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A smart copper (Ⅱ) responsive binuclear gadolinium (Ⅲ) complex based magnetic resonance imaging contrast agent

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A novel Gd-DO3A-type bismacrocyclic complex, **[Gd_2(DO3A)$_2$BMPNA]**, with a Cu^{2+} selective binding unit was synthesized as a potential "smart" copper (Ⅱ) responsive magnetic resonance imaging (MRI) contrast agent. The relaxivity of the complex was modulated by the presence or absence of the Cu^{2+}, that was, in the absence of Cu^{2+} the complex exhibited a relatively low relaxivity value of 6.40 $mM^{-1}s^{-1}$ while upon addition of Cu^{2+} triggered the relaxivity to 11.28 $mM^{-1}s^{-1}$, approximately a 76% enhancement in relaxivity. Moreover, this Cu^{2+} responsive contrast agent was highly selective response to Cu^{2+} over other biologically relevant metal ions. The influence of some common biological anions on the Cu^{2+} responsive contrast agent and luminescence lifetime measurement of the complex were also carried out, the results of the luminescence lifetime indicated that the enhancement in relaxivity mainly ascribed to the increased number of inner-sphere water molecules binding to the paramagnetic Gd^{3+} core upon addition of Cu^{2+}. Besides, the visualizing change of the significantly enhanced relaxivity by addition of Cu^{2+} was observed from T_1-weighted phantom images.

Introduction

Copper(Ⅱ) ion is a vital metal nutrient for metabolism of life and plays a critical role in various biological processes.[1,2] Its homeostasis is critical for the metabolism and development of living organisms.[3,4] On the other hand, disruption of its homeostasis may lead to a variety of physical diseases and neurological problems, such as Alzheimer's disease,[5] Menkes and Wilson's disease,[6] amyotrophic lateral sclerosis,[7,8] and prion disease.[9,10] Therefore, the assessment and understanding of biological copper distribution in living systems by noninvasive imaging should be crucial to provide more insight into copper homeostasis, as well as gain a better knowledge of the relationship between copper regulation and its physiological function.

A wide variety of organic fluorescent dyes have been exploited for optical detection of ions during the last decades.[11-13] However, optical imaging using organic fluorescent dyes has several limitations, such as photobleaching, light scattering, limited penetration, low spatial resolution and autofluorescence disturbing.[14] By comparison, magnetic resonance imaging (MRI) is an increasingly accessible technique used noninvasive clinical diagnostic modality for medical diagnosis and biomedical research.[15] It can provide a high spatial resolution three-dimensional anatomical image with information of physiological signals and biochemical events.[16] As a powerful diagnostics imaging tool in medicine, MRI can distinguish normal tissue from diseased tissue and lesions in a noninvasive manner,[17-19] which avoids the diagnostic thoracotomy or laparotomy surgery for medical diagnostic and improves the diagnostic efficiency greatly. Multiple imaging parameters of MRI can provide a wealth of diagnostic information. Besides, the desired cross-section to acquire multi-angle and multi-planar image of various parts of the whole body can be freely chosen by adjusting the magnetic field of MRI, which makes medical diagnostics, the body's metabolism and function studies more and more effective and convenient.

Contrast agents are often used in MRI examination to improve the resolution and sensitivity of MRI, image quality can be significantly improved by applying contrast agents which enhance the MRI signal intensity by increasing the relaxation rates of the surrounding water protons.[20] Due to the high magnetic moment (seven unpaired electrons) and slow electronic relaxation of the paramagnetic gadolinium(Ⅲ) ion, gadolinium(Ⅲ)-based MRI contrast agents are commonly applied to increase the relaxation rate of the surrounding water protons.[16,21] However, most of these contrast agents are nonspecific and provide only anatomical information. On the basis of Solomon-Bloembergen-Morgan theory,[22-24] several parameters can be manipulated to alter the relaxivity of gadolinium(Ⅲ)-based MRI contrast agents, including the number of coordinated water molecules (q); the rotational correlation time (τ_R); the residence lifetime of coordinated water molecules binding to paramagnetic Gd^{3+} center (τ_M). Adjusting any of these three factors gives the opportunity to design certain biochemical events "smart" MRI contrast agents.[25-27] In recent years, there have been many studies in the development of

responsive gadolinium(Ⅲ)-based MRI contrast agents, most of them are focused on the development of targeted, high relaxivity and bioactivated contrast agents. These responsive gadolinium(Ⅲ)-based MRI contrast agents can be modulated by a particular in vivo stimulus such as pH,[28-35] metal ion concentration[36-43] or enzyme-activity.[44-50] Notably, a number of copper responsive MRI contrast agents have been reported to detect fluctuations of copper ion *in vivo*.[51-58] These activated contrast agents exploit the modulation of coordinated water molecular numbers to generate distinct enhancement in longitudinal relaxivity in response to copper ion (Cu^{+} or Cu^{2+}).

In this study, we designed and synthesized a binuclear gadolinium-based MRI contrast agent, **[Gd_2(DO3A)$_2$BMPNA]**, which was specifically responsive to Cu^{2+} over other biologically relevant metal ions. The new copper-responsive MRI contrast agent comprised two Gd-DO3A cores connected by a 2,6-bis(3-methyl-1H-pyrazol-1-yl)isonicotinic acid scaffold[59,60] (**BMPNA**) which functioned as a receptor for copper-induced relaxivity switching. The synthetic strategy of **[Gd_2(DO3A)$_2$BMPNA]** was depicted in Scheme 1. Subsequently, T_1 relaxivity of **[Gd_2(DO3A)$_2$BMPNA]** was studied at 25℃ and 60 MHz in the absence or presence of Cu^{2+}. Moreover, the selectivity experiments of **[Gd_2(DO3A)$_2$BMPNA]** towards Cu^{2+} over other biologically relevant ions were carried out as well. Luminescence lifetime measurement was conducted to determine the number of coordinated water molecules (q) of **[Gd_2(DO3A)$_2$BMPNA]**in the absence or presence of Cu^{2+}. In addition, T_1-weighted phantom images were performed to visualize the relaxivity enhancement caused by Cu^{2+}, which suggested a potential application *in vivo*.

Experimental section

Materials and Instrument

All materials for synthesis were purchased from commercial suppliers and used without further purification. ^{1}H and ^{13}C NMR spectra were taken on an AMX600 Bruker FT-NMR spectrometer with tetramethylsilane (TMS) as an internal standard. Luminescence measurements were performed on a Hitachi Fluorescence spectrophotometer-F-4600. The time-resolved luminescence emission spectra were recorded on a Perkin-Elmer LS-55 fluorimeter with the conditions of excitation wavelength, 295 nm; emission wavelength, 545 nm; delay time, 0.02 ms, gate time 2.00 ms, cycle time 20 ms; excitation slit, 5 nm; emission slit, 10 nm. The luminescence lifetime was measured on a Lecroy Wave Runner 6100 Digital Oscilloscope (1 GHz) using a tunable laser (pulse width =4 ns, gate = 50 ns) as the excitation (Continuum Sunlite OPO). Mass spectra (MS) were obtained at an auto flex III TOF/TOF MALDI-MS and IonSpec ESI-FTICR mass spectrometer. Elemental analyses were performed on a Vario EL Element Analyzer.

Synthesis

Synthesis of Compound 3

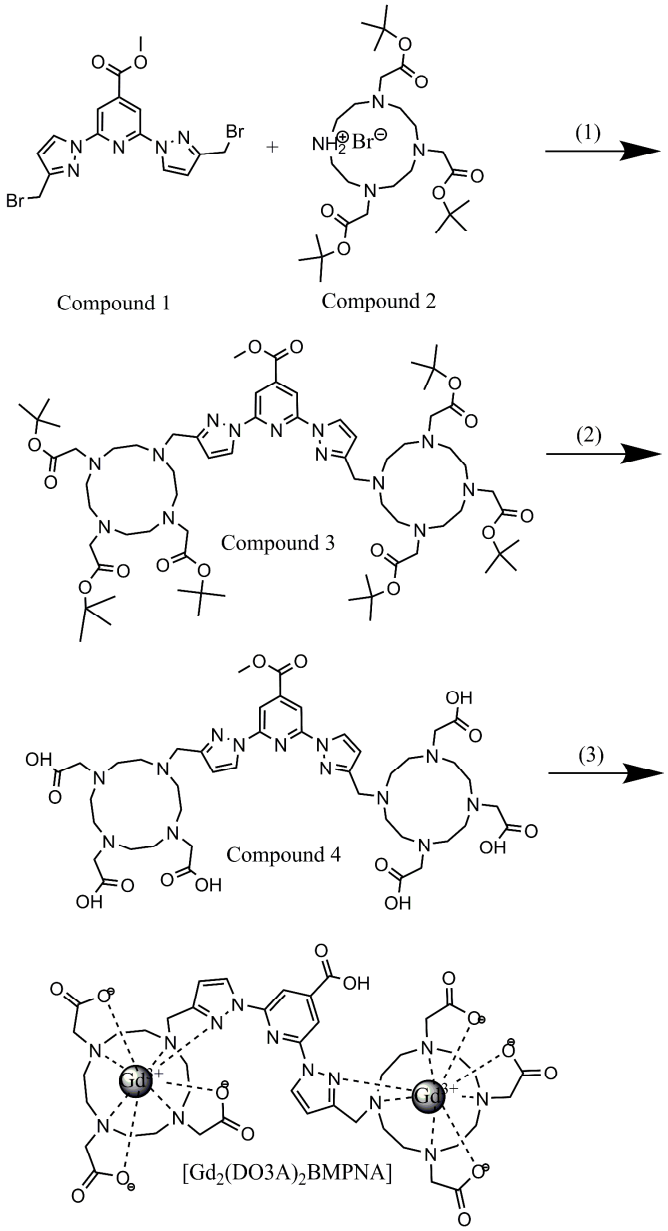
Methyl 2,6-bis(3-(bromomethyl)-1H-pyrazol-1-yl) isonicotinate (Compound 1)[59,60] and 4,7,10-tris(2-(tert-butoxy)-2-oxoethyl)-4,7,10-triaza-azoniacyclododecan-1-ium bromide (Compound 2)[61] were prepared following the reported methods. Compound 2 (0.25 g, 0.296 mmol) was suspended in 2 ml anhydrous acetonitrile with 6 equivalents of $NaHCO_3$ (0.1492 g) and the mixture was stirred at room temperature for 0.5 h. Compound 1 (0.0675 g, 0.148 mmol) was added and the mixture solution was slowly heated to reflux (80 °C) and stirred overnight. After the reaction was terminated and the mixture was cooled down to room temperature, the solution was filtered and the precipitate was washed with anhydrous acetonitrile several times. The collected filtrate solution was evaporated under reduced pressure. The residue was purified using silica gel column chromatography eluting with CH_2Cl_2/n-hexane/CH_3OH (10:3:1, *v/v/v*) to afford Compound 3 (0.1038g, 53%) as a pale yellow solid. ^{1}H NMR (600 MHz, DMSO) 8.22 (s, 2H), 8.15 (s, 2H), 6.62 (s, 2H), 4.53 (s, 4H), 3.82 (s, 3H), 3.42 (m, 4H), 2.98 (m, 8H), 2.85 (s, 8H), 2.71 (m, 24H), 1.33 (s, 54H) (Fig. S1). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.21, 172.44, 163.99, 152.38, 150.11, 143.13, 128.07, 109.83, 108.36, 82.59, 57.84, 56.52, 56.06, 55.56, 52.98, 50.55, 48.91, 47.30, 27.96 (Fig. S2). HRMS (ESI) *m/z* calc. for $C_{67}H_{111}N_{13}O_{14}$ $[M + 2H]^{2+}$ 661.92650, $[M + H + Na]^{2+}$ 672.91747, $[M + 2Na]^{2+}$ 683.90844, found $[M + 2H]^{2+}$ 661.92584, $[M + H + Na]^{2+}$ 672.91690, $[M + 2Na]^{2+}$ 683.90682 (Fig. S3).

Synthesis of Compound 4

Compound 3 (0.1 g, 0.0756 mmol) was stirred with trifluoroacetic acid in methylene chloride solution (2 ml) at room temperature for 24h. Then the solvent was evaporated under reduced pressure and the residue was washed three times in CH_3OH and CH_2Cl_2 to eliminate excess acid. The obtained residue was dissolved with minimum volume of CH_3OH and precipitated with cold Et_2O. The precipitate was filtered to afford a brown yellow solid (0.1022 g). ^{1}H NMR (600 MHz, DMSO) 9.06 (s, 2H), 8.17 (s, 2H), 6.84 (s, 2H), 4.33 (s, 4H), 3.98 (s, 3H), 3.56 (b, 20H), 3.09 (m, 24H) (Fig. S4). ^{13}C NMR (151 MHz, D_2O) δ 174.11, 169.13, 164.64, 150.75, 148.85, 142.10, 129.88, 109.75, 107.99, 55.69, 54.01, 53.10, 52.43, 51.15, 49.59, 48.22, 47.69 (Fig. S5). MALDI-TOF-MS spectrum (CH_3OH) *m/z* calc. for $C_{43}H_{63}N_{13}O_{14}$ $[M - H]^-$ 984.46, found 984.7. (Fig. S6). Anal Calc. for $C_{43}H_{63}N_{13}O_{14}$•$3CF_3COOH$•$2H_2O$, C, 43.14; H, 5.17; N, 13.35; found C, 42.34; H, 4.999; N, 13.29

Preparation of [Gd_2(DO3A)$_2$BMPNA] and [Tb_2(DO3A)$_2$BMPNA]

Compound 4 (0.05 mmol) was dissolved in 2 ml highly purified water. $GdCl_3$ or $TbCl_3$ (0.1 mmol) was dropwise added. The pH was maintained at 6.5-7.0 with NaOH during the whole process. The solution was then stirred at 75 °C for 24h. MALDI-MS (H_2O) *m/z* calc. for $C_{42}H_{55}N_{13}O_{14}Gd_2$ $[M + H]^+$ 1281.46, found 1281.4 (Fig. S7). MALDI-MS (H_2O) *m/z* calc. for $C_{42}H_{55}N_{13}O_{14}Tb_2$ $[M + H]^+$ 1284.3, found 1284.4 (Fig. S8).



Scheme 1 (1) $NaHCO_3$, CH_3CN, reflux; (2) CF_3COOH, CH_2Cl_2, rt. 24h; (3) $GdCl_3$ or $TbCl_3$, H_2O, pH=7.0.

T_1 measurements

The longitudinal relaxation times (T_1) of aqueous solutions of **[Gd$_2$(DO3A)$_2$BMPNA]** were measured on HT-MRSI60-25 spectrometer (Shanghai Shinning Globe Science and Education Equipment Co., Ltd) at **1.5 T**. All of the tested samples were prepared in HEPES-buffered aqueous solutions, at pH 7.4. All of the metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Fe^{2+}) were used as chloride salts. Concentrations of Gd^{3+} were determined by ICP-OES. Relaxivities were determined from the slope of the plot of $1/T_1$ vs. [Gd]. The data were fitted to the following equation (1)[20]:

$$(1/T_1)_{obs} = (1/T_1)_d + r_1[M] \quad (1)$$

Where $(1/T_1)_{obs}$ and $(1/T_1)_d$ were the observed values in the presence and absence of the paramagnetic species, and [M] was the concentration of the paramagnetic [Gd].

Luminescence measurements

Luminescence emission spectra were performed on a Hitachi Fluorescence spectrophotometer-F-4600. The luminescence lifetime was measured on a Lecroy Wave Runner 6100 Digital Oscilloscope (1 GHz) using a tunable laser (pulse width =4 ns, gate = 50 ns) as the excitation (Continuum Sunlite OPO).Samples were excited at 290 nm and the emission maximum (545 nm) was used to determine luminescence lifetimes. The Tb(III)-based emission spectra were measured using 0.1 mM solution of Tb complex analog in 100 mM HEPES buffer at pH 7.4 in H_2O and D_2O in the absence and presence of Cu^{2+}. The number of coordinated water molecules (q) was calculated according to the equation (2).[62,63]

$$q = 5(\tau_{H2O}^{-1} - \tau_{D2O}^{-1} - 0.06) \quad (2)$$

T_1-weighted MRI phantom images

Phantom images were collected on a **1.5 T** HT-MRSI60-25 spectrometer (Shanghai Shinning Globe Science and Education Equipment Co., Ltd). Instrument parameter settings were as follows: **1.5 T** magnet, matrix =256×256, slice thickness = 1 mm, TE = 13 ms, TR =100 ms, number of acquisitions = 1.

Results and discussion

Longitudinal relaxivity of [Gd$_2$(DO3A)$_2$BMPNA] in response to copper(II) ion

To investigate the influence of Cu^{2+} on the relaxivity of **[Gd$_2$(DO3A)$_2$BMPNA]**, the longitudinal relaxivity r_1 for **[Gd$_2$(DO3A)$_2$BMPNA]** contrast agent was determined using T_1 measurements in the absence or presence of Cu^{2+} at 60 MHz and 25℃ using 0.2mM Gd^{3+} solution of **[Gd$_2$(DO3A)$_2$BMPNA]** in 100mM HEPES buffer (pH 7.4) under simulated physiological condition. The concentrations of Gd^{3+} were determined by ICP-OES. The relaxivity r_1 was calculated from equation (1). In the absence of Cu^{2+}, the relaxivity of **[Gd$_2$(DO3A)$_2$BMPNA]** was 6.40 $mM^{-1}s^{-1}$, which was higher than that of $[Gd(DOTA)(H_2O)]^-$ (4.2 $mM^{-1}s^{-1}$, 20MHz, 25℃) and $Gd(DO3A)(H_2O)_2$ (4.8 $mM^{-1}s^{-1}$, 20 MHz, 40℃).[64] Upon addition of up to 1 equiv. of Cu^{2+}, the relaxivity of **[Gd$_2$(DO3A)$_2$BMPNA]** increased to 11.28 $mM^{-1}s^{-1}$ (Cu^{2+} triggered a 76% relaxivity enhancement). As shown in Fig. 1, the relaxivity gradually increased with the addition of copper ion concentration, and reached a maximum value at about 1.2 equivalents of Cu^{2+}. Due to the use of trifluoroacetic acid in the synthesis of compound 4, the trifluoroacetic acid residues produced CF_3COO^- in the [Gd_2(DO3A)$_2$BMPNA] solution, and CF_3COO^- could coordinate with partial Cu^{2+} to form " Chinese lantern" type of structure complex.[65] When more than 1.2 equiv. of copper ions was further added, the relaxivity maintained

substantially at the same level. The observed difference in Cu^{2+} triggered relaxivity enhancement established the ability of this contrast agent to sense Cu^{2+} *in vivo* by the means of MRI. Our designed contrast agent not only had a higher relaxivity, but also displayed a Cu^{2+} responsive relaxivity enhancement.

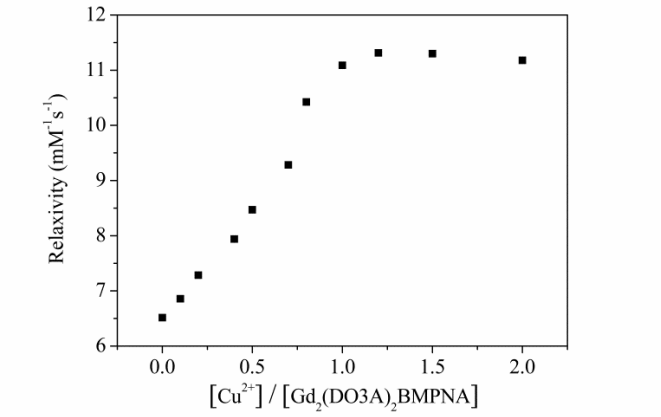


Fig. 1 Relaxivity response of 0.2 mM **[Gd$_2$(DO3A)$_2$BMPNA]** towards various concentrations of Cu^{2+}. T_1 measurements were performed at a proton Larmor frequency of 60 MHz at 25 °C in 100 mM HEPES buffer (pH 7.4).

Selectivity studies

The relaxivity response of **[Gd$_2$(DO3A)$_2$BMPNA]** exhibited excellent selectivity for Cu^{2+} over a variety of other competing, biologically relevant metal ions at physiological levels. As depicted in Fig. 2 (white bars), the addition of alkali metal cations (10 mM Na^+, 2 mM K^+) and alkaline earth metal cations (2 mM Mg^{2+}, 2 mM Ca^{2+}) did not activate an increase in relaxivity compared with copper ion turn-on response, even if introduction of d-block metal cations (0.2 mM Fe^{2+}, 0.2 mM Fe^{3+}, 0.2 mM or 2 mM Zn^{2+}) did not trigger relaxivity enhancements either. We noted that Zn^{2+} was also known to replace Gd^{3+} in transmetalation experiments, however, studies with analogous Gd^{3+}-DO3A complexes demonstrated that this ligand is more kinetically inert to metal-ion exchange.[66] To ensure the kinetic stability of the complex, we used MS to monitor [Gd$_2$(DO3A)$_2$BMPNA] in the presence of 1 equiv. of Zn^{2+}. No metal-ion exchange was observed at room temperature after 7 days (Fig. S13). Likewise, the interference experiments of relaxivity for **[Gd$_2$(DO3A)$_2$BMPNA]** in the presence of both Cu^{2+} (0.2 mM) and other biologically relevant metal ions were investigated, the results were shown as black bars in Fig.2, which indicated these biologically relevant metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}) had no interference on the Cu^{2+}-triggered relaxivity enhancement.

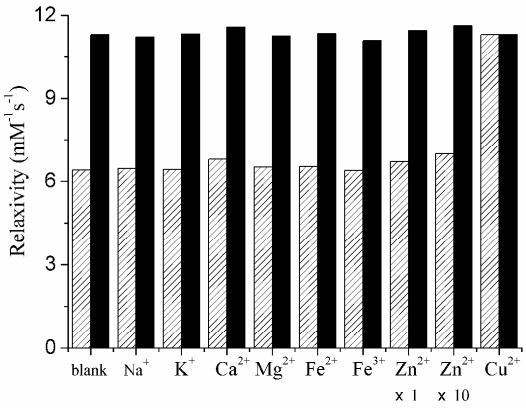


Fig. 2 Relaxivity of **[Gd$_2$(DO3A)$_2$BMPNA]** at 60 MHz and 25 ◦C in the presence of various metal ions. White bars represent the addition of the appropriate metal ion (10 mM for Na^+; 2 mM for K^+, Mg^{2+}, or Ca^{2+}; 0.2 mM for Fe^{2+}, Fe^{3+}) to 0.2 mM solutions of **[Gd$_2$(DO3A)$_2$BMPNA]**. Response to Zn^{2+} was measured both at 0.2 mM Zn^{2+} (Zn^{2+} × 1) and 2 mM Zn^{2+} (Zn^{2+} × 10). Black bars represent the subsequent addition of 0.2 mM Cu^{2+} to the contrast agent solution. All solutions were prepared in 100 mM HEPES buffer at pH = 7.4.

Besides, we also tested the Cu^{2+} response for **[Gd$_2$(DO3A)$_2$BMPNA]** in the presence of physiologically relevant concentrations of common biological anions to determine whether the Cu^{2+}-triggered relaxivity enhancement for **[Gd$_2$(DO3A)$_2$BMPNA]** was affected by biological anions at physiological levels. As previously mentioned, Cu^{2+} binding induced a relaxivity enhancement from 6.40 $mM^{-1}s^{-1}$ to 11.28 $mM^{-1}s^{-1}$ (a 76% relaxivity increase). As shown in Fig. 3, in the presence of citrate (0.13 mM), lactate (0.9 mM), $H_2PO_4^-$ (0.9 mM), or HCO_3^- (10 mM), Cu^{2+}-triggered relaxivity enhancement was approximately 61% (from 6.01 $mM^{-1}s^{-1}$ to 9.66 $mM^{-1}s^{-1}$), 66% (from 6.13 $mM^{-1}s^{-1}$ to 10.16 $mM^{-1}s^{-1}$), 20% (from 5.88 $mM^{-1}s^{-1}$ to 7.02 $mM^{-1}s^{-1}$), or 55% (from 6.15 $mM^{-1}s^{-1}$ to 9.55 $mM^{-1}s^{-1}$), respectively. Besides, 100 mM NaCl almost had no effect (about a 75% increase), in the simulated extracellular anion solution (EAS, contain 30 mM $NaHCO_3$,100 mM NaCl, 0.9 mM KH_2PO_4, 2.3 mM sodium lactate, and 0.13 mM sodium citrate, pH = 7),[67] Cu^{2+}-triggered relaxivity enhancement was about 26% (from 6.02 $mM^{-1}s^{-1}$ to 7.56 $mM^{-1}s^{-1}$). Generally, the results revealed that lactate, citrate, and HCO_3^- had a slight impact on Cu^{2+}-triggered relaxivity enhancement, while $H_2PO_4^-$ and EAS could influence the enhancement to some degree. As shown in Scheme 2, [Gd$_2$(DO3A)$_2$BMPNA] possessed two water molecules after addition of 1 equiv. amounts of Cu^{2+}, According to Dickins and coworkers' research, lanthanide complexes include two water molecules that can be partially displaced by phosphate, carbonate, acetate, carboxylate, lactate and citrate in different levels.[68-70]. The influence of these anions on Cu^{2+}-triggered relaxivity enhancement may be attributed to partial replacement of coordinated water molecules by these anions. It was likely that the relative high concentration of phosphate could replace the coordinated water molecules to reduce the increased number of water molecules surrounding the paramagnetic Gd^{3+} center induced by Cu^{2+}. As shown in Table 1, we measured the

number of water molecules in the first coordination sphere of Tb^{3+} in the presence of phosphate, the result suggested that the number of coordinated water molecules (q) decreased from 1.5 to 0.8.

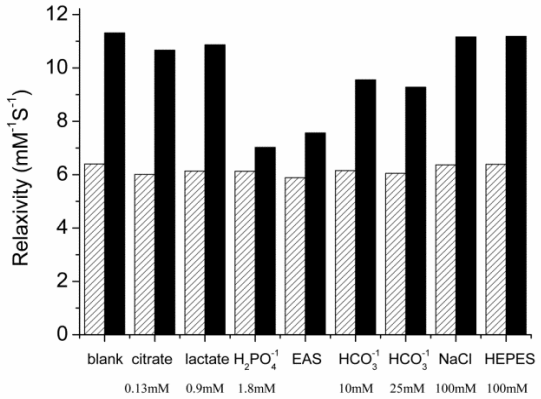


Fig. 3 Relaxivity response of 0.2mM **[Gd_2(DO3A)$_2$BMPNA]** to 1 equiv. of Cu^{2+} in the presence of biologically relevant anions. White bars represent **[Gd_2(DO3A)$_2$BMPNA]** relaxivities without Cu^{2+} in the presence of various anions. Black bars represent **[Gd_2(DO3A)$_2$BMPNA]** relaxivities with Cu^{2+} in the presence of different anions. Relaxivity measurements with HEPES, citrate, lactate, HCO_3^-, and $H_2PO_4^-$ were acquired at 60 MHz and 25 ◦C in 100mM HEPES buffer, pH = 7.4. EAS is 30 mM $NaHCO_3$, 100 mM NaCl, 0.9 mM KH_2PO_4, 2.3 mM sodium lactate, and 0.13 mM sodium citrate, pH = 7.4.

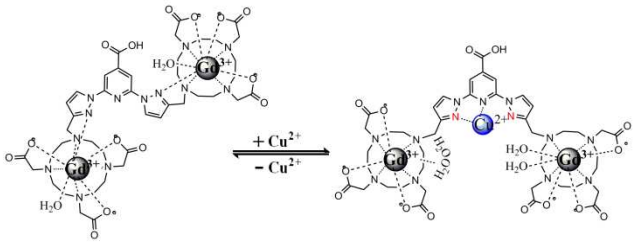
Coordination features

Luminescence lifetime experiments were performed to explore the mechanism of Cu^{2+}-triggered relaxivity enhancement. Luminescence lifetime measurements of lanthanide complexes have been widely used to quantify the number of inner-sphere water molecules.[71] Especially, Tb^{3+} and Eu^{3+} were commonly applied for lifetime measurements because their emission spectra were in the visible region when their 4f electrons relaxed from higher energy levels to the lowest energy multiplets.[72,73] Therefore, the Tb^{3+} analogue of **[Gd_2(DO3A)$_2$BMPNA]**, **[Tb_2(DO3A)$_2$BMPNA]**, was prepared according to the similar method, the luminescence lifetimes of the Tb^{3+} analogue in HEPES-buffered H_2O and D_2O in the absence and presence of Cu^{2+} were measured. As shown in Fig. S9, the luminescence decay curve of **[Tb_2(DO3A)$_2$BMPNA]** was fitting to obtain the luminescence lifetimes[74] (Table 1), the number of coordinated water molecules (q) was calculated according to the equation (2). The analysis results (Table 1) of **[Tb_2(DO3A)$_2$BMPNA]** in HEPES-buffered H_2O and D_2O in the absence and presence of Cu^{2+} indicated that q increased from 0.6 to 1.5 upon addition of 1 equiv. amounts of Cu^{2+}, which provided the strongest support that the Cu^{2+}-triggered relaxivity enhancement for **[Gd_2(DO3A)$_2$BMPNA]** was most likely due to the increased number of coordinated water molecules around Gd^{3+}ion upon Cu^{2+} binding to the pyrazole center as depicted in Scheme 2. After addition of Cu^{2+}, Cu^{2+} took off the pyrazole center N atom from the paramagnetic Gd^{3+} ion to generate an open coordination site available for a water molecule.

Luminescence emission titrations of **[Tb_2(DO3A)$_2$BMPNA]** towards Cu^{2+} were also performed to investigate the binding property of the contrast agent toward Cu^{2+}. Upon addition of 1 equiv. Cu^{2+}, the luminescence of **[Tb_2(DO3A)$_2$BMPNA]** at 545 nm decreased gradually and reached a minimum due to the quenching nature of the paramagnetic Cu^{2+} (Fig. S10). These titrations data indicated a 1:1 binding stoichiometry. (Scheme 2)

Table 1 Luminescence lifetimes (ms) and the number of water molecules (q)

[Tb_2(DO3A)$_2$BMPNA]	τH_2O / ms	τD_2O/ ms	q
without Cu^{2+}	1.91	2.91	0.6
with Cu^{2+}	0.85	1.21	1.5
with Cu^{2+} + $H_2PO_3^-$	1.74	2.08	0.8



Scheme 2 Schematic binding mode of **[Gd_2(DO3A)$_2$BMPNA]** with Cu^{2+}.

Copper- responsive T_1-weighted phantom MRI *in vitro*

To demonstrate the potential feasibility of this Cu^{2+}-responsive **[Gd_2(DO3A)$_2$BMPNA]** for copper-imaging applications, T_1-weighted phantom images of **[Gd_2(DO3A)$_2$BMPNA]** were acquired in the absence and presence of copper ions. The phantom images depicted in Fig. 4 displayed distinct image intensity increase in the presence of 1equiv. amounts Cu^{2+} compared with that without Cu^{2+} (Fig. 4, D). Moreover, some of other competing metal ions were also tested to further verify the selectivity of **[Gd_2(DO3A)$_2$BMPNA]** towards Cu^{2+}, whereas discernible differences were not observed upon addition of Mg^{2+} (Fig. 4, C), Zn^{2+} (Fig. 4, E), or Ca^{2+} (Fig. 4, F). Besides, we also tested the clinical contrast agent Magnevist (Fig. 4, G), the image intensity was a bit darker than that of our contrast agent.

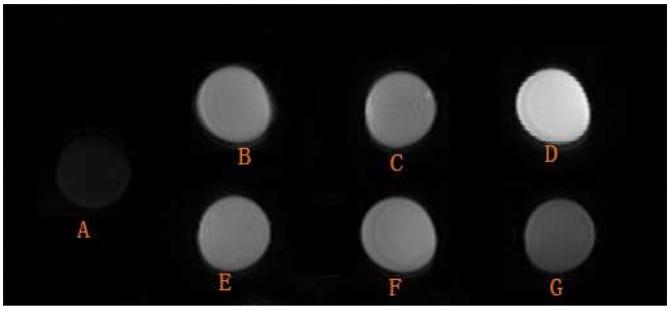


Fig. 4 T_1-weighted phantom MR images for **[Gd$_2$(DO3A)$_2$BMPNA]**. The images were recorded in a HEPES buffer (100 mM) solution of 0.2 mM **[Gd$_2$(DO3A)$_2$BMPNA]** at pH = 7.4. (A) HEPES buffer only; (B) [Gd$_2$(DO3A)$_2$BMPNA] ; (C) **[Gd$_2$(DO3A)$_2$BMPNA]** with 0.2 mM $MgCl_2$; (D) [Gd$_2$(DO3A)$_2$BMPNA] with 0.2 mM $CuCl_2$; (E) **[Gd$_2$(DO3A)$_2$BMPNA]** with 0.4 mM $ZnCl_2$; (F) [Gd$_2$(DO3A)$_2$BMPNA] with 0.4 mM $CaCl_2$; (G) 0.4 mM Magnevist (Gadopentetate Dimeglumine).

Conclusions

In conclusion, we designed and synthesized a novel bismacrocyclic DO3A-type Cu^{2+}-responsive MRI contrast agent, **[Gd$_2$(DO3A)$_2$BMPNA]**. The new Cu^{2+}-responsive MRI contrast agent comprised two Gd-DO3A cores connected by a 2,6-bis(3-methyl-1H-pyrazol-1-yl)isonicotinic acid scaffold (**BMPNA**) which functioned as a Cu^{2+} receptor switch to induce a distinct enhancement in relaxivity in response to Cu^{2+}, with a relaxivity increase up to 76%. Importantly, the complex exhibited high selectivity for Cu^{2+} over a range of other biologically relevant metal ions at physiological levels. Luminescence lifetime experiment results showed that the number of inner-sphere water molecules (q) increased from increased from 0.6 to 1.5 upon addition of 1 equiv. amounts of Cu^{2+}. When Cu^{2+} was coordinated in the central part of the complex, the donor N atom of the pyrazole center was removed from the paramagnetic Gd^{3+} ion and was replaced by a water molecule (Scheme 2). Consequently, the Cu^{2+}-triggered relaxivity enhancement could be ascribed to the increase in the number of inner-sphere water molecules. The designed contrast agent had a longitudinal relaxivity of 6.40 $mM^{-1}s^{-1}$, which was higher than that of $[Gd(DOTA)(H_2O)]^-$ (4.2 $mM^{-1}s^{-1}$, 20 MHz, 25℃) and $Gd(DO3A)(H_2O)_2$ (4.8 $mM^{-1}s^{-1}$, 20 MHz, 40℃). Besides, the visualizing change of the significantly enhanced relaxivity by addition of Cu^{2+} was observed from T_1-weighted phantom images.

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Notes and references

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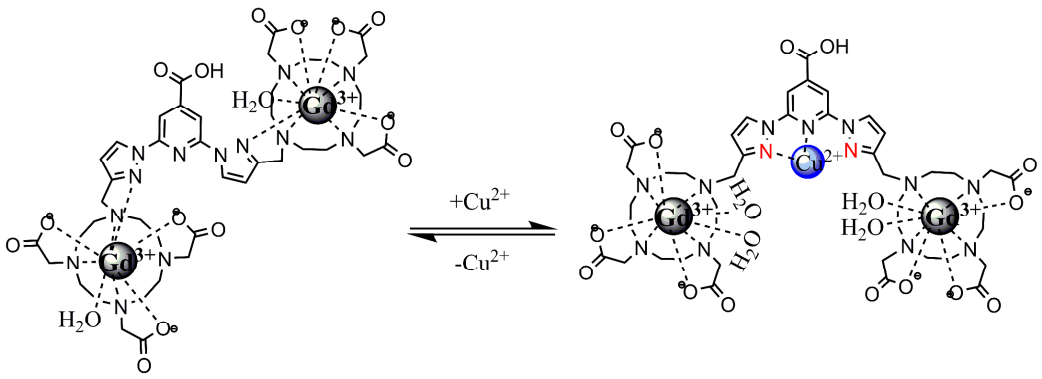

The relaxivity of the complex was modulated by the presence or absence of the Cu^{2+}, that was, in the absence of Cu^{2+} the complex exhibited a relatively low relaxivity value of 6.40 $mM^{-1}s^{-1}$ while upon addition of Cu^{2+} triggered the relaxivity to 11.28 $mM^{-1}s^{-1}$, approximately a 76% enhancement in relaxivity.