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Cu^{2+}-Responsive Bimodal (Optical/MRI) Contrast Agent for Cellular Imaging

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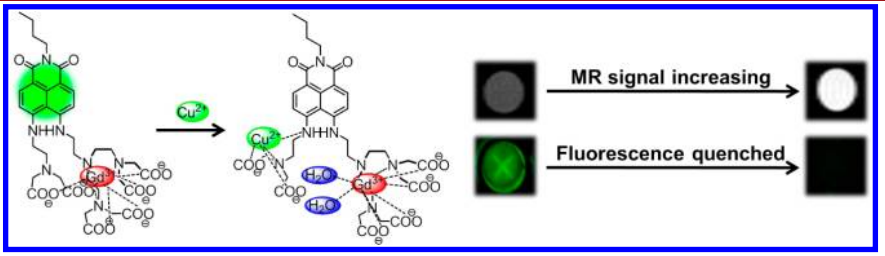
ABSTRACT



A water-soluble T_1 magnetic resonance imaging contrast agent (1) has been synthesized. The bimodal contrast agent 1 responds to the Cu^{2+} ion in living cells by enhancing the MRI modality signal whereas the optical signal gradually drops. This dual modality probe response depends on the cellular free copper ions in RAW 264.7 cells even at the micromolar level.

Copper is a ubiquitous and an indispensable trace element in the human body[1] and is necessary for the proper functioning of various organs and metabolic processes. However, like all essential elements and nutrients, too much or too little nutritional ingestion of Cu^{2+} ions can correspondingly result in copper ion excess or deficiency in the body, each of which has its own unique set of adverse health effects.[2] A copper imbalance induces several diseases, including neurodegenerative diseases[3] such as Alzheimer's,[4] Menkes' and Wilson's,[4] amyotrophic lateral sclerosis,[5] and prion diseases.[6]

Several chemical approaches have been established to elucidate the complex phenomenon between copper and normal healthy tissues and/or pathogens.[7] Among these approaches, copper-induced optical imaging has been applied for the past two decades.[8] Recently, the copper-induced magnetic resonance imaging (MRI) signal enhancement has gained much attention because it provides noninvasive three-dimensional images of the tissues.[9] Here, the number of coordinated water molecules first increases with activated contrast agents, which, in turn, increases the relaxivity to give a low relaxivity state ($q = 0$) before activation and a high relaxivity state ($q = 1$ or 2) after activation. Hence, the overall T_1-weighted magnetic resonance signal is increased.[10]

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MRI or optical imaging modality has its own strengths and weaknesses. Thus, different and complementary systems are combined to overcome inherent limitations associated with any individual technique. In particular, the dual modality of optical/MRI imaging may find preclinical and clinical applications. In the intraoperative procedure, diseased tissues can be localized by MRI, which is then biopsied for histologic validation by an optical method. Duan et al.[11] reported a copper-induced bimodal contrast agent. However, none of the cases with contrast agent toxicity *in vitro* has been evaluated so far.

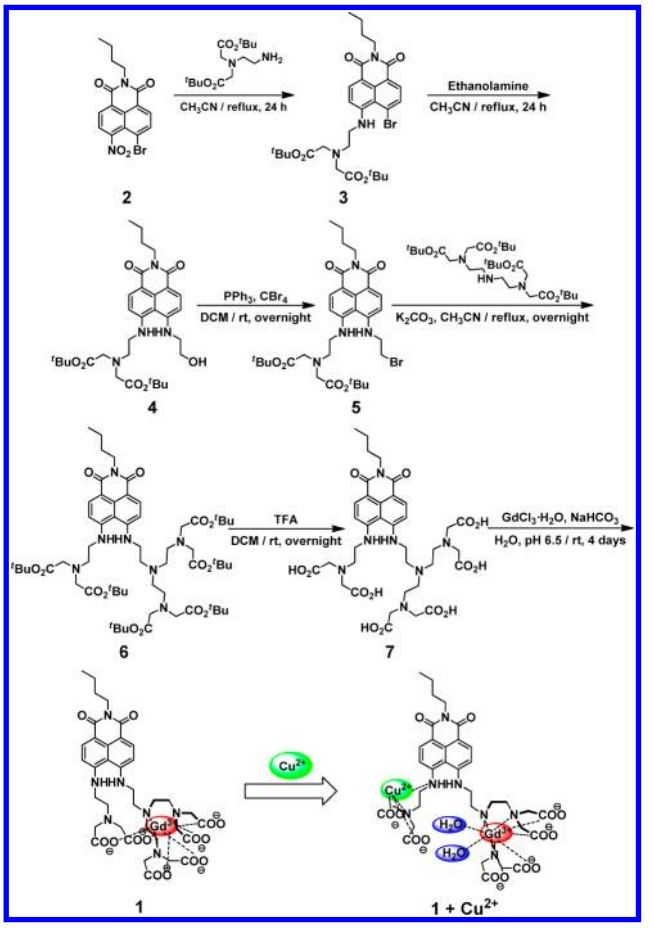
In this study, we report the synthesis and optical/MR properties of the bimodal contrast agent (**1**). Probe **1** showed selective optical changes in the copper ions (Cu^{2+}) level in physiological relevant milieus over other competitive cations. Additionally, T_1-weighted MR signal enhancement at 3 T, together with turning-off of the optical signal, depending on cellular copper ion level, suggested that the probe **1** could be a suitable candidate for the Cu^{2+} ion-induced bimodal imaging of cells.

Compound **2** was synthesized by the literature procedures.[12] As indicated in Scheme 1, **2** was reacted with *tert*-butyl 2,2′-(2-aminoethylazanediyl)diacetate (**10**) to give **3** in 18% yield. Compound **3** with ethanolamine was refluxed in acetonitrile to obtain **4**, whose bromination with PPh_3 and CBr_4 at room temperature gave **5** in 90% yield. Then, *tert*-butyl 2,2′,2″,2‴-(2,2′-azanediylbis(ethane-2,1-diyl)bis(azanetriyl))tetraacetate (**14**) was attached to **5**. The deprotection of the *tert*-butyl group by trifluoroacetic acid (TFA) in dichloromethane afforded **7**. The ligand **7** was complexed with $GdCl_3$ in deionized water at pH 6.5 to give the Gd^{3+} complex **1**. The detailed synthetic procedure and the corresponding spectroscopic data are available in the Supporting Information (SI).

To prove the chelating ability of compound **1** toward various metals ions, we investigated the fluorescence changes of **1** in the presence of various metal ions, including Cu^{2+} ions (Figure 1A). We observed that the fluorescence of probe **1** was selectively quenched by Cu^{2+} ions, because of the paramagnetic nature of the Cu^{2+} ions. No fluorescence changes were observed upon the addition of metal ions, except for Zn^{2+} ions. When the Zn^{2+} ions were added to the solution of **1**, the fluorescence intensity was moderately enhanced due to the Chelation Enhanced Fluorescence (CHEF) effect.[13] From the Cu^{2+} ion titration experiment, the binding constant of **1** for Cu^{2+} ions was calculated to be $9.30 \times 10^4\ M^{-1}$ based on 1:1 stoichiometry in the HEPES buffer solution (Figure 1B).

Then, we investigated the selectivity of Cu^{2+} ions toward **1** in the presence of other interfering cations such as Li^+, Zn^{2+}, Ba^{2+}, Mn^{2+}, Hg^{2+}, K^+, Na^+, Ca^{2+}, Sr^{2+}, and Mg^{2+} in the aqueous milieu. We also observed that the fluorescence change is unaltered with other metal ions, including Zn^{2+} ions (see Figure S3 in SI). It indicates that compound **1** is highly selective toward Cu^{2+} ions, which is in good

Scheme 1. Synthesis Scheme of Probe **1** and **1**−Cu^{2+} Complex



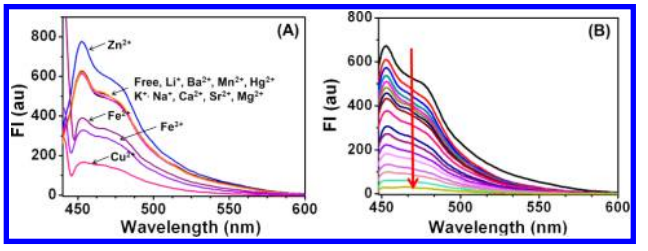


Figure 1. Fluorescence spectra of probe **1** (5 μM, λ_{ex} = 440 nm) in the presence of various metal ions (Li^+, Zn^{2+}, Ba^{2+}, Mn^{2+}, Hg^{2+}, K^+, Na^+, Ca^{2+}, Sr^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, and Cu^{2+}) (A), and different concentrations of Cu^{2+} ions (0−2500 μM) (B) in HEPES buffer (10 mM, pH 7.4).

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agreement with the previous study that the carboxylate group strongly binds with Cu^{2+} ions rather than other cations.[14]

The ability of Cu^{2+} ions to modulate the longitudinal relaxivity of **1** was determined by T_1 measurements at a proton frequency of 60 MHz (pH 7.4, HEPES buffer, at 37 °C). We observed the T_1 relaxivity of probe **1** without Cu^{2+} ions is 2.01 mM^{-1} s^{-1}, which is gradually modulated with Cu^{2+} ions and reaches 4.01 mM^{-1} s^{-1} in the presence of 2 equiv of Cu^{2+} ions. This increment (more than 2-fold) is considerably high compared to the previously reported contrast agent for Cu^{2+} ions.[11] In contrast, the relaxivity of **1** with Zn^{2+} ions marginally decreases (Figure 2). To rationalize the selectivity of **1** toward Cu^{2+} ions over biologically relevant metal ions, we measured the proton relaxation rate (R_1) of water coordinated to the Gd^{3+} ions. The relaxation rate increased only for Cu^{2+} ions according to its concentration. However, in other biologically relevant metal ions such as Na^+, K^+, Ca^{2+}, and Zn^{2+} ions, the relaxation rate remains unaltered with their variable concentration. These data confirm that the MR signal of **1** is selectively enhanced by Cu^{2+} ions.

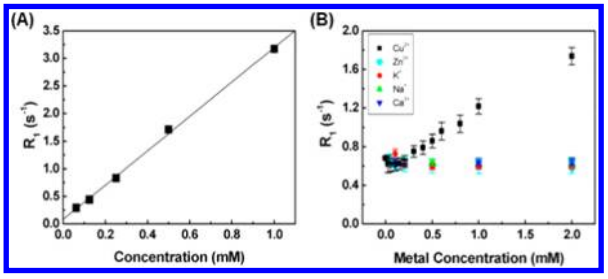

Figure 2. ^1H relaxation rate (R_1). (A) Various concentrations of probe **1** (0−1 mM), (B) change in ^1H relaxation rate (R_1) of probe **1** (0.2 mM) in the presence various metal ions (Cu^{2+}, Zn^{2+}, K^+, Na^+, and Ca^{2+}) (0−2 mM), at 25 °C and 60 MHz (1.4 T).

To directly visualize the enhanced relaxivity of **1** and the potential for increasing resolution, an MR phantom was imaged (Figure 3) using a 4.7 T MRI system (Bruker) with increasing concentrations of Cu^{2+} ions at a fixed concentration of probe **1**. The phantom images become gradually brighter by increasing the concentration Cu^{2+} ions, but remain unchanged in the case of Zn^{2+} ions. In general, the Gd^{3+} complex can be transmetallized by the Zn^{2+} ions,[15] which is fortuitous in the fact that neither relaxivity nor phantom images revealed the transmetalation by the Zn^{2+} ions of probe **1**. Thus, the new probe **1** can be used to estimate the cellular unbound Cu^{2+} ions.

A viability assay determines the ability of cells or tissues to maintain or recover its viability.[16] To demonstrate **1** as a sensitive MRI/FI cellular imaging probe for Cu^{2+} ions, we first analyzed the cell biocompatibility of **1** using a murine

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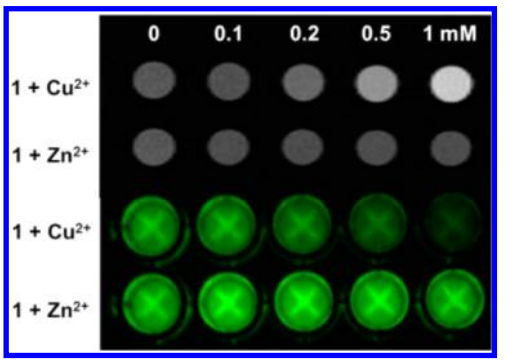

Figure 3. T_1-weighted phantom MR image (*up*) at 4.7 T, and fluorescence phantom image (*down*) of **1** (0.2 mM) in the presence of Cu^{2+} or Zn^{2+} ions with various concentrations (0−1 mM).

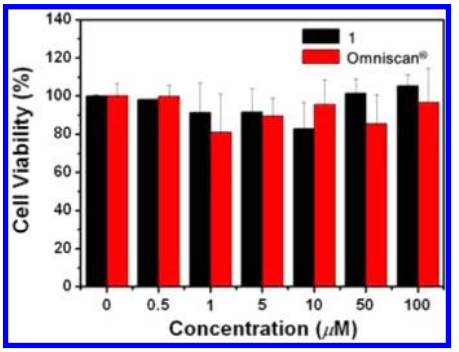

Figure 4. Comparative cell viability data of probe **1** in RAW 264.7 cell lines with clinically used MRI T_1 contrast agent, Omniscan.

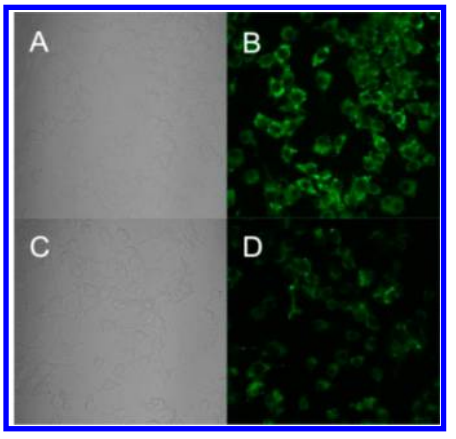

Figure 5. (A) Bright-field transmission image and (B) fluorescence image of **1** (12.5 μM). (C) Bright-field transmission image and (D) fluorescence image of probe **1** (12.5 μM) with 1 equiv of Cu^{2+} ions in RAW 264.7 cell lines; λ_{ex} = 400−460 nm, λ_{em} = 463 nm.

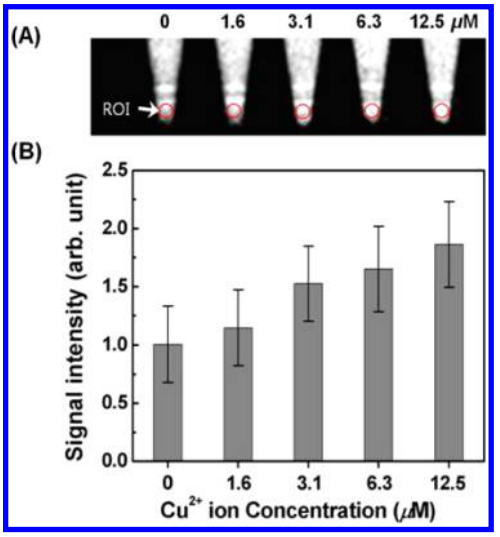

Figure 6. (A) Positive contrast in T_1-weighted MR image of RAW 264.7 cells (1×10^7 each) labeled with probe **1** (12.5 μM) and Cu^{2+} ions. The cells labeled with probe **1** were incubated with variable Cu^{2+} ion concentrations in a culture medium for 1 h and washed. A 3 T human MRI scanner was used with TE/TR = 13/200 ms. (B) Signal intensity in the region of interest increases with Cu^{2+} ion concentration.

macrophage cell line (RAW 264.7). In Figure 4, the cell viability of probe **1** treated cell lines is compared with commercially used Omniscan up to a 100 μM concentration of Gd^{3+} ions.

Then, we obtained the fluorescence images based on the multiproton confocal laser scanning microscope system (LSM510-Meta NLO, Karl Zeiss, Germany) with a 60× objective lens excited with blue light (400−460 nm). As shown by our fluorescence imaging results (Figure 5), labeling of RAW 264.7 cells with a 12.5 μM solution of probe **1** at room temperature for 12 h results in obvious intracellular green fluorescence (Figure 5B). When the cells labeled with probe **1** (12.5 μM) were incubated with 1 equiv of Cu^{2+} ions in a culture medium for 1 h and washed, obvious quenching of the fluorescence intensity was observed. The results firmly implicate that living cells are permeable to **1** and the Cu^{2+} ions could be detected in them.

In aqueous solution, probe **1** showed Cu^{2+} ion-induced turn-on of MR signal changes and depleted optical changes in cellular milieus. Thus we investigated the ability of **1** to detect changes in the cellular Cu^{2+} ion levels using MRI. To validate the possible application of probe **1** in MRI, we recorded T_1-weighted MR images of RAW 264.7 cells labeled with probe **1** and variable Cu^{2+} ion concentrations, using a 3 T human MRI scanner. Figure 6 illustrates that the contrast enhancement gradually increases with Cu^{2+} ion concentration even at the micromolar (μM) level. In Wilson's disease, the dysfunction of copper accumulation is due to loss of functionality of the copper transport gene of ATP7B,[17] resulting in the excess chelatable Cu^{2+} overload in extrahepatic tissues.[18] Moreover in the cerebral fluid, the copper ion concentration also belongs to the micromolar level;[19] thus this new chelatable probe **1** may deplete the copper storage of certain organs.

In summary, we have synthesized a Cu^{2+} triggered bimodal MRI contrast agent (**1**) comprising 1,8-naphthalimide as a fluorescence reporter and a diethylenetriamine tetraacetic acid (DTTA) unit as a chelator for Gd^{3+} ions. In the presence of Cu^{2+} ions, the fluorescence intensity of **1** decreased because of the paramagnetic nature of Cu^{2+} ions. In contrast, with the addition of Cu^{2+} ions, first, the total number of water molecules coordinated to **1** increased, and then the T_1-weighted MR signal increased. The new bimodal contrast agent was nontoxic and able to provide bimodal, i.e., fluorescence and T_1-weighted MRI, imagings in the cellular medium in the presence of Cu^{2+} ions. This new contrast agent can be used to detect free Cu^{2+} ions in living cells and inspire researchers to develop new probes to detect free metal ions *in vivo*.

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Supporting Information Available. Text and figures giving details of UV−vis and fluorescence study, NMR, and MASS data of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.