A facile "click" reaction to fabricate a FRET-based ratiometric fluorescent Cu²⁺ probe

Zhang-Jun Hu, Jiwen Hu, Yang Cui, Guannan Wang, Xuanjun Zhang, Kajsa Uvdal and Hong-Wen Gao

Linköping University Post Print

N.B.: When citing this work, cite the original article.

Original Publication:

Zhang-Jun Hu, Jiwen Hu, Yang Cui, Guannan Wang, Xuanjun Zhang, Kajsa Uvdal and Hong-Wen Gao, A facile "click" reaction to fabricate a FRET-based ratiometric fluorescent Cu²⁺ probe, 2014, Journal of materials chemistry. B, (2), 28, 4467-4472.

<http://dx.doi.org/10.1039/c4tb00441h>

Copyright: Royal Society of Chemistry

<http://www.rsc.org/>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-109291>

ARTICLE

A Facile “click” reaction to fabricate a FRET-based ratiometric fluorescent Cu²⁺ probe†

Cite this: DOI: 10.1039/x0xx00000x

Zhangjun Hu,^{†,a,b} Jiwen Hu,^{†,a} Yang Cui,^a Guannan Wang,^b Xuanjun Zhang,^{*b} Kajsa Uvdal,^b and Hong-Wen Gao^aReceived 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

A facile one-step Cu(I)-catalyzed “click” reaction, between a dansyl-azide and a propargyl-substituted rhodamine B hydrazide, is employed to fabricate a novel FRET ratiometric “off-on” fluorescent probe. The sensitive emission of the donor, a dansyl group, overlaps perfectly with the absorption of acceptor, xanthene in the open-ring rhodamine. The proposed probe shows high selectivity towards Cu²⁺. The ratio of the emission intensities at 568 and 540 nm (*I*₅₆₈/*I*₅₄₀) exhibits a drastic 28-fold enhancement upon addition of Cu²⁺. The probe shows an excellent linear relationship between emission ratios and the concentrations of Cu²⁺ from 10 to 50 μM, with a detection limit (*S/N* = 3) of 0.12 μM. The preliminary cellular studies demonstrated that the probe is cell membrane permeable and could be applied for ratiometric fluorescence imaging of intracellular Cu²⁺ with almost no cytotoxicity. The ingenuity of the probe design is to construct a FRET donor-acceptor interconnector and a selective receptor simultaneously by “click” reaction. The strategy was verified to have great potential for developing novel FRET probes for Cu²⁺.

Introduction

The copper ion (Cu²⁺) is one of the most abundant essential trace elements and plays diverse roles in human physiopathology.¹ However, under overloading, copper exhibits toxicity, by which it causes oxidative stress and related symptoms, which may lead to diabetes and many neurodegenerative disorders such as Alzheimer's, Parkinson's, Menkes, and Wilson's diseases.² Therefore, sensitive techniques to trace and visualize Cu²⁺ are highly demanded. So far, the fluorogenic method has been regarded as a preferable approach because fluorimetry is rapidly performed, non-destructive, highly sensitive, and suitable for high-throughput screening applications.³ Cu²⁺ presents an inherent problem for fluorescent sensing because of the likely quenching of fluorescence by mechanisms inherent to paramagnetic species.⁴ Alternatively, reactivity-based detections were applied, which utilize chemical reactions to transform non-emissive precursors to fluorescent products and track time-dependent cumulative effects of metal exposure.⁵

Even though these “turn-on” probes have high sensitivity, a major limitation is that variations in the sample environment might influence the fluorescence intensity measurements. In principle, this issue can be alleviated by using ratiometric fluorescent probes.⁶ Förster resonance energy transfer (FRET) is the commonly exploited mechanism for design of these ratiometric probes because the FRET-based “off-on” module is

inherently more sensitive owing to its zero background.⁷ For an effective FRET, a substantial spectral overlap between the donor emission and the acceptor absorption is required, which sometimes restricts the design and development of these kinds of probes. So far, only few successful FRET-based ratiometric fluorescent Cu²⁺ probes have been constructed.⁸ Cu²⁺ induced ratiometric small molecule probes that are compatible with aqueous environments are still exceedingly rare, which further highlights ongoing challenges in this research area.⁹

Recently, the Lin group has developed a versatile platform for “second-generation” FRET rhodamine-based probes, profiting from their high levels of synthesis experience.¹⁰ Meanwhile, by facilitating synthesis, the other strategized ones are still very robust platforms for the species extension of the ratiometric probe.^{8b-c,9a,11} Rhodamine hydrazide is well known as a metal-ions-inducible recognition substrate, whose selectivity or sensitivity is further tuneable by appending other ancillary groups.¹² Among them, heterocyclic have been frequently used, due to their strong coordinating abilities.^{12b} The “click triazole” has recently been reported as an effective site in the recognition receptors to trigger the ring opening of spirolactam in rhodamine-based probes,¹³ whereas the “click triazole” is well known as an interconnector between two entities in the architecture of molecules.¹⁴ Therefore, it becomes possible to use “click triazole” to construct a FRET-based ratiometric probe, in which the triazole functions as not only a donor-acceptor interconnector but also an ancillary site in the

Scheme 1 Design and synthesis of rhodamine-based fluorescent Cu^{2+} probe **RN**.

recognition receptor.¹⁵ Herein, a rhodamine derivative (**RN**) was synthesized by bearing a dansyl fluorochrome, which has been often used as fluorescent donor in the FRET-based probes.^{8b,16} Different from the reported strategy,¹⁵ the donor moiety and triazole and were readily introduced to form a ‘O–N–N’ chelating receptor simultaneously by a one-step Cu(I)-catalyzed “click reaction” between a dansyl-azide and a propargyl-substituted rhodamine B hydrazide (Scheme 1). In addition, **RN'**, which does not contain the donor, was synthesized for studying the affinity and FRET pathway (in Scheme S1, ESI†). Both **RN** and **RN'** display selective colorimetric and fluorimetric responses towards Cu^{2+} in the aqueous media.

Experimental Section

Synthetic materials and methods

Rhodamine B, hydrazine hydrate (85%), 2-(2-(2-chloroethoxy)ethoxy) ethanol, dansyl chloride, N-Methylethanolamine, sodium hydride, sodium azide, N,N-diisopropylethylamine and other solvents were pursued from Sigma-aldrich (<http://www.sigmaaldrich.com/>). Proton nuclear magnetic resonance (^1H) and carbon nuclear magnetic resonance (^{13}C) was recorded on Varian 300 MHz or 500 MHz spectrometers. Electrospray ionisation mass spectra (ESI-MS) were recorded on a Thermo Finnigan Surveyor MSQ. Elemental analysis was performed with a Perkin–Elmer 240 analyzer.

Synthesis of 1

A mixture of dansyl chloride (120 mg, 0.45 mmol), N-methyl-N-(2-chloroethyl)amine hydrochloride (87 mg, 0.68 mmol) and anhydrous triethylamine (232 μL , 1.11 mmol) in 15 mL methanol was stirring overnight. The organic solvent was evaporated under vacuum. The residue was purified by flash chromatography using ethyl acetate/heptane (1/2, v/v), yielding a yellow solid **1** (140 mg, 96 %). ^1H NMR (300 MHz, CDCl_3) δ (ppm) = 8.56 (d, J = 8.5 Hz, 1H), 8.33 (d, J = 8.7 Hz, 1H), 8.18

(d, J = 7.3 Hz, 1H), 7.54 (dd, J = 17.7, 8.5 Hz, 2H), 7.19 (d, J = 7.5 Hz, 1H), 3.70–3.50 (m, 4H), 2.95 (s, 3H), 2.89 (s, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm) = 152.00, 134.00, 130.82, 130.33, 130.06, 128.34, 123.25, 119.60, 115.47, 51.47, 45.56, 41.52, 35.86 ppm. MS (ESI): m/z (%): 326.9 (100) M^+ . Anal. Calcd for $\text{C}_{15}\text{H}_{19}\text{ClN}_2\text{O}_2\text{S}$ (%): C, 55.12; H, 5.86; N, 8.57. Found: C, 55.14; H, 5.96; N, 8.58.

Synthesis of 2

A slurry of **2** (135 mg, 0.41 mmol) and sodium azide (53 mg, 0.82 mmol) in 5 mL DMF was stirring at 100°C overnight. After cooling to room temperature, 10 mL dichloromethane was added and the mixture was washed with brine (3 $\times$ 20 mL). The organic phase was dried over anhydrous MgSO_4 , filtered and evaporated under vacuum. The crude was purified by chromatography using ethyl acetate/heptane (1/2, v/v), yielding a yellow solid **2** (130 mg, 95 %). ^1H NMR (300 MHz, CDCl_3) δ (ppm) = 8.56 (d, J = 8.5 Hz, 1H), 8.34 (d, J = 8.7 Hz, 1H), 8.18 (dd, J = 7.3, 1.3 Hz, 1H), 7.54 (ddd, J = 10.7, 8.6, 7.5 Hz, 2H), 7.19 (d, J = 7.6 Hz, 1H), 3.52–3.34 (m, 4H), 2.93 (s, 3H), 2.88 (s, 6H); ^{13}C NMR (75 MHz, CDCl_3) δ (ppm) = 151.94, 133.88, 130.78, 130.29, 130.05, 128.31, 123.21, 119.52, 115.42, 50.12, 48.86, 45.51, 35.71; MS (ESI): m/z (%): 334.0 (100) $\text{M}^+ + \text{H}$. Anal. Calcd for $\text{C}_{15}\text{H}_{19}\text{N}_5\text{O}_2\text{S}$ (%): C, 54.04; H, 5.74; N, 21.01. Found: C, 54.03; H, 5.76; N, 21.07.

Synthesis of 3

To a solution of rhodamine B hydrazide (912 mg, 2 mmol) in THF (50 mL), sodium hydride (160 mg, 4 mmol) in THF (5 mL) was added drop-by-drop at 0°C and stirred for 30 min. A solution of propargyl bromide (260 mg, 2.2 mmol) in THF (5 mL) was then added slowly and the mixture was stirred overnight under reflux. The reaction mixture was quenched with water (100 mL) and extracted with ethyl acetate (3 $\times$ 100 mL). The combined organic phase was dried over anhydrous Na_2SO_4 , filtered and evaporated under vacuum. The residue was purified by flash chromatography using ethyl

acetate/heptane (4/1, v/v), yielding a pale solid **3** (544 mg, 55%). ^1H NMR (500 MHz, CDCl_3), δ (ppm) = 7.92 (dd, J = 6.0, 2.2 Hz, 1H), 7.52–7.41 (m, 2H), 7.10 (dd, J = 6.1, 2.0 Hz, 1H), 6.47 (d, J = 8.8 Hz, 1H), 6.41 (d, J = 2.6 Hz, 2H), 6.28 (d, J = 2.6 Hz, 2H), 6.26 (d, J = 2.6 Hz, 2H), 4.58 (t, J = 6.5 Hz, 1H), 3.34 (q, J = 7.1 Hz, 8H), 3.30 (dd, J = 6.6, 2.5 Hz, 2H), 2.10 (s, 1H), 1.16 (t, J = 7.1 Hz, 12H). ^{13}C NMR (126 MHz, CDCl_3), δ (ppm) = 166.75, 153.80, 151.91, 148.93, 132.87, 129.99, 128.64, 128.22, 124.10, 122.99, 107.94, 105.74, 98.10, 80.24, 72.49, 65.53, 44.47, 40.57, 12.77. MS (ESI): m/z (%): 495.2 (100) $\text{M}^+ + \text{H}$. Anal. Calcd for $\text{C}_{31}\text{H}_{34}\text{N}_4\text{O}_2$ (%): C, 75.28; H, 6.93; N, 11.33. Found: C, 75.25; H, 6.96; N, 11.32.

Synthesis of RN

A slurry of **3** (178 mg, 0.36 mmol), **2** (120 mg, 0.36 mmol), CuI (7.6 mg, 0.04 mmol) and N,N -diisopropylethylamine (47 mg, 0.36 mmol) in 15 mL THF was stirring at room temperature overnight. The reaction mixture was quenched with 5 mL brine, and extracted with EtOAc (3×20 mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered and evaporated under vacuum. The crude was by flash column chromatography ethyl acetate/methanol (20/1, v/v) yielding an apricot solid **RN** (283 mg, 95%). ^1H NMR (300 MHz, CDCl_3) δ (ppm) = 8.50 (d, J = 8.5 Hz, 1H), 8.20 (d, J = 8.7 Hz, 1H), 8.09 (d, J = 6.9 Hz, 1H), 7.86 (dd, J = 5.5, 2.4 Hz, 1H), 7.53 – 7.35 (m, 4H), 7.18 – 6.98 (m, 2H), 6.49 – 6.32 (m, 4H), 6.22 (dd, J = 8.9, 2.4 Hz, 2H), 4.39 (t, J = 6.4 Hz, 2H), 3.86 (d, J = 5.5 Hz, 2H), 3.59 (t, J = 6.3 Hz, 2H), 3.30 (q, J = 7.0 Hz, 8H), 2.84 (s, 6H), 2.68 (s, 3H), 1.13 (t, J = 7.0 Hz, 12H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm) = 166.86, 153.88, 151.86, 151.59, 148.86, 145.69, 133.42, 132.77, 130.84, 130.26, 130.16, 130.14, 129.97, 128.60, 128.36, 128.19, 124.12, 123.31, 123.14, 122.83, 119.26, 115.40, 107.92, 105.85, 97.91, 65.73, 60.39, 49.81, 49.00, 46.64, 45.44, 44.38, 35.91, 21.07, 14.26, 12.71; MS (ESI): m/z (%): 828.3 (100) $\text{M}^+ + \text{H}$. Anal. Calcd for $\text{C}_{46}\text{H}_{53}\text{N}_9\text{O}_4\text{S}$ (%): C, 66.72; H, 6.45; N, 15.22. Found: C, 66.83; H, 6.23; N, 15.09.

Spectroscopic materials and methods

Most of the used chemicals are analytical grade. All solvents used were AR and purchased from Sigma-aldrich (<http://www.sigmaaldrich.com/>). All the aqueous solutions were prepared with deionized water. Stock solutions of inorganic salts (1.00 mM) were prepared by dissolving appropriate amount of ions in deionized water. The cation solutions are prepared from $\text{Cu}(\text{NO}_3)_2$, NaNO_3 , KNO_3 , $\text{Mg}(\text{NO}_3)_2$, $\text{Ca}(\text{NO}_3)_2$, $\text{Ba}(\text{NO}_3)_2$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{La}(\text{NO}_3)_3 \cdot n\text{H}_2\text{O}$, $\text{Al}(\text{NO}_3)_3$, $\text{Co}(\text{NO}_3)_2$, $\text{Ni}(\text{NO}_3)_2$, CuCl , $\text{Cd}(\text{NO}_3)_2$, $\text{Zn}(\text{NO}_3)_2$, $\text{Cr}(\text{NO}_3)_3$, $\text{Pb}(\text{NO}_3)_2$ and $\text{Hg}(\text{NO}_3)_2$; and anions solutions are obtained from NaF, NaCl, NaI, NaBr, NaOH, NaAcO, Na_2SO_4 , $\text{Na}_2\text{S}_2\text{O}_8$, Na_2SiO_3 , Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, NaNO_3 , NaNO_2 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, Na_2HPO_4 , $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ in the deionized water. Stock solution of probe **RN'** and **RN** (1.00 mM) was prepared by CH_3CN . The dilutions were carried out

by using $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM).

The pH was measured on a PHS-3C meter. Fluorescent and UV-vis absorption spectra were collected on a HITACHI F-4500 fluorescence spectrometer and a Shinco S-4100 Uv-vis Spectrophotometer, respectively. All fluorescence measurements were carried out at room temperature. The excitation and emission slit widths are both 10 and 5 nm, respectively.

Cell culture, cytotoxicity and imaging

The living HeLa cells (human cervical cancer cell) were provided by the School of Medicine, Tongji University (Shanghai, China). HeLa cells were incubated in DMEM (Dulbecco modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) and 1% Penicillin-Streptomycin in an atmosphere of 5% CO_2 and 95% air at 37°C . The cells were plated confocal dish and allowed to adhere for a night. The second day before the experiments, the cells were washed with phosphate-buffered saline (PBS) buffer. To test the cytotoxic effect of the probe in cells over a 24 h period, the MTT (5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay was performed as the reported methods.¹⁷ Cytotoxicity assays show that **RN** is safe enough for confocal microscopy at low concentrations, so that the cells were incubated with **RN** (10 μM) in the culture medium containing 1% DMSO for 30 min at 37°C , then washed with PBS three times, and the fluorescence images were acquired through with a leica TCS SP5 Laser Scanning Confocal Microscope. Subsequently, after incubating with Cu^{2+} (50 μM) for another 30 min at 37°C , the HeLa cells were rinsed with PBS three times, and the fluorescence images were acquired.

Results and discussion

RNs were prepared by the synthetic routes outlined in [Scheme 1](#) and [Scheme S1](#).[†] Rhodamine-B hydrazide and N -methyl- N -(2-chloroethyl) aminehydro-chloride salt were synthesized by following published procedures, respectively.^{5a, 18} **3** was obtained by reacting rhodamine-B hydrazide with propargyl bromide in THF. **1** was highly yielded from dansyl chloride and N -methyl- N -(2-chloroethyl) amine in dichloromethane through a substitution reaction. **2** and **4** were obtained by azide substitution of the corresponding chlorides in DMF. The gentle Cu(I)-catalyzed “click reaction” was further employed to produce final **RNs** in high yields (> 90%). All chemical structures of were confirmed by ^1H and ^{13}C NMR spectroscopy, as well as ESI mass spectrometry (detailed in experimental section and ESI[†]).

To fabricate an effective probe, the first important thing is to construct sensitive recognition receptor in the probe molecule. **RN'** was synthesized to study the binding event of the new designed ‘O-N-N’ receptor towards metal ions. As shown in [Fig. S1](#),[†] only Cu^{2+} induced significant absorbance around 555 nm and fluorescence around 570 nm changes. The other tested

ions didn't promote any noticeable changes. The results show that **RN'** with new 'O-N-N' receptor has high selectivity towards Cu^{2+} , which signifies that **RN'** could be further developed into a FRET-based probe by "click" reaction using a suitable donor fluorophore. As mentioned above, a substantial spectral overlap for the donor emission and acceptor absorption bands is required for an effective FRET. A commercial fluorophore dansyl was chosen as the donor to verify the strategy, due to its sensitive emission around 540 nm (dansyl in **2**), even in the presence of Cu^{2+} .^{8b} It overlaps perfectly with the absorption of the acceptor (xanthene in open-ring rhodamine, **RN'** + Cu^{2+}) (Fig. 1), which might facilitate an efficient intermolecular energy transfer (FRET) from dansyl to xanthene.

Fig. 1 Normalized absorption of **RN'** and emission of **4** ($\lambda_{\text{ex}} = 420$ nm), **RN'** ($\lambda_{\text{ex}} = 510$ nm) and **RN** ($\lambda_{\text{ex}} = 420$ nm) in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM) in the presence of Cu^{2+} (5 equiv). The inset shows the visual color and fluorescence (under illumination at 365 nm) of **RN** (10 μM) before (left) and after (right) addition of 50 μM of Cu^{2+} , in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM).

As expected, in the free **RN**, the FRET pathway is completely suppressed, and only an emission around 540 nm is observed when excited at 420 nm; because the rodamine moiety exists in a spirolactam form that has almost no absorption in the visible region, and thus cannot act as an acceptor. In the presence of Cu^{2+} , the binding of the receptor to Cu^{2+} induces the FRET process to result in intense red emission (Fig. 1), caused by the ring-opening which increases the overlap integral between the emission of dansyl and the absorption of xanthene. The ratiometric "off-on" fluorescence change occurs visually and the colour also changes to be reddish-pink (insets of Fig. 1). Completely similar to **RN'**, the metal induced ring-opening of **RN** is highly selective towards Cu^{2+} and does not reveal any noticeable spectral changes for other tested ions (Fig. 2).

Fig. 2 Fluorescence spectra of **RN** (5 μM) in the presence of various metal ions (25 μM): Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Al^{3+} , Cr^{3+} , Pb^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Ba^{2+} , Hg^{2+} , Mn^{2+} , La^{3+} , Zn^{2+} , Cd^{2+} , Cu^+ and Cu^{2+} , in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM).

To gain an insight into the potential of **RN** as a probe for Cu^{2+} , a titration experiment was performed (Fig. 3). Accordingly, as shown in Fig. 3a, upon excitation at 420 nm, the fluorescence intensity around 540 nm decreases along with the incremental addition of Cu^{2+} , and simultaneously a new emission band around 568 nm gradually increases. These changes in the fluorescence spectrum stopped when the addition amount of Cu^{2+} reached 24 equiv. of the probe (Fig. 3b). At this amount, the ratio of the emission intensities at 568 and 540 nm (I_{568}/I_{540}) exhibits a drastic change from 0.6 in the absence of Cu^{2+} to 16.8, a 28-fold variation in the ratios. Noticeably, **RN** shows an excellent linear relationship between the emission ratios and the concentrations of Cu^{2+} from 10 to 50 μM (Fig. S2†), suggesting that the **RN** is potentially useful for quantitative determination of Cu^{2+} . And the detection limit ($S/N = 3$) of the ratiometric **RN** was determined to be 0.12 μM . Linear fitting of the fluorescence titration curve (Fig. S3†) exhibited a binding for Cu^{2+} and **RN**, with an association constant of $K_a = 2.07 \times 10^4 \text{ M}^{-1}$. The relatively modest emission response in the short wavelength was observed in the titration process, which might be caused by the large difference in extinction coefficient between the donor ($\epsilon = 1.90 \times 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$) and acceptor ($\epsilon = 1.06 \times 10^5 \text{ M}^{-1}\cdot\text{cm}^{-1}$) moieties in this buffer system (Fig. S4†).

Fig. 3 a) Emission spectra ($\lambda_{\text{ex}} = 420$ nm) of **RN** (5 μM) in the presence of various amounts of Cu^{2+} (0–50 μM), in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM); b) The ratio (I_{568}/I_{540}) of **RN** (5 μM) upon addition of increasing amounts

of Cu^{2+} (0–120 μM), in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM).

Furthermore, the fluorescence responses of **RN** towards various metal ions and the emission that occurred after the addition of Cu^{2+} were measured. The results revealed that the Cu^{2+} -induced ratiometric fluorescence response was almost unaffected in the presence of common metal cations (Fig. 4a). Thus, the Cu^{2+} -selective binding and FRET “off-on” response could take place in the coexistence of the competitive metal ions. In other words, **RN** can selectively sense Cu^{2+} in a ratiometric fashion. To assess the performance of **RN** in complicated systems, the fluorescence responses of **RN** towards Cu^{2+} on the addition of several anions was also investigated. The results (Fig. 4b) showed that the enhancement resulting from the addition of Cu^{2+} is not interfered by the subsequent addition of these anions.

Fig. 4 a) Ratio (I_{568}/I_{540}) of **RN** (5 μM) in the presence of Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Ca^{2+} (1 mM); Ba^{2+} , Mn^{2+} , Fe^{2+} , Fe^{3+} , La^{3+} , Al^{3+} (75 μM) and Co^{2+} , Ni^{2+} , Cd^{2+} , Zn^{2+} , Cr^{3+} , Pb^{2+} , Hg^{2+} , Cu^+ (15 μM) (gray bars) and subsequent addition of Cu^{2+} (15 μM) (red bars), in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM); b) Ratio (I_{568}/I_{540}) of **RN** (5 μM) in the presence of Cu^{2+} (15 μM) upon addition of anions (75 μM), in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1, v/v) buffered with hepes (pH 7.4, 20 mM).

The effect of pH on the fluorescence response of **RN** to Cu^{2+} was also evaluated. As shown in Fig. 5, in the absence of Cu^{2+} , almost no change in ratio of I_{568}/I_{540} was observed for the free **RN** over a wide pH range of 5.0–9.0, indicating that the free sensor was stable in the wide pH range. Upon treatment

with Cu^{2+} , the maximal ratio was observed in the pH range of 4.0–7.5, which means that **RN** can work in the weak acidic and neutral media. In the pH of 7.4, the selectivity and sensitivity were investigated in the experiments mentioned above, and so at physiological pH, it suggests that **RN** is promising for applications in the complicated biological systems

Fig. 5 Ratio (I_{568}/I_{540}) of free probe **RN** (5 μM) and **RN** (5 μM) in the presence of Cu^{2+} (25 μM) at different pH values.

The significant ratiometric fluorescent “off-on” response of **RN** towards Cu^{2+} inspired us to evaluate its potential application as an imaging probe for Cu^{2+} in the bio-systems. Potential cytotoxic effects of **RN** would impede its use for *in vivo* applications. To address this issue, cell proliferation was first evaluated by MTT assays with HeLa cells (Fig. 6). Six different concentrations of **RN** were incubated with HeLa cells for 24 h. The cell viability obtained by the MTT assay was expressed as a fraction of viable cells and normalized to that of cells without co-incubation with **RN** (control). The increased concentration of **RN** gradually gave rise to the more pronounced inhibition of the cell proliferation, observed as decreases in the cell viability. Cell viabilities were maintained at 90% on incubation for 24 h with a concentration up to 50 μM . For the further evaluation **RN** as a probe for Cu^{2+} imaging, 10 μM **RN** was used, in which the cell viability remains more than 95%.

Fig. 6 Cell viability of HeLa cells incubated with various concentration of **RN** for 24 h.

Fig. 7 Fluorescence Images of HeLa cells. a) Microscopic image of HeLa cells treated with **RN** (10 μM) for 30 min in the absence of Cu^{2+} (control); b) Fluorescence image of (a) in green channel at 515 ± 10 nm, excited at 405 nm; c) Fluorescence image of (a) in red channel at 580 ± 10 nm, excited at 405 nm; d) Merge of (a-c); e) Microscopic image of HeLa cells further treated with Cu^{2+} (50 μM) for 30 min; f) Fluorescence image of (e) in green channel at 515 ± 10 nm; g) Fluorescence image of (e) in red channel at 580 ± 10 nm; h) Merge of (e-g).

To demonstrate the potential application in the biogeochemical systems, the ratiometric probe **RN** was preliminarily applied for ratiometric fluorescence imaging of intracellular Cu^{2+} in living cells. The dual-channel fluorescence images recorded at 515 ± 10 nm and 580 ± 10 nm with excitation at 405 nm were shown in Fig. 7. The HeLa cells incubated with **RN** (10 μM) for 30 min at 37°C displayed a strong green fluorescence of dansyl group (Fig. 7b) and almost no red fluorescence in the emission of rhodamine (Fig. 7c). By contrast, after incubated with Cu^{2+} (50 μM) for 30 min further, the HeLa cells gave a decrease in the green emission (Fig. 7f), but a dramatic enhancement in the red emission (Fig. 7g). The phenomenon was in good agreement with the emission profile in aqueous solution. Thus, these preliminary results demonstrate that the probe is cell membrane permeable and could be applied for ratiometric fluorescence imaging of intracellular Cu^{2+} with almost no cytotoxicity.

Conclusion

In summary, we have described a FRET “off-on” fluorescent ratiometric Cu^{2+} probe **RN** by using a facile “click” reaction. The proposed **RN** shows high selectivity towards Cu^{2+} . The imaging experiment confirmed that the ratiometric imaging ability of **RN** to trace intracellular Cu^{2+} in living cells. Although a relatively modest emission response in the short wavelength was observed for **RN**, the strategy was verified to have great potential for developing novel FRET probes for Cu^{2+} . The development of new probes with both large emission shifts and ratiometric responses could be further promoted by developing and appending new efficient donors. This strategy was also expected to have possibility and potential to give the Cu^{2+} probes other specific natures by “click”-linking specific moieties.

Acknowledgements

This work was financially supported by the Foundation of State Key Laboratory of Pollution Control and Resource Reuse, China (PCRRK11003) and Swedish government strategic faculty grant in material science (SFO, MATLIU) in Advanced Functional Materials (AFM).

Notes and references

^a State Key Laboratory of Pollution Control and Resource Reuse, Tongji University, Siping Rd 1239, Shanghai, 200092, P.R. China.

^b Division of Molecular Surface Physics & Nanoscience, IFM, Linköping University, Linköping 58183, Sweden.

* Corresponding authors. huzjun@tongji.edu.cn or xuazh@ifm.liu.se.

† Z. Hu and J. Hu contributed equally to this work.

† Electronic Supplementary Information (ESI) available: details of any supplementary information available should be included here. See DOI: 10.1039/b000000x/

- 1 a) H. Tapiero, D. M. Townsend, K. D. Tew, *Biomed. Pharmacother.*, 2003, **57**, 386-398; b) M. C. Linder, M. Hazegh-Azam, *Am. J. Clin. Nutr.*, 1996, **63**, 797-811; c) R. Uauy, M. Olivares, M. Gonzalez, *Am. J. Clin. Nutr.* 1998, **67**, 952-959.
- 2 a) G. Muthaup, A. Schlicksupp, L. Hess, D. Beher, T. Ruppert, C. L. Masters, K. Beyreuther, *Science*, 1996, **271**, 1406-1409; b) R. A. Løvstad, *BioMetals*, 2004, **17**, 111-113.
- 3 a) X. Li, X. Gao, W. Shi, H. Ma, *Chem. Rev.*, 2014, **114**, 590-659; b) A. P. Silva, H. Q. N. Gunaratne, T. H. Gunnlaugsson, J. M. Allen, C. P. McCoy, J. T. Rademacher, T. E. Rice, *Chem. Rev.*, 1997, **97**, 1515-1566; c) X. Chen, X. Tian, I. Shin, J. Yoon, *Chem. Soc. Rev.*, 2011, **40**, 4783-4804.
- 4 R. Bergonzi, L. Fabbrizzi, M. Licchelli, C. Mangano, *Chem. Rev.*, 1998, **170**, 31-47.
- 5 a) V. Dujols, F. Ford, A. W. Czarnik, *J. Am. Chem. Soc.*, 1997, **119**, 7386-7387; b) J. Jo, H. Y. Lee, W. Liu, A. Olasz, C.-H. Chen, D. Lee, *J. Am. Chem. Soc.*, 2012, **134**, 16000-16007; c) W. Lin, L. Long, B. Chen, W. Tan, W. Gao, *Chem. Commun.*, 2010, **46**, 1311-1313; d) Y. Ruan, C. Li, J. Tang, J. Xie, *Chem. Commun.*, 2010, **46**, 9220-9222; e) Z. Shi, X. Tang, X. Zhou, J. Cheng, Q. Han, J. Zhou, B.

- Wang, Y. Yang, W. Liu, D. Bai, *Inorg. Chem.*, 2013, **52**, 12668-12673.
- 6 A. P. Demchenko, *J. Fluoresc.*, 2010, **20**, 1099–1128.
- 7 K. Kikuchi, H. Takakusa, T. Nagano, *Trend Anal. Chem.*, 2004, **23**, 407-415.
- 8 a) L. Yuan, W. Lin, B. Chen, Y. Xie, *Org Lett.*, 2012, **14**, 432-435; b) M. H. Lee, H. J. Kim, S. W. Yoon, N. J. Park, J. S. Kim, *Org. Lett.*, 2008, **10**, 213-216; c) C. Kar, M. D. Adhikari, A. Ramesh, G. Das, *Inorg. Chem.*, 2013, **52**, 743-752; d) G. He, X. Zhang, C. He, X. Zhao, C. Duan, *Tetrahedron*, 2010, **66**, 9762-9768; e) C. Kaewtong, J. Noiseephum, Y. Uppa, N. Morakot, N. Morakot, B. Wanno, T. Tuntulanic, B. Pulpoka, *New J. Chem.*, 2010, **34**, 1104; f) D. Maitya, D. Karthigeyan, T. K. Kundu and T. Govindaraju, *Sens. Actuators, B*, 2013, **176**, 831.
- 9 a) J. Fan, P. Zhan, M. Hu, W. Sun, J. Tang, J. Wang, S. Sun, F. Song, X. Peng, *Org. Lett.*, 2013, **15**, 492-495; b) Z. Liu, C. Zhang, X. Wang, W. He, Z. Guo, *Org. Lett.*, 2012, **14**, 4378-4381; c) R. K. Pathak, V. K. Hinge, P. Mondal, C. P. Rao, *Dalton Trans.*, 2012, **41**, 10652-10660.
- 10 L. Yuan, W. Lin, K. Zheng, S. Zhu, *Acc. Chem. Res.*, 2013, **46**, 1462-1473.
- 11 J. F. Zhang, Y. Zhou, J. Yoon, Y. Kim, S. J. Kim, J. S. Kim, *Org Lett.*, 2010, **12**, 3852-3855.
- 12 a) W. Shi, H. Ma, *Chem. Commun.*, 2012, **48**, 8732-8744; b) X. Chen, T. Pradhan, F. Wang, J. S. Kim, J. Yoon, *Chem. Rev.*, 2012, **112**, 1910–1956.
- 13 a) H. Kim, S. Lee, J. Lee, J. Tae, *Org. Lett.*, 2010, **12**, 5342-5345; c) K. Sreenath, R. J. Clark, L. Zhu, *J. Org. Chem.*, 2012, **77**, 8268-8279.
- 14 a) J. E. Moses, A. D. Moorhouse, *Chem. Soc. Rev.*, 2007, **36**, 1249–1262; b) F. Amblard, J. H. Cho, R. F. Schinazi, *Chem. Rev.*, 2009, **109**, 4207–4220.
- 15 N. R. Chereddy, S. Thennarasu, A. B. Mandal, *Analyst*, 2013, **138**, 1334-1337.
- 16 J. Jia, H. Ma, *Chinese Sci. Bull.*, 2011, **56**, 3266-3272.
- 17 X. Meng, S. Wang, Y. Li, M. Zhu, Q. Guo *Chem. Commun.*, 2012, **48**, 4196-4198
- 18 K. M. Fries, C. Joswig, R. F. Borch, *J. Med. Chem.*, 1995, **38**, 2672-2680.