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Rapid Detection of Copper in Biological Systems Using Click Chemistry

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A fast (1 min), straightforward but efficient, click chemistry-based system that enables the rapid detection of free copper (Cu) ions in either biological fluids or living cells without tedious pretreatment is provided. Cu can quickly induce the conjugation between graphene oxide (GO) and a fluorescent dye via click reaction. On the basis of the high specificity of bioorthogonal reaction and the effective quenching ability of GO, the assay studied in this paper can respond to Cu ions in less than 1 min with excellent selectivity and sensitivity, which is the fastest sensor for Cu as far as it is known. In addition, the application of this system is verified by performing assays in living cells and untreated urine samples from patients suffering from Wilson's Disease. Such a Cu detection system shows great promises in both fundamental research and routine clinical diagnostics.

1. Introduction

Copper (Cu), as an essential element for life and the third most abundant transition metal in human bodies, is closely involved in many biological processes such as metalloprotein composition and gene expression.[1–3] However, the amount of Cu in living systems must be strictly regulated, the alteration of Cu in physiological systems can trigger aberrant oxidative damage and toxicity and is associated with many diseases such as anemia, cancer, cardiovascular disorders, Wilson's Disease, and other neurodegenerative diseases.[4–9] For instance, Wilson's disease that can be diagnosed through detection of Cu^{2+} in the urine, is one of the very few hereditary neuropathic diseases that can be efficiently treated if promptly diagnosed. Unfortunately, a majority of patients suffer from permanent mental damage or even death due to failure in early diagnostics.[10] As such, a tool for convenient monitoring of Cu ions in biological system such as biological fluidics and living cells is of great significance for both scientific research and clinical diagnostics.

Existing methods for Cu analysis have several major limitations. The gold standards for the quantification of Cu including inductively coupled plasma mass spectroscopy (ICP-MS), atomic absorption/emission spectroscopy (AAS/AES) and are currently employed in early screen of Cu metabolism-related diseases in clinics.[11–13] Unfortunately, these methods usually rely on expensive instruments and complicated pre-treatment procedures with skilled operators and are not accessible in most hospitals or clinics, especially those in remote areas, nor could these assays be employed to develop point-of-care-testing (POCT) assays. In comparison, electrochemical assays can enable rapid detection of copper with good selectivity, and simplified operation due to the development of ion-selective membrane.[14–17] Very recently, colorimetric assays based on the surface plasmon resonance of nanoparticles have gained popularity since they can enable the rapid, even naked-eye detection of metallic ions in aqueous solutions,[18] with many rapid Cu sensors being developed.[18–22] However, none of these methods can satisfy the demand of intercellular imaging, which can give more critical information for fundamental research.[23–25] Currently there are few fluorescent sensors for copper imaging and a majority of them are based on synthetic organic molecules.[25] In order to obtain tight binding affinity to Cu, these molecules mostly require rigorous synthetic procedures and purifications, which limits their widespread application.[26–29] Thus, a straightforward, rapid, inexpensive but effective method for Cu detection in biological samples is thus in urgent needs.

To address these issues, we turn to the catalytic activity of Cu for click reaction based on our previous study that click-chemistry occurs on surface of nanomaterials can enable copper detection

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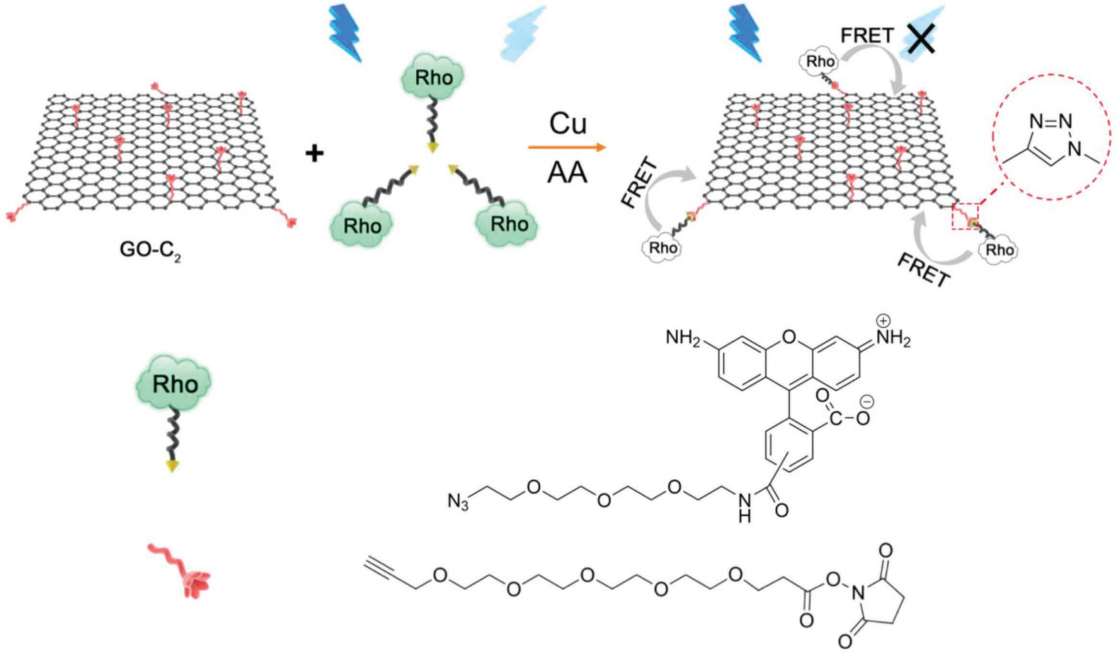

Figure 1. Schematic illustration of the Cu detection system based on click chemistry and FRET between GO-C_2 and Rho-N_3. GO-C_2: graphene oxide functionalized with alkyne. Rho: carboxyrhodamine110; AA: sodium ascorbate.

in aqueous,[13] and demonstrate a graphene oxide (GO)-based system for Cu detection in biological fluids and living cells based on Förster resonance energy transfer (FRET) (**Figure 1**),[30,31] since previous studies indicate that graphene oxide can quench the fluorescence on its surface by FRET.[32–34] Click chemistry is Cu-catalyzed bio-orthogonal reaction between alkyne and azide groups.[35] In this study, we functionalize the GO with ligands presenting alkyne group and mix the alkyne-functionalized GO (GO-C_2) and carboxyrhodamine110-azide (Rho-N_3). The addition of Cu^{2+} and a reductant which converts Cu^{2+} to Cu^{+}, the effective catalyst for click chemistry then induces the linkage of Rho-N_3 to the surface of GO-C_2. GO is an ideal energy acceptor that can quench the fluorescence of Rho-N_3 efficiently.[36,37] As the Cu-catalyzed click chemistry is a very fast reaction, and has high specificity and efficiency for Cu, the sensor would present rapidity, selectivity, specificity and sensitivity.[35,38] We further apply this platform for Cu sensing in biological systems including living cells and patient urine samples.

2. Result and Discussion

2.1. Characterization of GO-C_2

After the conjugation of acetylene-tetraethylene glycol *N*-hydroxysuccinimide ester (C_2-PEG_4-NHS) on the surface of amine-functionalized graphene oxide (GO-NH_2) in a one-step reaction, the obtained GO-C_2 is capable of quenching the fluorescence of Rho-N_3 in the presence of Cu^{2+} and sodium ascorbate (AA), which suggests the successful modification of alkyne groups on the surface of GO. On the basis of Cu(I)-catalyzed click reaction, neither Cu^{2+} without AA nor AA by itself would induce the quenching of the fluorescence (**Figure 2**a). The obtained GO-C_2 is well dispersed in phosphate buffer saline (PBS) with a narrow size distribution (Figure 2b). A shift in the UV–vis spectra (Figure 2c) and the increase of surface zeta potential (Figure 2d) also confirm the surface functional group variation of GO. These results indicate the successful preparation of GO-C_2 and its further possible capability for Cu detection.

2.2. Condition Optimization for Assaying Cu^{2+}

To understand the response rate of this sensing system to Cu^{+}, we measure the fluorescence spectra change of our system at different time intervals. After the addition of Cu^{2+} (50×10^{-6} M) and excess AA (1×10^{-3} M), the fluorescence decreases rapidly within 1 min and the reaction completes within 5 min (**Figure 3**a,b). The fast reaction rate is most likely due to the rapid kinetics of Cu(I)-catalyzed click reaction. In addition, the high surface-to-volume ratio of graphene also plays an important role in the fast rate of reaction. The result reveals that this system can provide a rapid way to detect Cu ions in aqueous solutions.

In order to obtain the most efficient detection, we optimize the ratio of GO-C_2 to Rho-N_3 that causes the most obvious relative fluorescence reduction in the presence of Cu. We perform this study because if the GO-C_2 were in excess, the initial fluorescence intensity would be too low as a result of nonspecific adsorption-induced quenching. On the contrary, insufficient amount of GO-C_2 cannot quench enough fluorescence for signal generation. We add 50×10^{-6} M Cu^{2+} and 1×10^{-3} M AA to 1 mL mixtures of GO-C_2&Rho-N_3 with different mass ratio (GO-C_2 with a constant concentration of 50 mg L^{-1}), and use the value of the decreased fluorescence intensity (I_d) at 525 nm versus the fluorescence intensity (I_0) without Cu addition to compare the relative reduction in fluorescence. The relative reduction in fluorescence increases

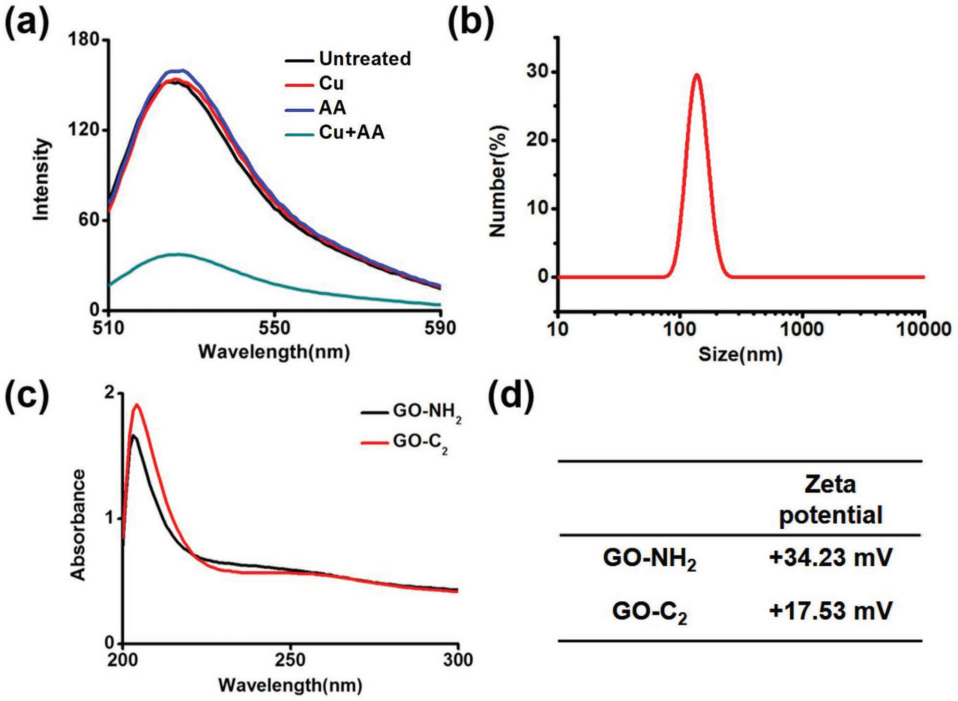

Figure 2. Confirmation of the conjugation of C_2 on the surfaces of GO-NH_2. a) Fluorescent spectra of untreated GO-C_2&Rho-N_3 and GO-C_2&Rho-N_3 in the present of AA (1×10^{-3} M), Cu^{2+} (50×10^{-6} M), or Cu^{+} (50×10^{-6} M); Cu^{+} is obtained by reducing Cu^{2+} with AA (1×10^{-3} M). b) Size distribution of GO-C_2 measured by dynamic light scattering (DLS). c) UV–vis spectra of GO-C_2 and GO. d) Zeta potential of GO-C_2 and GO-NH_2 measured by DLS.

from 34% to 81% with the increasing mass ratio of GO-C_2 to Rho-N_3, indicating that this system shows obvious response to Cu at high mass ratio of GO-C_2 to Rho-N_3. We observe no significant difference after the mass ratio increases past 100–400 (Figure 3c). To obtain maximum quenching fluorescence in the following study we use the mass ratio (GO-C_2:Rho-N_3 = 100:1) to evaluate the response of our system to Cu.

2.3. Sensitivity of GO-C_2&Rho-N_3 for Assaying Cu^{2+}

To explore the minimum detectable concentration of Cu^{2+} in PBS by our assay, we add different concentrations of Cu into mixtures of GO-C_2&Rho-N_3 with final concentrations of 0, 2.5×10^{-9}, 5×10^{-9}, 25×10^{-9}, 50×10^{-9}, 250×10^{-9}, 500×10^{-9} M, 2.5×10^{-6}, 5×10^{-6}, 25×10^{-6}, and 50×10^{-6} M. We add AA to a final concentration of 1×10^{-3} M to allow the reduction of Cu^{2+} to Cu^{+}. After mixing and keeping the mixtures in darkness for 5 min, we record the fluorescent spectra of these mixtures (**Figure 4**a). The fluorescence intensities at 525 nm of the mixtures decrease gradually depending on the increasing concentration of Cu^{2+} (Figure 4b). In addition, our assay exhibits a broad dynamic range ranging (from 50×10^{-9} M to 50×10^{-6} M), and the limit of detection for Cu^{2+} by fluorescence intensity change is as low as 50×10^{-9} M (S/N = 3). Due to the fact that click reaction is a catalytic reaction, the response is not being

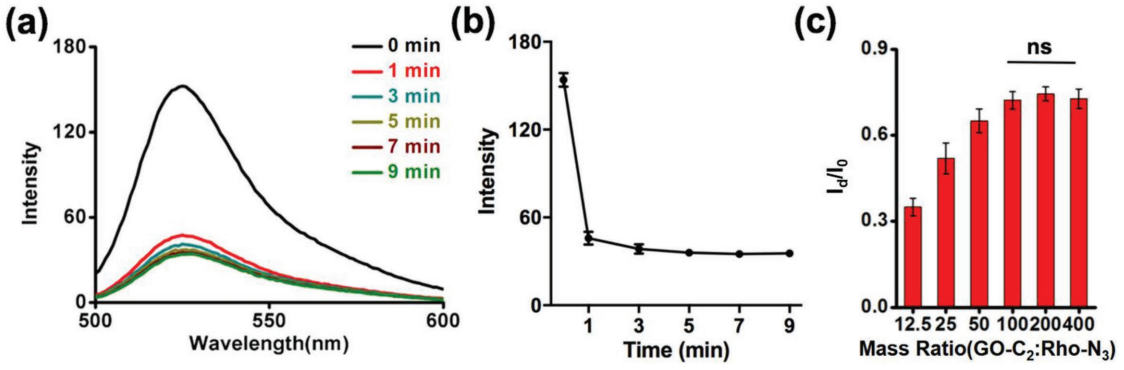

Figure 3. Kinetics and condition optimization for Cu detection. a) Fluorescence spectra of GO-C_2&Rho-N_3 versus time after the addition of 50×10^{-6} M Cu^{+}. b) Fluorescence intensity at 525 nm of GO-C_2&Rho-N_3 versus time after the addition of 50×10^{-6} M Cu^{+}. c) Fluorescence decrease of GO-C_2&Rho-N_3 mixtures with different mass ratio in the presence of 50×10^{-6} M Cu^{+}, GO-C_2 keeps a constant concentration of 50 mg L^{-1}. I_d: decreased fluorescence intensity, I_0: fluorescence intensity (I_0) without Cu addition, mean ± SD, $n = 3$.

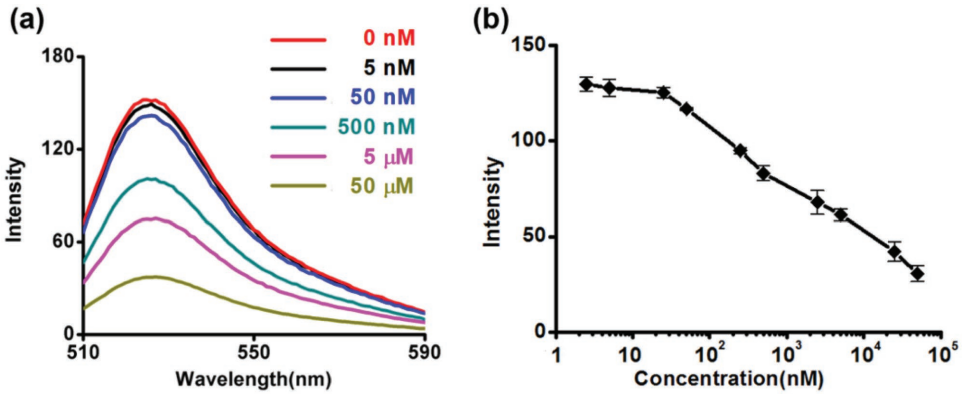

Figure 4. Sensitivity of GO-C_2&Rho-N_3 for assaying Cu^{2+} a) fluorescence spectra of GO-C_2&Rho-N_3 in the presence of different concentrations of Cu^{2+} and 1×10^{-3} M AA. b) fluorescence intensity at 525 nm of GO-C_2&Rho-N_3 with different concentrations of Cu^{2+} and 1×10^{-3} M AA, mean ± SD, $n = 3$.

linear with concentration of the targets, which is in accordance with our previous report and other work employ catalytic reaction for detection.[39–41] The result indicates the capability of this system in detecting trace amount of Cu^{2+} in aqueous solutions.

2.4. Selectivity for Assaying Cu^{2+}

To demonstrate that the GO-C_2&Rho-N_3 system has good selectivity for Cu^{2+}, we test the response of GO-C_2&Rho-N_3 to other metallic ions including Fe^{3+}, Al^{3+}, Cr^{3+}, Pb^{2+}, Hg^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Sn^{2+}, Fe^{2+}, Mg^{2+}, and Ag^{+}, Na^{+} and K^{+}. We also add AA (1×10^{-3} M) to every mixture except for the reducible ions Fe^{3+} and Cu^{2+}. At a concentration of 1×10^{-3} M, these metal ions cannot cause the decrease of fluorescence intensity compared to the control (**Figure 5**), suggesting the good selectivity of our assay toward Cu detection.

Next, we evaluate the specificity of the detection system by performing our assay in the presence of mixtures containing four other different metal ions in PBS (Figure S1, Supporting Information). we use four groups of random mixtures of metallic ions for this experiment. Without the addition of Cu^{2+} (50×10^{-6} M) and AA (1×10^{-3} M), none the four groups of mixtures with different other metal ions can induce the fluorescence change of the detection system. However, a significant fluorescent reduction occurs when we add Cu^{2+} (50×10^{-6} M) and AA (1×10^{-3} M) to these mixtures. The result implies that other metallic ions have negligible influence toward Cu detection. The good selectivity is due to the high specificity of the Cu(I)-catalyzed click reaction, which endows the detection system the capability to perform assays in complex samples without tedious pretreatment.

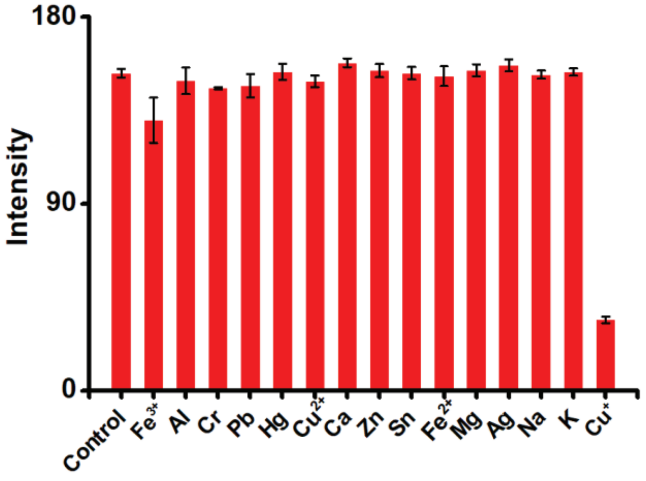

Figure 5. Selectivity of GO-C_2&Rho-N_3 for assaying Cu^{2+}, fluorescence intensity at 525 nm of GO-C_2&Rho-N_3 in the presence of different metal ions, mean ± SD, $n = 3$.

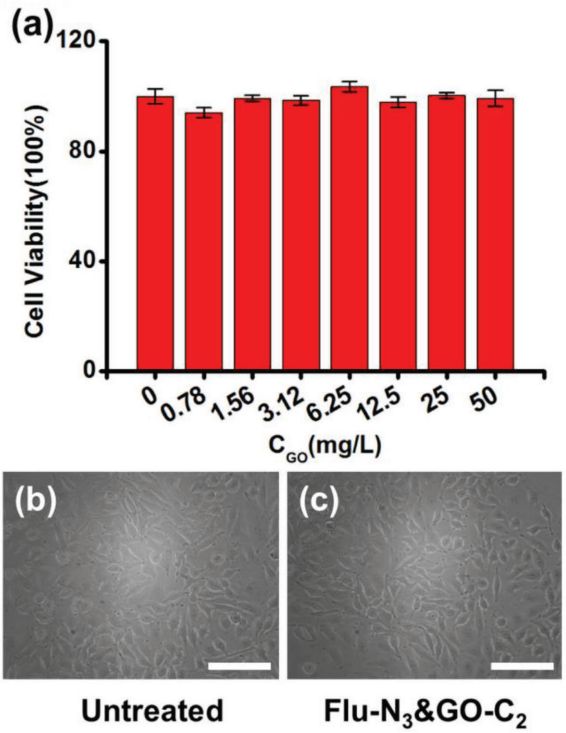

Figure 6. Cytotoxicity of the GO-C_2&Rho-N_3 based sensing system. a) Cell viability of HUVEC after incubation with different amounts of GO-C_2&Rho-N_3. The mass ratio between GO-C_2 and Rho-N_3 is 100:1, mean ± SD, $n = 3$. b) Phase-contrast microscopy image of untreated HUVESs. c) Contrast image of HUVECs after incubating with GO-C_2&Rho-N_3 (50 mg L^{-1} for GO-C_2, 1 mg L^{-1} for Rho-N_3) for 24 h, scale bar: 100×10^{-6} M.

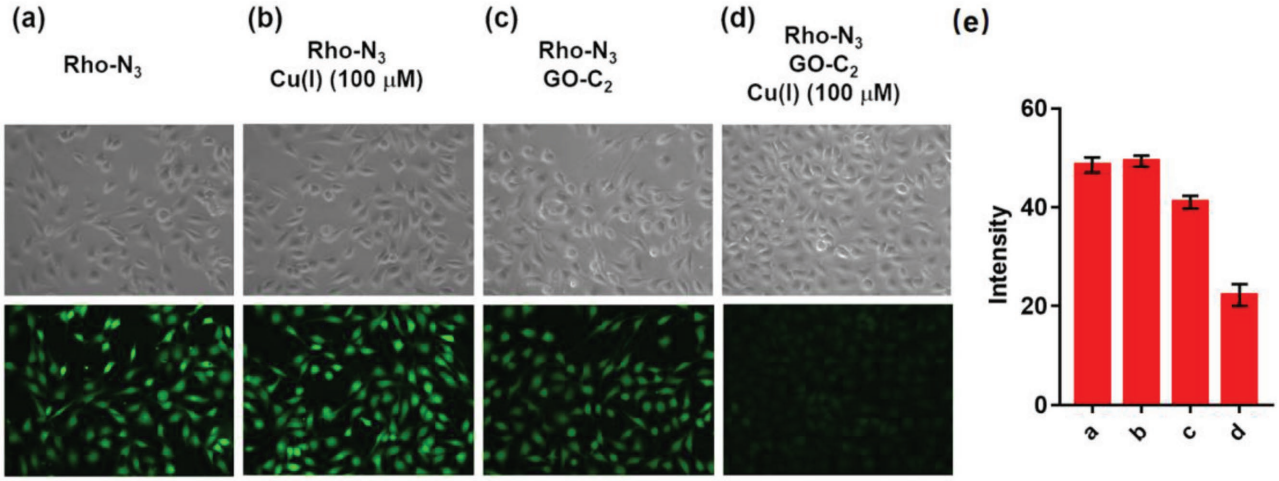

Figure 7. Phase contrast and fluorescence images of HUVECs in the presence of a) Rho-N_3, b) Rho-N_3&Cu, c) Rho-N_3&GO-C_2, and d) Rho-N_3&GO-C_2&Cu, scale bar: 100×10^{-6} M. e) Quantified mean fluorescence intensity inside cells from group (a)–(d), mean ± SD, $n = 3$.

2.5. Cytotoxicity of the GO-C_2&Rho-N_3

We test the toxicity of our sensing systems, since an ideal fluorescent sensor for intracellular Cu should be biocompatible and nontoxic.[42] We thus evaluate the cytotoxicity of Rho-N_3&GO-C_2 by using human umbilical vein endothelial cells (HUVECs) as a model. After incubating HUVECs with different concentrations of Rho-N_3&GO-C_2 for 24 h, we test the cell viability using standard Counting Kit-8 (CCK-8) protocols (**Figure 6**a). At the concentration for intracellular imaging, the viability of HUVECs shows no differences compared to these without Rho-N_3&GO-C_2. In addition, HUVECs exhibit typical morphology and density after incubation (Figure 6b,c). These results indicate the good biocompatibility of our system for cell imaging.

2.6. Intracellular Cu Imaging

Intracellular imaging of copper can provide much fundamental information for scientific research, while few probe for copper imaging are generally accessible due to their costs in both synthesis and purification, which limits their widespread application. In our assay, the materials used are inexpensive, commercially available, and the total procedure for synthesize and purification are very easy to perform. We thus evaluate if the detection system can assay Cu in living cells for future research. Previous study indicates that both GO and Rho-based materials and can rapidly enter living cells for intercellular imaging.[43–46] In this study, to test if Rho-N_3 and GO-C_2 can penetrate living cells, we incubate HUVECs with Rho-N_3 or Rho-N_3&GO-C_2 for 30 min, and observe the strongest fluorescence in Rho-N_3 treated cells (**Figure 7**a,e), as well as a slightly weakened fluorescence in Rho-N_3&GO-C_2 treated cells (Figure 7c,e). The weakened fluorescence is due to the exogenous copper by observing the increased fluorescence of cell that previously treated with buthionine sulfoximine (BSO), which can reduce the level of glutathione to lower the concentration of endogenous labile Cu(I) in living cells (Figure S2, Supporting Information).[47,48] These results confirm the penetration of these compounds into the living cells and the potential of our assay in sensing endogenous Cu. We evaluate the performance of our assay to imaging Cu in living HUVECs according to previous reports. In the absence of GO-C_2, Cu (would not significantly quench the fluorescence of Rho-N_3 (Figure 7b,e). However, reduction of fluorescence intensity is obvious in HUVECs loaded with both Rho-N_3&GO-C_2 and Cu(I) (Figure 7d,e). To further test the response rate to Cu in living cells, we add Cu^+ (100×10^{-6} M) to HUVECs pre-loaded with Rho-N_3&GO-C_2. After the addition we observe obvious quench of fluorescence within 1 min, which is also in accordance with our results in PBS (Movie S1, Supporting Information), indicating our assay can rapidly respond exogenous copper within 1 min. We attribute the fast response to the click reaction. The fast reaction is critical for monitoring transient variation of Cu. These results verify the capability of the detection system in Cu imaging of living cells and the potential of our assay as a robust tool for basic research in physiology.

2.7. Analysis Cu^{2+} in Patient Urine Sample

To test the practicability of our assay for clinical use, we perform our assay in detecting Cu in patient (Wilson's Disease) urine samples. Detecting Cu content in urine is an effective way for the screening of Wilson's Disease at early stage, which can greatly improves the cure rate and quality of life of patients.[49,50] Unfortunately, few hospitals can carry out this test due to the reliance on bulky instrument, such as ICP optical emission spectrometry (OES). In comparison, the simplicity and rapidity thus endow our assay the potential for developing POCT assays in resource-poor areas. To test this hypothesis, we add the urine samples from healthy people/patients and AA to a final concentration of 1×10^{-3} M to the mixtures of Rho-N_3&GO-C_2, after addition we observe a stronger reduction in the fluorescence signal for patient samples whose Cu content is higher than those for healthy individuals (**Figure 8**a). We employ artificial urine to calibrate for quantification (Figure S3, Supporting Information). In addition, we integrate our system with a smartphone by a

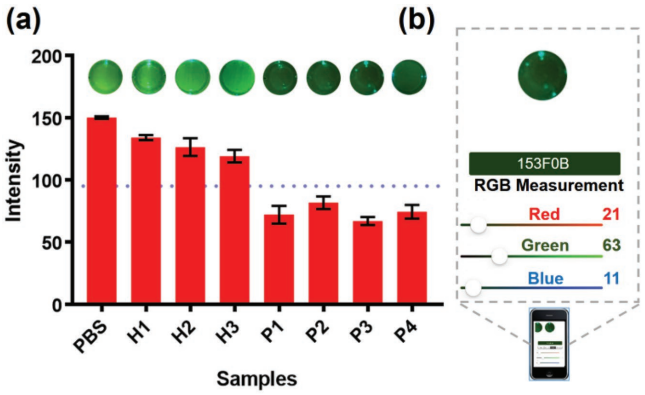

Figure 8. Detection Cu in patient samples. a) Photos under UV lamp and fluorescence intensity at 525 nm of GO-C_2&Rho-N_3 in the presence of urine samples and AA (1×10^{-3} M), mean ± SD, $n = 3$. b) Detection copper with GO-C_2&Rho-N_3 by reading the green value of the photo of the samples using the smartphone.

color scan software. We scan the photos of the solutions of GO-C_2&Rho-N_3 in the presence of urine samples and AA (1×10^{-3} M), the values from patient samples decrease significantly compare to the normal samples (Figure S4, Supporting Information). When comparing our result with those obtained by ICP-OES, the gold standard for the quantification of metallic ions, our assay shows good consistency (**Table 1**). The result indicates the practicability of our assay in developing strategies for early screening of Cu-related metabolism diseases.

3. Conclusion

We develop a fluorescent assay to detect Cu that offers advantages of simplicity, rapidity, high sensitivity, and selectivity. The GO-C_2&Rho-N_3 system based on click chemistry and FRET can response Cu within 1 min, and detects Cu as low as 50×10^{-9} M with good selectivity as well as anti-interference activity. Moreover, our method can detect Cu in living cells and patient urine samples without tedious treatment. In our system, the materials used are inexpensive, commercially available and free of complicated instruments, which gives our assay great potential as a robust tool in both research and clinical uses. The large surface-to-volume ratio of graphene could also provide additional modification to enable the possibility of targeted imaging, multiimaging or theranostics in the future. We hope that this kind of assay will be useful for many settings, like assays based on lab-on-chip format or lateral flow devices, where sensitive and selectivity assays requiring no advanced instruments are highly desired.

Table 1. Concentration of Cu in human urine samples determined by ICP and our assays.

Doner	ICP-OES [μg L^{-1}]	Quantitative detection with fluorescence [μg L^{-1}]	Qualitative detection with smartphone [μg L^{-1}]
Healthy 1	24.06 (N)	8.64 (N)	N
Healthy 2	30.06 (N)	16.64 (N)	N
Healthy 3	59.25 (N)	30.1523 (N)	N
Patient 1	473.5 (P)	422.14 (P)	P
Patient 2	285.5 (P)	186.69 (P)	P
Patient 3	625.1 (P)	627.80 (P)	P
Patient 4	580.72(P)	530.62 (P)	P

4. Experimental Section

Materials and Instrumentation: Most chemicals were purchased from major suppliers such as Alfa Aesar and Sigma–Aldrich unless otherwise noted, C_2-PEG_4-NHS and carboxyrhodamine110-Azide (Rho-N_3) were from Click Chemistry Tools Bioconjugate Technology Company (USA) and amine functionalized graphene oxides (GO-NH_2) are from Nanjing FAME Bearing Co., Ltd. (China). Patient urine samples were received from Beijing ChaoYang Hospital, and all the experiments were performed in compliance with the hospital guidelines (The Ethics Guidelines for Research Involving Human Subjects or Human Tissue from the Beijing ChaoYang Hospital). The fluorescence spectra on an RF-5301PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) operating at an excitation wavelength at 488 nm were collected; the fluorescence intensities at 525 nm were also collected. The excitation and emission slit widths were both 5 nm. The UV–vis measurement with an UV-2450 spectrophotometer (Hitachi, Tokyo, Japan) were performed. The hydrated radius and zeta potential with a zeta sizer nano ZS were measured (Malvern Zetasizer 3000HS and He/Ne laser at 632.8 nm and scattering angles of 90°). Barring special annotations, all the measurements were performed at room temperature. Cell image was carried out with a Leica microscope 6100A (Germany).

Preparation of Alkynes Functionalized GO: The functionalization of GO was performed in a one-step reaction. Amine functionalized GO was dissolved in MeOH/H_2O (10 vol%) solution under ultrasound for 3 h, GO-NH_2 (1 μg,1 mL) and acetylene-PEG_4-NHS Ester (10 μg) were mixed in 10 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (0.1 M pH 7.4). The reaction was continued for 2 h to allow the formation of C_2-PEG_4-functionalized graphene oxide (GO-C_2); the free molecules were removed by three rounds of centrifugation (7000 × *g*, 10 min). The as-prepared GO-C_2 was dissolved in PBS buffer with a final concentration of 50 μg mL^{-1} (transparent) for further use.

Experimental Procedure for Assaying Cu^{2+} in PBS Buffers: A typical procedure for assaying Cu^{2+} is as follows: GO-C_2 (50 μg mL^{-1}, 500 μL) was mixed with Rho-N_3 (500 μL, resolved in PBS); 10 μL solutions of different metal ions were added with a given concentration to 1 mL mixture of GO-C_2&Rho-N_3; excess amount of sodium ascorbate (AA, 10 μL) was added to a final concentration of 1×10^{-3} M. After mildly stirring the mixture in darkness for 5 min, the fluorescence measurements were performed.

To evaluate the sensitivity of this system to the detection of Cu^{2+}, PBS was used to dissolve $CuCl_2$ to prepare a stock solution, then different concentrations of $CuCl_2$ solutions were added (10 μL) to the mixture of GO-C_2&Rho-N_3 (1 mL), and the final concentrations of Cu^{2+} ranged from 2.5×10^{-9} M to 50×10^{-6} M, 10 μL AA was also added to a final concentration of 1×10^{-3} M and allow the reduction of Cu^{2+} to Cu^{+}.

To investigate the selectivity of GO-C_2&Rho-N_3, 10 μL solutions of different metal ions and AA (0.1 M) were added to the mixture of GO-C_2&Rho-N_3 (1 mL), the metal ions included Fe^{3+}, Al^{3+}, Cr^{3+}, Pb^{2+}, Hg^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Sn^{2+}, Fe^{2+}, Mg^{2+}, and Ag^{+}, Na^{+}, and K^{+}, each with a final concentration of 1×10^{-3} M, except that the concentration of Cu^{2+} is 50×10^{-6} M.

Cell Culture and Imaging: HUVECs (ATCC, US) were cultured in Dulbecco's modified Eagle medium (Gibco, American) supplemented with 10% fetal bovine serum (Gibco, American), glutamine (21×10^{-3} M), and 1% penicillin–streptomycin (PS, Invitrogen); the cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. To detect Cu in living cells, HUVECs were seeded on a 12 well plate and cultured for 24 h to allow attachments; afterward, 100×10^{-6} M Cu^{2+} or BSO was added to these cells and incubate for 30 min at 37 °C. After three rounds of washing to remove the excess Cu, PBS buffer that contained

GO-C_2&Rho-N_3 (50 mg L^{-1} for GO-C_2, 1 mg L^{-1} for Rho-N_3) and AA (100×10^{-6} M) was added and further incubated at 37 °C for 3 h. Then, another three rounds of washing were performed and cell imaging was carried out with a Leica microscope 6100A. To further test the response rate to Cu^+, HUVECs were cultured in the presence of GO-C_2&Rho-N_3 (50 mg L^{-1} for GO-C_2, 1 mg L^{-1} for Rho-N_3) for 3 h, and perform cell imaging by adding Cu^+ and AA (100×10^{-6} M) after washing. For all fluorescence images, it was confirmed that the microscope settings including brightness, contrast, and exposure time were constant in order to compare the relative intensity of intracellular fluorescence. Image processing was performed using the program of Leica Application Suite Advanced Fluorescence (LAS-AF) v4.8 software.

Cytotoxicity: The cytotoxicity of GO-C_2&Rho-N_3 was investigated via commercialized CCK-8 (Dojindo, Japan) and morphology observation. Briefly, 100 µL HUVECs were seeded in a 96 well plate (5000 per well) and cultured for 24 h to allow attachment. These cells were incubated with different concentrations of GO-C_2&Rho-N_3 in medium for 24 h. The medium was replaced with 200 µL fresh media containing 20 µL of CCK-8 solution and incubated for additional 3 h. The absorbance was measured at 450 nm measured using a microplate reader.

The absorbance from the cells on untreated plates was defined as a negative control (A_n), and the absorbance of solution containing only the same volume of the CCK-8 without HUVECs (A_b) was defined as blank control. Cell viability was calculated with the following equation

$$\text{Cell viability (\%)} = (A - A_b)/(A_n - A_b) \times 100\% \quad (1)$$

Similarly, GO-C_2&Rho-N_3 treated cell was imaged with calcium green (Invitrogen, USA) before recording with a Leica microscope 6100A (Germany) to investigate the morphological changes of cells.

Analysis of Cu^{2+} in Patient Urine Samples: To detect the free Cu ion in patient urine sample, 10 µL mixture of GO-C_2&Rho-N_3 was added to 990 µL urine samples, and the final concentrations made for GO-C_2 and Rho-N_3 were 50 mg L^{-1} and 500 µg L^{-1}, respectively. Excess amount of AA (10 µL) was further added to a final concentration of 1×10^{-3} M to convert Cu^{2+} to Cu^+. All the results were then recorded with a fluorescence spectrophotometer.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

click chemistry, Cu detection, graphene

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