Cytotoxicity, tumor targeting and PET imaging of sub-5 nm KGdF₄ multifunctional rare earth nanoparticles†

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Ultrasmall sub-5 nm KGdF₄ rare earth nanoparticles were synthesized as multifunctional probes for fluorescent, magnetic, and radionuclide imaging. The cytotoxicity of these nanoparticles in human glioblastoma U87MG and human non-small cell lung carcinoma H1299 cells was evaluated, and their application for in vitro and in vivo tumor targeted imaging has also been demonstrated.

Rare earth nanoparticles (REs) have recently attracted enormous attention in the field of biological imaging owing to their unique optical properties, such as narrow emission bandwidths, large Stokes shifts, long fluorescence lifetimes and photostability.1-6 In particular, REs can be excited with near-infrared (NIR) to emit in both the visible and infrared region of the electromagnetic spectrum, through the up-conversion and down-conversion process, respectively. Up-conversion luminescence occurs during the excitation of trivalent rare earth ions by the sequential absorption of two or more NIR photons, and such a unique luminescence mechanism excludes both conventional luminescent labels and endogenous fluorescent substances. REs are also capable of generating short-wavelength infrared emissions (SWIR, 1000–2300 nm) with large Stokes shifts after NIR excitation through down-conversion fluorescence mechanisms.6

Furthermore, REs are also useful for multimodal in vivo imaging because simple variations in the composition of the lattice atoms and dopant ions integrated into the REs can be easily implemented, yielding various distinct biomedical activities relevant to magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single-photon emission computed tomography (SPECT), and photoacoustic imaging.7-13 These multiple functions embedded in a single type of REs play a crucial role in precise disease diagnosis. In particular, with their increasing bioapplications, the potential dissemination of REs and their interactions in the human body have increased.14-16 The studies on the toxicity of REs were mostly limited to NaMF₄, (M = Y³⁺, Gd³⁺, Lu³⁺) hosts. The previous results demonstrated that those REs exhibited a low toxicity effect on cells and animals in most cases.17-19 However, there are few reports on the toxicity of REs based on the KGdF₄ host.20,21

In addition, the particle size is a key factor requiring consideration to realize the application of REs in biomedical imaging. Current REs are typically larger than 10 nm, which is not optimal for use as a bioimaging probe. It is recently demonstrated that the nanoparticles with size less than 10 nm are easily taken up and excreted, and show longer blood circulation times in comparison with larger ones.22-25 However, the size of the particle and the upconversion emission intensity are mutually dependent parameters, and in general a smaller nanoparticle size will result in a weaker emission. So it is a big challenge to test the in vivo behavior of ultrasmall sub-5 nm REs by optical imaging technology. To overcome this deficiency, herein, PET is chosen to detect the in vivo biodistribution and tumor imaging of ultrasmall REs because it shows unlimited tissue penetration compared with fluorescent imaging and exhibits higher sensitivity than both MRI and CT.

In this work, we report REs based on the KGdF₄ host as nanoprobe for in vitro and in vivo tumor imaging for the first time. The prepared KGdF₄ REs were sub-5 nm in diameter, and exhibited up/down-conversion luminescence by doped Yb³⁺/Tm³⁺ and Eu³⁺, respectively. Moreover, these REs were applied to target imaging of human glioblastoma U87MG cells by conjugating with the RGD peptide, and no obvious cytotoxicity was detected. Furthermore, to visualize the in vivo behavior

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of KGdF₄ by PET imaging, ¹⁸F⁻ was labeled with KGdF₄, and ¹⁸F⁻ labeled KGdF₄ REs were able to image U87MG and H1299 tumors in living mice after intravenous injection.

The KGdF₄ host possesses several attractive merits as multifunctional REs such as the tendency to form ultrasmall size nanoparticles (∼10 nm), the absence of phase change down to ∼3.7 nm, and the intrinsic magnetic and luminescence properties.⁴⁶ Therefore, we chose KGdF₄ as a host to obtain the ultrasmall sub-5 nm multifunctional REs. Oleic acid (OA)-capped KGdF₄ REs were synthesized by a modified hydrothermal route. Due to the presence of oleic acid on the surface of KGdF₄ REs, the KGdF₄-OA sample was well dispersed in nonpolar solvents such as cyclohexane, chloroform, and dichloromethane. Therefore, surface functionalization of OA-capped KGdF₄ REs is required prior to biological application. Herein, using PAA coating methods by a modified ligand exchange procedure, hydrophobic KGdF₄-OA was easily converted into the hydrophilic species. Following the exchange with oleic acid, the resultant PAA-conjugated KGdF₄ possessed two properties: (I) good dispersibility in aqueous solutions, and (II) carboxyl functional groups on the surface of REs to allow conjugation with biological molecules (such as peptides) for further targeted in vitro and in vivo studies.

As shown in Fig. 1, transmission electron microscopy (TEM) images showed that the KGdF₄ REs were quite monodisperse with an average diameter of 3.79 nm. High-resolution TEM images suggested that the KGdF₄ REs were single crystals with an interplanar spacing of 3.1 Å, which could be indexed as the (110) lattice planes. Furthermore, the energy-dispersive X-ray analysis (EDXA) patterns confirmed the presence of K, Gd, and F elements in the as-synthesized samples (Fig. S1†). The crystal structure of the as-synthesized KGdF₄ REs was identified using powder X-ray diffraction (XRD) analysis (Fig. S2†). The broad XRD peaks imply that the obtained particles fall within the nano domain. Although XRD peak intensities are very weak, the crystal phase could still be identified. The XRD patterns could be indexed as the cubic phase of NaGdF₄ (JCPDS No. 27-0697), which was in good agreement with that reported by Capobianco et al.⁴⁶ The dynamic light scattering (DLS) measurement indicated that the effective hydrodynamic diameter of the OA-capped KGdF₄ REs was ∼4.9 nm (Fig. 1D). After PAA coating, the FTIR spectrum showed the stretching mode of the –COOH group at 1727 cm⁻¹, suggesting PAA bonding to the particle surface (Fig. S3†). And the effective hydrodynamic diameter of the PAA-coated KGdF₄ REs reached ∼30 nm (Fig. 1E). This increase in hydrodynamic diameter was attributed to the linkage of the PAA polymer to the surface of KGdF₄ REs. The zeta potential of the PAA-coated KGdF₄ REs in water was about −13 mV (Fig. S4†). Thermogravimetry analysis (TGA) showed that the percentage of PAA on the KGdF₄ REs was approximately 13% (Fig. S5†). In addition, DLS analysis exhibited that PAA-coated KGdF₄ REs were stable in water for weeks without aggregation (Fig. S6†).

The up/down-conversion luminescence of KGdF₄ REs was obtained by doped Yb³⁺/Tm³⁺ and Eu³⁺, respectively. As shown in Fig. 2A, under the excitation of a CW laser at 980 nm, the
up-conversion luminescence spectrum of the KGdF4:Yb3+, Tm3+ sample exhibited three Tm3+ emission bands. The up-conversion luminescence bands at 476, 694 and 803 nm originated from 2F7/2→3H6, 3F4→3H6 and 3H4→3H6 transitions of Tm3+, respectively.

The down-conversion luminescence properties of the KGdF4:Eu3+ were characterized by excitation and emission spectra (Fig. 2B). The excitation spectra consisted of the characteristic absorption peaks of Eu3+ corresponding to the direct excitation from the europium ground state into the higher excited states of the Eu3+ f-electrons. The most intense peak was centered at 393 nm, which can be assigned to the 7F0→5L6 transitions of Eu3+ ions. Under excitation at 393 nm, the emission spectra were composed of three strong emission peaks at about 591 nm, 611 nm, and 698 nm, which can be attributed to the 5D0→7F2, 5D0→7F1 and 5D0→7F0 transitions of Eu3+, respectively. The intensity of electric dipole transition (5D0→7F2) at 611 nm was slightly higher than that of magnetic dipole transition (5D0→7F1) at 591 nm.

The longitudinal relaxation time (T1) was measured in aqueous solutions with different Gd3+ concentrations. To evaluate the ionic relaxivities, the Gd3+ concentration of the KGdF4 REs was determined using ICP-MS, after digesting the KGdF4 REs in concentrated nitric acid. From the slope of the plot of 1/T1 versus the Gd3+ concentration (Fig. 3), the ionic longitudinal relaxivity (r1) was determined to be 3.05 ± 0.32 s−1 mM−1.

The cytotoxicity of the KGdF4 REs was evaluated by the CCK-8 assay in human non-small cell lung carcinoma H1299 and human glioblastoma U87MG cells (Fig. 4). The viability of the cells above a 10–1000 μg mL−1 concentration of KGdF4 REs was slightly decreased, and the difference was statistically significant. After 12 h of incubation with KGdF4 REs, the cellular viability was estimated to be greater than 96% for both cell lines. After 24 h of incubation with KGdF4 REs, the cells maintained greater than 94% and 88% cell viabilities for H1299 and U87MG cells, respectively. Even after 48 h of incubation with KGdF4 REs, more than 76% of H1299 cells and 62% of U87MG cells were viable, respectively. These results demonstrated the weak toxic effects of KGdF4 REs on cell viability under these conditions.

Integrin αvβ3 plays a pivotal role in tumor angiogenesis and is a receptor for the extracellular matrix proteins with the exposed RGD tripeptide sequence.3,27,28 Herein, c(RGDFK) was chosen as a target ligand for further application in targeted imaging of cancer cells based on KGdF4:Eu3+ REs. The covalent coupling of c(RGDFK) to the surface of PAA-coated KGdF4:Eu3+ REs was facilitated by EDC, which activated the carboxyl groups of KGdF4:Eu3+ REs and led to the formation of amide bonds. To evaluate the αvβ3 integrin specificity of the RGD-conjugated KGdF4:Eu3+ REs, U87MG cells (expressing high levels of integrin αvβ3) were chosen for target-specific imaging, whereas H1299 cells (expressing low levels of integrin αvβ3) were used in the control experiments. The living cells were incubated with KGdF4:Eu3+ REs (~20 μg mL−1) for 2 h at 37 °C. Cell imaging was then performed by confocal luminescence microscopy. As shown in Fig. 5B, intense red luminescence signals were detected within the U87MG cells after 2 h of incubation with RGD-conjugated KGdF4:Eu3+ REs at 37 °C, and no aggregation of REs was observed. Bright-field measurements after treatment with KGdF4:Eu3+ REs confirmed that the cells were viable throughout the imaging experiments. In con-
contrast, probe controls (PAA-coated KGdF$_4$:Eu$^{3+}$ REs) showed weak luminescence emission (Fig. 5A). In addition, the luminescence signal of RGD-conjugated KGdF$_4$:Eu$^{3+}$ REs was mainly observed in the cytoplasmic region of the U87MG cells (Fig. 5B), while the luminescence signal of PAA-coated KGdF$_4$:Eu$^{3+}$ REs was mainly detected on the cell membrane (Fig. 5A). The integrin receptor specificity of KGdF$_4$:Eu$^{3+}$ REs was further evaluated by the cell control assay; slightly weaker luminescence signals were detected in the control H1299 cells (Fig. 5C, D) compared with that detected in the U87MG cells after RGD-conjugated KGdF$_4$:Eu$^{3+}$ RE incubation (Fig. 5B). And no obvious luminescence intensity changes were observed between the PAA-coated KGdF$_4$:Eu$^{3+}$ REs (Fig. 5C) and the RGD-conjugated KGdF$_4$:Eu$^{3+}$ REs (Fig. 5D) in the control H1299 cells. Z scanning analysis showed that the luminescence signals of both PAA- and RGD-conjugated KGdF$_4$:Eu$^{3+}$ REs were mainly observed in the perinuclear cytoplasmic region of the H1299 cells (Fig. S7†).

Fluorine-18 ($^{18}$F) is often used for PET imaging due to its ease in production in high quantities on a medical cyclotron and an ideal half-life of about 110 min, but its labeling reaction generally requires multiple synthetic steps often under harsh conditions and tedious purification processes.$^{29-31}$ Recently, the reaction between fluoride and rare-earth metal ions has been applied to label REs with $^{18}$F$^{-}$. Therefore, $^{18}$F$^{-}$ was chosen to label PAA-coated KGdF$_4$:Eu$^{3+}$ REs for PET imaging. $^{18}$F-labeling was carried out by simply mixing $[^{18}$F]KF solution with the aqueous solutions of KGdF$_4$ REs at room temperature followed by 10 min incubation, and free $^{18}$F$^{-}$ was easily removed by centrifugation. The $^{18}$F-labeling yield for KGdF$_4$ REs was estimated to be $\sim$50%. Under the same conditions, the $^{18}$F-labeling yield for the large size NaYF$_4$ REs with an average diameter of $\sim$25 nm (Fig. S8†) was $\sim$80%, which is higher than that for the sub-5 nm KGdF$_4$ REs.

For in vivo imaging studies, athymic nude mice bearing a U87MG or H1299 tumor on the left shoulder (stomach position) were administered the $^{18}$F-labeled KGdF$_4$ REs ($\sim$60 μCi/2.22 MBq) through tail-vein injection. At 1 h after injection, the mice were imaged using the MicroPET/CT imaging system. The strong uptake of $[^{18}$F]KGdF$_4$ REs in the lung could be clearly visualized (Fig. 6), indicating the aggregation of some sub-5 nm KGdF$_4$ REs. Long-time and high-speed centrifugation may lead to the aggregation of sub-5 nm KGdF$_4$ REs in the purification process of removing the free $^{18}$F$^{-}$. Under the same conditions, nearly no uptake in the lung of mice was observed for the large size NaYF$_4$ REs ($\sim$25 nm) from the MicroPET/CT imaging (Fig. S9†). These data indicated that small size REs are more likely to aggregate than large size REs.

The region of interest (ROI) analysis of the U87MG tumor-bearing mouse showed that the mean standardized uptake value (SUV) of $[^{18}$F]KGdF$_4$ REs in the lung, liver, bladder and bone was 13.6, 2.7, 6.6 and 2.4, respectively. For the H1299 tumor-bearing mouse, the SUV of $[^{18}$F]KGdF$_4$ REs in the lung, liver, bladder and bone was 13.6, 2.7, 6.6 and 2.4, respectively. For the H1299 tumor-bearing mouse, the SUV of $[^{18}$F]KGdF$_4$ REs in the lung,
liver, bladder and bone was 7.5, 2.3, 16.3 and 1.7, respectively. In addition, accumulation of the $[^{18}F]$KGdF$_4$ REs was also visualized in the tumor regions, which is likely due to the enhanced permeability and retention effect. The uptake of the $[^{18}F]$KGdF$_4$ REs in the U87MG tumor was slightly higher than that in the H1299 tumor. The SUV was 0.13 in the U87MG tumor and 0.085 in the H1299 tumor, respectively.

The accurate amount of KGdF$_4$ REs in the main organs (heart, liver, spleen, lung, kidneys, bone, urine and blood) was measured by ICP-MS analysis (Fig. S10). A high uptake of the KGdF$_4$ REs was detected in the liver, spleen, lung and blood at 1 h post injection. Slight uptake in the bone and area was also detected, and the value is lower than that obtained from the SUV result, suggesting that a low level of defluorination of $[^{18}F]$KGdF$_4$ REs may occur in vivo. Additionally, the in vivo MR imaging was also carried out to support the biodistribution of KGdF$_4$ REs. The pre-contrast and post-contrast $T_1$-weighted MR images were recorded before and after 1 h injection of 400 µL KGdF$_4$ REs (∼50 µg). It was noteworthy that the KGdF$_4$ REs could induce an efficient positive-contrast enhancement in the liver, spleen, lung, heart, and kidney (Fig. S11, Table S1†), which was consistent with the ICP-MS results.

In conclusion, we report sub-5 nm REs based on the KGdF$_4$ host as nanoprobes for in vitro and in vivo imaging. The prepared KGdF$_4$ REs exhibited the up/down-conversion luminescence by doped Yb$^{3+}$/Tm$^{3+}$ and Eu$^{3+}$, respectively. Moreover, these KGdF$_4$ REs showed low cytotoxicity on U87MG and H1299 cells. After conjugating with the RGD peptide, these KGdF$_4$ REs were applied to target imaging of U87MG cells in vitro. In addition, $^{18}$F$^-$ was labeled with KGdF$_4$ REs for PET imaging, and these $^{18}$F$^-$ labeled KGdF$_4$ REs were able to image U87MG and H1299 tumors in living mice after intravenous injection. To the best of our knowledge, this is the first successful demonstration of sub-5 nm REs for in vivo tumor imaging. This study provides a foundation for the development of the whole-body tumor imaging based on the use of ultrasmall REs as multifunctional nanoprobes.

Author contributions

Liqin Xiong conceived the project, analyzed the data and wrote the manuscript. Xinmin Cao, Fengwen Cao, Yixiao Guo, and Yimin Zhang prepared and characterized the nanoparticles. Xinmin Cao performed the relaxation study and analyzed the data. Fengwen Cao performed the cell imaging studies and analyzed the data. Xi Cai performed the cytotoxicity studies and analyzed the data. Liqin Xiong performed tumor xenografts. Xinmin Cao and Wangxi Hai performed the MicroPET/CT imaging studies and analyzed the data. Biao Li helped with the supply of the $[^{18}F]$KF aqueous solution. Tianye Cao, Yang Yang, and Fuyou Li provided advice on nanoparticle preparation, discussed the TEM and DLS results and commented on the project.

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References


