Communication

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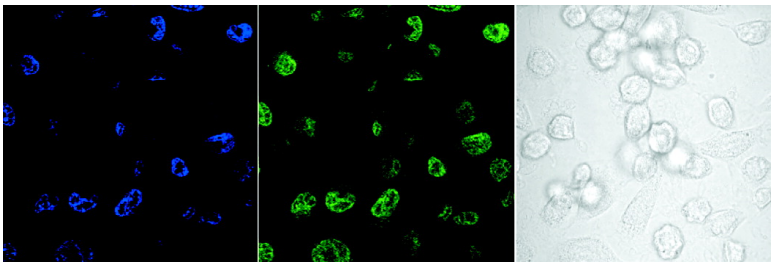
J. Am. Chem. Soc., **2008**, 130 (12), 3714-3715 • DOI: 10.1021/ja710418d



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Published on Web 03/06/2008

Emissive Terbium Probe for Multiphoton *in Vitro* Cell Imaging

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Multiphoton excitation allows molecules that typically absorb in the ultraviolet region to be excited with red or near-infrared light. The photoluminescence processes of organic−lanthanide complexes are usually induced by an organic chromophore ligand that absorbs incoming light and transfers the excitation energy to the lanthanide ion.[1] Previously, only a few multiphoton processes had been observed in organic−lanthanide complexes, owing to the limitations of experimental measurements.[2] To date, there are very few organic−lanthanide dyes with two-photon-induced f−f emission that can be used for live cell imaging.[3a] Most of the commercially available two-photon dyes are organic compounds.[3b,c] These dyes have broad emission spectra, and their lifetimes are in the nanosecond range, which means that the signals can be easily confused with biological autofluorescence in the cell or culture medium.[4] Live cell imaging that uses vital stains is an essential tool for improving our understanding of the way in which diseases occur in biological models and systems.[5] Rather than use commercial organic dyes, we have undertaken an advantageous approach that uses lanthanide as the emitter with energy transfer from the ligand to demonstrate a very specific and narrow emission band and a longer emission lifetime. However, there are still problems with quantum efficiency, the solubility of the complexes, toxicity, and the localization of the multiphoton lanthanide dyes. Here, we report the synthesis of a novel organic−terbium (Tb) complex. This modified complex demonstrates multiphoton-induced f−f emission in *in vitro* live cell imaging with a high three-photon cross section and low cytotoxicity.

The tripodal ligand *N*-[2-(bis{2-[(3-methoxybenzoyl)amino]-ethyl}amino)ethyl]-3-methoxybenzamide (**L**) was treated with Tb salts to give the complexes [Tb(**L**)$(NO_3)_3$] (**1**) (Figures 1, S1). Single crystals of Tb**L**$(NO_3)_3$, are suitable for X-ray analysis (Figure 1). Similar polymeric structures of Tb complexes have been reported with a similar ligand.[2] The appropriate long lifetime (∼3.56 ms) in the solution of HEPES, high quantum yield ($\phi_{solid} = 0.17$ and $\phi_{MeOH} = 0.11$), and the enhancements of the multiphoton excitation absorption cross sections of these complexes are around 3.1 GM (two-photon) and 1.9 GM (three-photon), which are much higher than those of our previously reported Tb complexes (2.3 GM-two-photon and 0.9 GM-three-photon)[6] and have potential for biological applications in live specimens.[7]

Human lung carcinoma (A549), cervical carcinoma (HeLa), and human nasopharyngeal carcinoma (HONE1) cells were exposed to the Tb complexes at 20 μg/mL (1:99 = DMSO/H_2O) for different time durations (0 to 60 min and 24 h). After 1−2 min of exposure, pale green signals were observable in the cytoplasm, which were revealed as a punctuated pattern in a small number of HONE1 cells under 800 nm laser excitation. After a further 1 min of exposure, more cells were found to contain green luminescence in the cytoplasm (Figure 2a). At an exposure time of up to 60 min, more than 95% of the cells exhibited green luminescence, as was observed in the cytoplasmic foci around the cell nucleus (Figure 2b). Similar observations were made in the A549 and HeLa cell lines (Figures 3a and 4). All three cell types were viable over the 24-h examination period, as exemplified by the presence of intact cell membranes with bright field images (Figures 2b, 2d, 3b, S6).

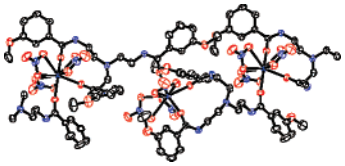

Figure 1. A diagram showing the linear polymer chain conformation of the terbium complex from the ligand along the *c*-axis.

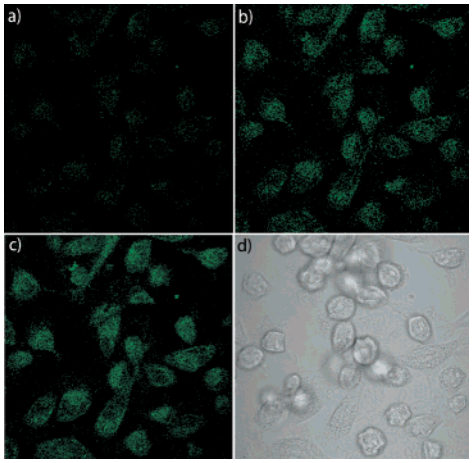

Figure 2. Three-photon confocal fluorescent microscopy images of HONE1 human nasopharyngeal carcinoma cells after (a) 3, (b) 45, and (c) 60 min of exposure to the terbium complexes (20 μg/mL in the culture medium, $\lambda_{ex} = 800$ nm, filter BP for $\lambda_{em} = 480-615$ nm), showing the Tb emission above 480 nm (green). (d) Bright field image of HONE1 cells.

To show that emission is via a multiphoton process, it can be observed that neither the ligand nor the complexes have absorption in the spectral range from 400 to 800 nm. However, the green luminescence from the Tb complexes can be seen under excitation at 800 nm, as generated by a Ti:Sapphire laser. Referring to the absorption band in the Supporting Information, a ligand requires at least three photons to reach the absorption state under 800 nm excitation, and the three-photon process was confirmed by a power-dependence experiment (Figure 3d).[8] Figure 3c shows the only multiphoton-induced f−f emission spectra of the Tb complexes, as recorded by a photomultiplier tube (PMT) with a lambda scan from Zeiss 510 multiphoton confocal microscopy under 800 nm excitation. The emission spectra in Figure 3c were taken from several parts of the cytoplasm in the different cells shown in Figure

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10.1021/ja710418d CCC: $40.75

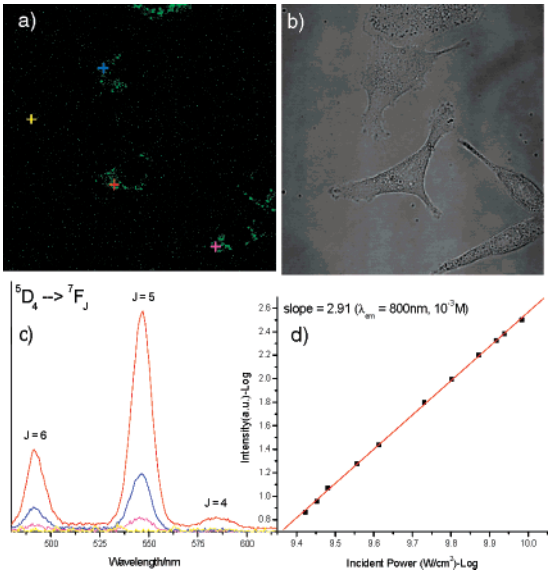

Figure 3. Confocal fluorescent microscopy images of human lung carcinoma A549 cells exposed to the Tb complexes ((a) dark field; (b) bright field; 20 μg/mL in the growth medium; λ_{ex} = 800 nm, filter band-pass for emission 480−615 nm). (c) The emission spectra of the four spots: (a) the green, blue, and pink spots are the f−f emission of the Tb complex in the cell, and the yellow spots are from the medium (lamba, Zeiss 510). (d) The power-dependence experiment (λ_{ex} = 800 nm) of the three-photon-induced f−f (Tb) emission (λ_{em} = 545 nm, $^5D_4 \rightarrow {}^7F_5$).[8]

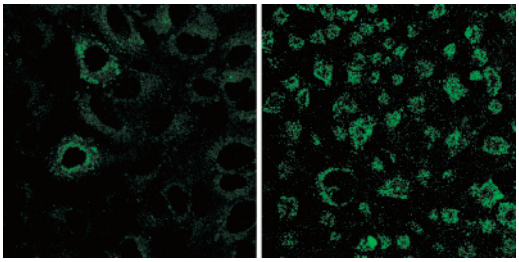

Figure 4. Three-photon confocal fluorescent microscopy images of human lung carcinoma A549 (left) and human cervical carcinoma HeLa cells (right) after 1 h of exposure to the terbium complexes (20 μg/mL in the growth medium, λ_{ex} = 800 nm, filter band-pass for emission 480−615 nm), showing the Tb emission above 480 nm (green).

3a. The structured emission bands of Tb**L**$(NO_3)_3$ that arise from the transitions of the $^5D_4 \rightarrow {}^7F_J$ (J = 6−4) states are shown in Figure 3c.[9] As a control, there was no f−f emission from the Tb complexes that were directly excited under the same experimental conditions outside of the cell shown in Figure 3c. It can thus be concluded that the three-photon processes occurred inside the cell. In addition, confocal microscopy of the cells with Hoechst 33342-labeled nuclei showed that the Tb complexes were internalized in the cytoplasm but not in the nuclei (Figure S5). The green emission of the Tb complexes and the green color of the Hoechst dye can both be observed due to the filter cutting at 480 nm; this is due to the broad emission band of the Hoechst. Thus, some of the emission is also observed toward the green spectral region.

No cytotoxicity was evident in the preliminary observations of the cells that were exposed to the terbium complexes for 24 h, and the results of the MTT assay using 10−20 μg/mL concentrations of the Tb complexes dosed for imaging showed that the viability of the cells was similar to that of the controls (Figure S7). The low cytotoxicity of our organic−lanthanide material and its unusual multiphoton luminescent properties make it a potential candidate as a new and novel luminescence material for live cell imaging. This probe would be particularly applicable to the study of signal transduction, cytokinesis during cancer cell division, antibody conjugation, and fluorescence recovery after photobleaching (FRAP). The impact on cell penetration of tailoring the morphology and dimensions of these materials is now being explored, and microscopy based on multiphoton excitation is clearly a powerful tool when applied in life science. The enhanced spatial resolution is a particularly attractive attribute. Further studies will explore this possibility in more detail.

Unlike condensed heterocyclic dyes or organic fluorophores, which possess short-lived excited states with lifetimes in the nanosecond range, we have provided evidence that our Tb probe can be used for the multiphoton imaging of long-term cellular processes with reduced photodamage, compared to UV-excited imaging. This Tb probe exhibits good cell uptake, and it is resistant to interference from light scattering in the cell culture medium and the natural autofluorescence of the biomolecules. It is also stable for storage and photodegradation. This enhances the potential of lanthanide complexes for use as biological probes for multiplex imaging and for multiphoton microscopy, in which bright and photostable probes are desired for various complex multi-imaging tasks.

Acknowledgment. This work was funded by grants from The Hong Kong Research Grants Council (HKU705307P), and the University of Hong Kong, City University of Hong Kong (Project Nos 7002002). K.L.W. acknowledges the Research Enhancement Scholarship Scheme from City University of Hong Kong. This work was also supported by the Area of Excellence Scheme of the University of Grants Committee (Hong Kong). Dedicated to Professor The Lord Lewis on the occasion of his 80th birthday.

Supporting Information Available: Experimental details of co-staining, MTT assays, and UV−vis absorption. Crystallographic information is available as CCDC 662083. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA710418D