text{ tritonX-100}}]}{[H_{\text{saline}} - H_{1\% \text{ tritonX-100}}]} \times 100,$$





where H is the amount of lysed hemoglobin. The experiments were performed thrice.

### *In vivo* $T_2$ MR image measurements

*In vivo* animal imaging experiments were conducted in accordance with the rules and regulations and permission of the animal research committee of the Korea Institute of Radiological and Medical Sciences (IACC number = 2021-0078). A 3.0 T MRI scanner (Magnetom Trio Tim, Siemens, Munich, Bayern, Germany) was used to obtain *in vivo*  $T_2$  MR images. Two balb/c male mice weighing 25–27 g were used for each aqueous nanoparticle suspension sample. The mice were anesthetized using 1.5% isoflurane in oxygen. Measurements were made before and after injecting the nanoparticle suspension sample into mice's tail veins. The injection dose was approximately 0.087–0.1 mmol Ho or Tm kg<sup>-1</sup>. The fast spin-echo sequence was used to obtain  $T_2$  MR images. The typical parameters for coronal (or axial) image measurements were as follows:  $H = 3.0$  T, echo time (TE) = 37 (36) ms, repetition time (TR) = 1620 (1629) ms, echo train length = 13 (13) mm, pixel bandwidth = 197 (197) mm, flip angle = 120 (120) degree, width = 60 (60) mm, height = 60 (30) mm, number of acquisitions (NEX) = 3 (4), slice thickness = 1.0 (1.2) mm, and slice gap = 1.1 (3.0) mm, where the numbers in parentheses are the parameters used for axial image measurements.

### *In vivo* biodistribution study

PEGD250-coated Ho<sub>2</sub>O<sub>3</sub> and PEGD600-coated Tm<sub>2</sub>O<sub>3</sub> nanoparticles which were used for *in vivo* MRI experiments were injected into normal balb/c mice tail veins with a 0.1 mmol Ho or Tm kg<sup>-1</sup> dosage (19–20 g, 6 weeks old, male,  $n = 3$ ). To obtain organ samples (*i.e.*, lung, heart, liver, intestine, and kidney), the mice were anesthetized and exsanguinated 12 h after injection. The extracted organs were digested with 65% nitric acid and 30% hydrogen peroxide at 180 °C for 2 h. The digested samples were diluted with 3% nitric acid to a defined weight to measure Ho or Tm concentrations using an ICP-AES. Then, the Ho or Tm concentration was converted into the injected dose per gram of organ (ID%/g) with normalization to a 20 g mouse using the formula: ID%/g = (weight of Ho or Tm in the organ/weight of organ)  $\times$  100  $\times$  (weight of mouse/20).

### Histological analysis

PEGD250-coated Ho<sub>2</sub>O<sub>3</sub> and PEGD600-coated Tm<sub>2</sub>O<sub>3</sub> nanoparticles which were used for *in vivo* MRI experiments were injected into normal balb/c mice tail veins with a 0.1 mmol Ho or Tm kg<sup>-1</sup> dosage (19–20 g, 6 weeks old, male,  $n = 3$ ). The mice were anesthetized using 1.5% isoflurane in oxygen and exsanguinated to obtain the kidney and the liver 24 h after injection. The organ samples were fixed in 4% paraformaldehyde for 72 h and treated with ethanol (concentration gradient 50, 70, 95, 100%), xylene (Junsei Chemical, Japan), and paraffin for 30 min. The organs were sectioned into 5  $\mu$ m thickness and then treated with xylene for 1 h and ethanol (concentration gradient 100, 95, 70, and 50%) for 10 min at 65 °C. Hematoxylin and eosin (H&E) staining (BBC Biochemical, Mount Vernon,

WA, USA) was performed according to the manufacturer's instructions and the stained samples were observed using a microscope (ECLIPSE Ti, Nikon, Tokyo, Japan) to assess acute *in vivo* toxicity.

## Conclusions

In summary, various hydrophilic and biocompatible ligand-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> and Tm<sub>2</sub>O<sub>3</sub> nanoparticles were synthesized *via* a one-pot polyol method (ligand = PEGD250, PEGD600, and PAA1800), and their  $r_1$  and  $r_2$  values and *in vivo*  $T_2$  MR images at a 3.0 T MR field were measured to investigate their potential as a new class of efficient  $T_2$  MRI contrast agents. The results are summarized below:

- (1) The average particle diameters were approximately 2.1 nm for all nanoparticle samples.
- (2) The negligible  $r_1$  ( $< 0.2$  s<sup>-1</sup> mM<sup>-1</sup>) and appreciable  $r_2$  values were observed for all nanoparticle samples, owing to 4f-electron orbital motion contributions of Ho<sup>3+</sup> and Tm<sup>3+</sup> to nanoparticle magnetic moments. The  $r_2$  value increased with increasing nanoparticle magnetic moments [from 1.73 (Tm<sub>2</sub>O<sub>3</sub>) to 4.64 (Ho<sub>2</sub>O<sub>3</sub>) emu g<sup>-1</sup> at 2.0 T and 300 K] and decreased with increasing ligand-size (PEGD250 < PEGD600 < PAA1800). These two factors explained the observed  $r_2$  values such that 30.39 s<sup>-1</sup> mM<sup>-1</sup> (PEGD250-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> nanoparticles) > 11.33 s<sup>-1</sup> mM<sup>-1</sup> (PEGD600-coated ultrasmall Ho<sub>2</sub>O<sub>3</sub> nanoparticles) > 5.79 s<sup>-1</sup> mM<sup>-1</sup> (PEGD600-coated ultrasmall Tm<sub>2</sub>O<sub>3</sub> nanoparticles) > 1.03 s<sup>-1</sup> mM<sup>-1</sup> (PAA1800-coated ultrasmall Tm<sub>2</sub>O<sub>3</sub> nanoparticles).
- (3) Owing to the above  $r_1$  and  $r_2$  values, appreciable negative contrast enhancements were observed in *in vivo*  $T_2$  MR images at a 3.0 T MR field, which demonstrated the potential of the ultrasmall Ho<sub>2</sub>O<sub>3</sub> and Tm<sub>2</sub>O<sub>3</sub> nanoparticles as a new class of efficient  $T_2$  MRI contrast agents.

## Author contributions

S. L. synthesized and characterized the samples and wrote the draft manuscript; T. T., H. Y., S. L. H., M. Y. A., A. K. A. A. S., D. Z., and Y. L. assisted with the synthesis and characterization of the samples; S. K. and J. A. P. obtained *in vivo* MR images; A. B. performed the hemolytic assay, H&E staining and biodistribution; S. K. measured relaxivities and map images; S. H. Y., D. W. H., and K.-S. C. measured cellular cytotoxicities; S.-W. N. assisted with the project; Y. C. and G. H. L. led the project; and G. H. L. wrote the manuscript.

## Conflicts of interest

There are no conflicts to declare.

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