Small-Molecule Lanthanide Complexes Probe for Second Near-Infrared Window Bioimaging

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Supporting Information

ABSTRACT: Over the past few years, significant efforts have been made to create new fluorescent probes operating at longer wavelengths, particularly in the second near-infrared (NIR-II) window from 1000 to 1700 nm, offering enhanced tissue penetration compared to light in the visible and first near-infrared window (700–900 nm). However, most of the reported NIR-II fluorophores meet such dilemmas; they are excreted slowly and largely retained within the reticuloendothelial system. Here, we report a rapidly excreted NIR-II lanthanide complex Nd-DOTA (over 50% excreted through the kidneys within 3 h postinjection) with a molecular mass only 0.54 kDa. The NIR-II imaging quality of Nd-DOTA was far superior to that of clinically approved ICG with good photostability and deep tissue penetration (7 mm). Superior tumor-to-normal tissue ratio was successfully achieved to facilitate the abdominal ovarian metastases surgical delineation. Metastases with ≤1 mm can be completely excised under NIR-II bioimaging guidance. Significantly, since the Nd-DOTA structure is same to the clinically approved magnetic resonance imaging (MRI) contrast Gd-DOTA, it will speed up the clinical translation for this novel kind of NIR-II probes in the future.

Intraoperative detection of microscopic residual cancer in the tumor bed could be used to decrease the risk of a positive surgical margin, reduce rates of reexcision, and tailor adjuvant therapy.1 However, during surgery, palpation and visual inspection are not always sufficient for discriminating between malignant and normal tissue types and can lead to incomplete resections or the unnecessary removal of healthy tissue.2 Radiologic approaches such as X-ray, CT, MRI, and ultrasound have been considered for use in assisting surgical procedures; however, these methods usually suffer from disadvantages, such as nontumor specificity, low sensitivity, and high expense.3 Furthermore, they are generally not applicable for intraoperative applications such as surgical inspection and practice.4

Over the past several years, intraoperative imaging using near-infrared (NIR) fluorescent light has been used to fill the gap between preoperative imaging and intraoperative reality by way of its improved contrast and depth of tissue penetration relative to visible light.5,6 At present there are only two clinically approved nonspecific NIR probes, methylene blue (MB), and indocyanine green (ICG),7 both of which can be excreted with small molecular size. The mean tumor-to-background ratio (TBR) with these imaging agents ranges from 1.2 to 8.5 in mouse and human studies.8–10 However, their fluorescence emission lies within the first near-infrared window (NIR-I; 750–900 nm), the penetration depth merely reaches 1–3 mm.11–15 Recently, significant efforts have been made to create new fluorescent probes operating at longer wavelengths, particularly in the second near-infrared (NIR-II) window from 1000 to 1700 nm11,14–18 with low absorbance and tissue autofluorescence compared to NIR-I but up to a 1000-fold reduction in scattering losses, offering enhanced tissue penetration (~5–20 mm) compared to light in the first near-infrared window.11–19 The power to achieve sub-10–μm high-resolution imaging up to centimeters of depth makes NIR-II fluorescence imaging promising for clinical applications ranging from noninvasive diagnosis to image-guided surgery.

Thus, far, inorganic nanomaterials, such as carbon nanotubes,14,15 quantum dots (e.g., Ag,S),16,20,21 lanthanide nano-
particles, and other nanoparticles are the major probes that have been used for biological imaging in the NIR-II region. Owing to unknown long-term toxicity concerns, it would be desirable to develop small-molecule NIR-II probes to facilitate FDA approval and clinical translation.

Currently, only a small number of organic molecules are known with fluorescence in the >1000 nm region, all of which are highly hydrophilic, water-insoluble cyanine, and a handful of polymethine dyes or thiopyrilium dyes. Furthermore, most of them must be encapsulated in a polymer matrix for bioimaging with increased particle size over the renal filtration threshold (~40 kDa). Most recently, a small-molecule CH1055 derivatives have been reported. CH1055 derivatives have been reported.30

**Synthesis of Nd-DOTA.** NdCl₃ (36.6 mg, 0.1 mmol) was slowly added to a solution of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (40.3 mg, 0.1 mmol) in deionized water (4 mL), followed by slow adjustment of the pH to 6.5 using an aqueous 0.4 M NaOH solution. The solution was stirred for 24 h at room temperature. The reaction mixture was concentrated in vacuum, and the residue was purified by precipitate with anhydrous ether and then filtered to give a bright white solid Nd-DOTA.

**Synthesis of Nd-DOTA-NHS.** The synthesis of Nd-DOTA-NHS was similar to that of Nd-DOTA complex. NdCl₃ (18.9 mg, 0.05 mmol) was slowly added to a solution of 2,2',2''-(10-((2-(1,2-dithiolan-3-yl)pentanamido)ethyl)amino-1-carboxy-4-oxobutyl)-1,4,7 triyl) tricarboxylic acid (DOTA-NHS) (38.5 mg, 0.05 mmol) in deionized water (2 mL), followed by slow adjustment of the pH to 6.5 using an aqueous 0.4 M NaOH solution. The solution was stirred for 24 h at room temperature. The reaction mixture was concentrated in vacuum, and the residue was purified by precipitate with anhydrous ether and then filtered to give a bright white solid Nd-DOTA-NHS.

**Lifetime and Quantum Yield of Nd-DOTA.** All fluorescence was collected using an external semiconductor laser (808 nm). Absorbance was collected on Lambda 750 S PerkinElmer instrument. The optical density values associated each spectra corresponds to the absorbance at 808 nm. The quantum yield was calculated in the following manner:

\[
QY_{sample} = \frac{QY_{IR26}}{n_2} \times \frac{A_{IR26}}{A_{IR26} - A_{IR26}} \times \frac{I_{sample}}{I_{IR26}}
\]

where \(QY_{sample}\) and \(QY_{IR26}\) are the quantum yields of the sample to be determined (Nd-DOTA) and the referenced standard sample (IR26), respectively; \(n_1\) and \(n_2\) are the average refractive index of the solvent (\(H_2O\) for Nd-DOTA, \(CH_2Cl_2\) for IR26) used for dissolving measured sample (Nd-DOTA) and referenced standard sample (IR26), respectively; \(A_{sample}\) and \(A_{IR26}\) are absorption value under 808 nm by Nd-DOTA and IR-26, respectively; \(I_{sample}\) and \(I_{IR26}\) are the emission intensity under 808 nm excitation for Nd-DOTA and IR-26, respectively; \(k_{sample}\) and \(k_{IR26}\) are the slopes for Nd-DOTA and IR-26 with the integrated fluorescence intensity at a single concentration to that of the reference, respectively. According to the reported method, instead of comparing the integrated fluorescence intensity at a single concentration to that of the reference, 5 difference concentrations at or below OD 0.1 (roughly OD 0.1, 0.0072, 0.0184, 0.0212, 0.0235, 0.0341 for Nd-DOTA and 0.0165, 0.0331, 0.0424, 0.0639, 0.0764 for IR-26) were measured and the integrated fluorescence was plotted against absorbance for both IR-26 and Nd-DOTA. Comparison of the slopes led to the determination of the quantum yield of Nd-DOTA.

**Cytotoxicity of Nd-DOTA/Nd-DOTA-FA Complex.** The cytotoxicity was measured by using the Cell Counting Kit-8 (CCK-8) assay in CaOV3 cell. The cells (1 × 10⁴) were incubated in each well of a 96-well plate at 37 °C under 5% CO₂ for 24 h. Then cells were incubated and cultured with the Nd-DOTA/Nd-DOTA-FA complex at different concentrations (0, 100, 200, 300, 400, 500 μg/mL) at 37 °C under 5% CO₂ for another 24 h. Finally, 10 μL/well of CCK-8 in PBS solution was added to each well and incubated at 37 °C for 4 h. The quantity was determined calorimetrically by using a multi reader (TECAN, Infinite M200). The measurements were based on the absorbance values at 450 nm. The following formula was used to calculate the viability of cell growth:

\[
\text{Viability} = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} \times 100\%
\]

where \(OD_{sample}\) and \(OD_{standard}\) are the average absorbance at 450 nm for the sample and standard, respectively. \(OD_{blank}\) is the absorbance at 450 nm of blank samples. 

**Experimental Section**

**Synthesis of Nd-DOTA.** NdCl₃ (36.6 mg, 0.1 mmol) was slowly added to a solution of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (40.3 mg, 0.1 mmol) in deionized water (4 mL), followed by slow adjustment of the pH to 6.5 using an aqueous 0.4 M NaOH solution. The solution was stirred for 24 h at room temperature. The reaction mixture was concentrated in vacuum and the residue was purified by precipitate with anhydrous ether and then filtered to give a bright purple solid Nd-DOTA.

**Synthesis of Nd-DOTA-NHS.** The synthesis of Nd-DOTA-NHS was similar to that of Nd-DOTA complex. NdCl₃ (18.9 mg, 0.05 mmol) was slowly added to a solution of

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viability(%) = (mean of absorbance value of treatment group/absorbance value of control) × 100

 Establishment of the Tumor Model. Subcutaneous Tumor Model. Animal procedures were in agreement with the guidelines of the Institutional Animal Care and Use Committee. CaOV3 cells (∼10⁶/dish) were seeded in cell culture flask in 8 mL of DMEM medium supplemented with 10% FBS and 1% antibiotics and incubated in CO₂ for 24 h at 37 °C. Then CaOV3 tumor cells were harvested by centrifugation and resuspended in sterile PBS. CaOV3 cells (5 × 10⁶ cells) were implanted subcutaneously into the right fore arm of 5-week-old mice. When the tumors reached 0.2–0.7 cm in diameter (12–28 days after implant), the tumor-bearing mice were subjected to imaging studies.

Peritoneal Ovarian Metastasis Model. CaOV3 cells (∼10⁶/dish) were seeded in cell culture flask in 8 mL of DMEM medium supplemented with 10% FBS and 1% antibiotics and incubated in CO₂ for 24 h at 37 °C. Then CaOV3 tumor cells were harvested by centrifugation and resuspended in sterile PBS. CaOV3 cells (5 × 10⁶ cells) were intraperitoneally injected into 5-week-old mice. Tumor size was observed under scarified mice from the first week.

Magnetic Resonance Imaging. In vivo MRI was carried out on a 3.0-T clinical MRI instrument (Siemens Magnetom TriO Tim 3.0 T MRI) in Shanghai Huashan Hospital. T₁-weighted MR images of the tumor sections were acquired with the turbo spin echo (TSE) sequence: TR = 2500 ms; TE = 76 ms; slice thickness = 1.5 mm; fat suppress, none; water suppress, none. MRI was taken after NIR (Supplementary Figure S-2). All collected tissues were harvested by centrifugation and resuspended in sterile PBS for 2 h (Figure 1c). Consistent with the previous study,33 the relative fluorescent brightness of Nd-DOTA was investigated by matching the absorbance at 808 nm (ε for 1060 nm excitation) and showed two NIR-II peaks around 1060 and 1330 nm, respectively, with large stokes-shift and narrow full-width at half-maximum (hw) (~25–30 nm), which is much narrower than that of previous reported organic dyes (~30–50 nm), or quantum dots (~100 nm). The lifetime of 1060 nm emission is ~5.5 μs (Supplementary Figure S-6).

Figure 1. (a) Chemical structure of Nd-DOTA and the one-step synthesis of Nd-DOTA. (b) Absorbance and fluorescent emission spectra of Nd-DOTA. The fluorescent emission spectrum was obtained with an 808 nm excitation laser. (c) Photostabilities of Nd-DOTA and ICG in a variety of biological media under continuous 808 nm exposure for 2 h at a power density of 0.24 W cm⁻², respectively. (d) Fluorescence images of capillaries of Nd-DOTA (NIR II) and ICG (NIR I) at depths of 0–7 mm in Intralipid excited at 808 nm. The Nd-DOTA sample shows less feature spread than that of the ICG sample. (e) Intensity decay of ICG and Nd-DOTA as a function of depth in Intralipid. (f) Feature width of Nd-DOTA and ICG capillary images as a function of depth in Intralipid, showing increased loss of feature integrity for ICG compared to Nd-DOTA. Error bars are derived from the uncertainty in the fitting of feature width. (g) Signal to background ratios of Nd-DOTA and ICG as a function of tissue phantom depth. Error bars, mean ± s.d. (n = 3).
In Vivo Pharmacokinetics. We performed whole-body imaging of nude mice after intravenous injection of a solution of Nd-DOTA molecular probe (150 μL) in deionized water at an injected dose of approximately 0.75 mg mL\(^{-1}\). After 10 min postinjection, fluorescence signals were observed within the bladder and the liver, lungs, and kidneys of the mouse also could be observed as the probe passed through these organs with the blood flow (Figure 2a). The strongest fluorescent signals in the bladder could be confirmed after 20 min postinjection, while the signals in other organs faded quickly and only the fluorescence in bladder could be observed after 100 min (Figure 2a,c). For comparison, same amount of ICG deionized water solution was injected to determine the difference in biodistribution and the long-term fate of each probe. The liver and spleen were clearly visualized almost immediately after the injection of ICG probe and no bladder fluorescence was noted (Figure 2a,d). According to a previous report,\textsuperscript{37} ICG shows reversible binding to serum proteins. Because it has both lipophilic and hydrophilic properties, ICG is 98% protein-bound \textit{in vivo}. Although it was previously thought to bind primarily to serum albumin,\textsuperscript{38} ICG molecule has also been shown to bind to serum globulins such as alpha1-lipoprotein (a high-density lipoprotein).\textsuperscript{39}

Based on the time-dependent variation of the NIR-II fluorescence throughout the body, we applied principal component analysis (PCA) to convert the time-dependent variation of the fluorescence intensity at various locations into spatially resolved components. PCA combines image pixels with a similar time dependence into a distinct principal component and assigns a pseudo color;\textsuperscript{40} this technique thus allows the facile delineation of different inner organs of the mouse, including the liver, lungs, and kidneys (Figure 2b). According to the PCA results for Nd-DOTA and ICG, respectively, the fast renal clearance of the Nd-DOTA through urine could be further confirmed in comparison to the long-term liver retention for the ICG probe (Figure 2b).

The pharmacokinetics were investigated by intravenously injected nude mice with 150 μL of Nd-DOTA and collecting urine and blood over the course of 9 h. With a molecular weight of 0.54 kDa, well below the renal filtration threshold of \(\sim 30-50\) kDa,\textsuperscript{33} ~50% of the Nd-DOTA probe was excreted through the urine within 3 h postinjection (Figure 2e). The half-life of Nd-DOTA in blood circulation was found to be approximately 4 min (Supplementary Figure S-9a). From the urine excretion data we estimated a renal elimination rate constant of 0.37 min\(^{-1}\) (Supplementary Figure S-9b). It is noteworthy that the pharmacokinetic property of Nd-DOTA is very similar to the clinically approved MRI contrast Gd-DOTA with the half-life of 4.6 min in blood circulation.\textsuperscript{41} A preliminary cellular toxicity assay shows no observable toxicity of Nd-DOTA even at relatively high doses up to 500 μg/mL (Supplementary Figure S-10).

Molecular Imaging of Tumor and \textit{in Vivo} Biodistribution of Nd-DOTA-FA. Encouraged by the excellent optics and pharmacokinetics properties of Nd-DOTA probe, we tried image-guided surgery for the ovarian tumor. The...
prognosis in advanced-stage ovarian cancer remains poor. The overall 5-year survival rate is 45%, and for stages III and IV it is only 20−25%. Currently, cytoreductive surgery followed by combination chemotherapy is regarded as the most effective treatment. The degree of cytoreduction, in which minimal residual disease is defined as tumor deposits <1 cm, is one of the few prognostic factors that can be actively influenced by the surgeon. The overexpression of folate receptor-α (FR-α) in 90−95% of epithelial ovarian cancers (EOC) prompted the investigation of intraoperative tumor-specific fluorescence imaging in ovarian cancer surgery using an FR-α-targeted fluorescent agent. In this study, folate conjugated to Nd-DOTA (Nd-DOTA-FA) (Supplementary Figures S-11 and S-12) for targeting FR-α is used together with a real-time NIR-II intraoperative fluorescence imaging system. The fluorescent emission spectrum of Nd-DOTA-FA was taken with the 808 nm excitation and showed NIR-II peak around 1060 nm with comparable emission intensity to Nd-DOTA, indicating the FA conjugation had little effect on the NIR-II optical properties (Supplementary Figure S-13). Cellular toxicity assay showed no obvious toxicity of Nd-DOTA-FA even at relatively high doses up to 500 μg/mL (Supplementary Figure S-14). The photostability comparison between Nd-DOTA-FA and ICG under continuous 808 nm laser irradiation for various time indicated that, compared with the clinically approved ICG probe, superior photostabilities of Nd-DOTA-FA were observed in water (Supplementary Figure S-15). The FA conjugated probe was then intravenously injected (0.75 mg mL⁻¹) in immune deficient mice possessing xenograft human EOC tumors. Nd-DOTA-FA showed rapid passive tumor uptake, and the tumor fluorescence was clearly observed at 0.5 h postinjection (1000 LP, 300 ms, Figure 3a), which is far superior to that of ICG (3.5 h postinjection), then at 3.5 h postinjection the T/NT ratio reached 11.27 (Figure 3b), significantly higher than that of the approved ICG probe. Significantly, according to the Rose criterion, which states that a T/NT ratio of 5 is needed to distinguish image features with 100% certainty, Nd-DOTA-FA is favorable for stable image-guided tumor surgery. The exact Nd-DOTA-FA accumulated in tumor and other organs were latterly investigated by the inductively coupled plasma atomic emission spectrometer (Figure 3c), exhibiting long-term retention in tumor from 0.5 to 6 h.

Surgical Resection under NIR-II Fluorescence Bioimaging. Tumor delineating effects of NIR-II fluorescence bioimaging were further evaluated by using common preclinical imaging modalities such as MRI and histopathological analyses. As shown in Figure 4b, the tumor margin can be readily distinguished by MRI. The tumor profile detected by the NIR-II imaging at 1 h postinjection (Figure 4a) exhibited excellent consistency with that of MRI result (Figure 4c). The tumor size ratio of MRI and NIR-II fluorescence bioimaging after intravenous injection and the corresponding merged images of NIR-II fluorescence and MR images. (d) H & E staining of the tumors resected at 1 h postinjection under NIR-II fluorescent bioimaging guidance. (e–g) Surgical resection of abdominal tumor under NIR-II fluorescence bioimaging at 30 min postinjection (e, f) and the enlargement of the NIR-II fluorescence bioimaging results of the peritoneal metastatic tumors (g). (h) T/NT ratios plotted as a function of different labeled peritoneal metastatic tumors, red dotted line is according to the Rose criterion. Error bars, mean ± s.d. (n = 3). The injected dose was approximately 0.75 mg mL⁻¹ for both of Nd-DOTA-FA.

Figure 3. (a) NIR-II fluorescence bioimaging of a murine epidermal ovarian tumor in the nude mice after an intravenous injection of Nd-DOTA-FA (top) and ICG (down) under 808 nm NIR irradiation. The injected dose was approximately 0.75 mg mL⁻¹ for both of Nd-DOTA-FA and ICG. (b) The corresponding T/NT ratio of Nd-DOTA-FA (top) and ICG (down). (c) Time-dependent biodistribution of Nd-DOTA-FA complex in mice. Error bars, mean ± s.d. (n = 3).
metastatic lesions (nos. 1−5) are capable of being identified by NIR-II fluorescence bioimaging (Figure 4fg). T/NT ratios of all the tumors were still kept on the ~8−11 (Figure 4h) similar to that of the epidermal tumor (Figure 3). On the other hand, tumors were removed at 30 min postinjection (Supplementary Video 1 and 2) and H & E staining was studied after tumors were removed under NIR-II fluorescence bioimaging, confirming the precise delineation of the tumor margin (Figure 4i−m). Significantly, ≤1 mm metastatic lesions were thoroughly removed, further demonstrating that the novel tumor targeting strategy were able to correctly identify eye-visible cancerous metastases.

■ CONCLUSION

In this study, we reported the novel Nd-DOTA NIR-II probe with a facile one-step synthesis approach. Nd-DOTA shows superior aqueous solubility, photostability, and a high level of renal clearance, and Nd-DOTA is capable of detecting a tumor through molecular imaging when conjugated to a folic acid targeting agent. The high degree of imaging clarity in conjunction with a high T/NT ratio at a penetration depth of 7 mm demonstrated the possible benefits obtained by imaging within the NIR-II window. In small-animal models with extensive peritoneal carcinomatosis, tumor deposits with ultrasmall size (≤1 mm) were detected using the NIR-II Nd-DOTA-FA probe, resulting in the discrimination between malignant and normal tissue types which avoid incomplete resections or the unnecessary removal of healthy tissue. The Nd-DOTA structure and component are same to the clinically approved MRI contrast Gd-DOTA.43 Actually, lanthanide Gd3+ complexes have been extremely successful for MRI in clinical applications. Since the approval of Gd-DTPA in 1988,41 approximately 30% of MRI exams include the use of contrast agents currently.44 For this kind of lanthanide complexes, when proper ligands are chosen, they actually do remain chelated in the body and are excreted intact. It had been demonstrated that the off-the-shelf ligands such as DTPA and DOTA form complexes strong enough so that, for the period that the agent is in the body, there was no detectable dissociation. Therefore, due to the similar structure to the clinically approved Gd3+ complexes, we believe it will speed up the clinical translation for this novel kind of Nd-DOTA NIR-II probe in the near future.

■ ASSOCIATED CONTENT

6 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b00603.

Movie of tumor removal (AVI)
Movie of tumor removal (AVI)
Materials and methods; additional spectra and characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES