ORGANOMETALLICS

Rhodamine–Ferrocene Conjugate Chemosensor for Selectively Sensing Copper(II) with Multisignals: Chromaticity, Fluorescence, and Electrochemistry and Its Application in Living Cell Imaging

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Supporting Information

ABSTRACT: A ferrocene unit is introduced into the salicylaldehyde Rhodamine 6G hydrazone with an alkynyl group to constitute a new chemosensor, R6G-FC, for specifically sensing Cu2+ over other metal ions with multisignals. Upon interaction with Cu²⁺ in aqueous solution, R6G-FC expresses a detectable fluorescence enhancement along with changes in UV-vis absorption spectra and electrochemical parameters due to the binding-induced spirolactam ringopening process in Rhodamine 6G. The complexation process between R6G-FC and Cu²⁺ can be carried out within a wide pH range in a short response time and with a good reversibility. R6G-FC also exhibits an excellent performance in sensing Cu²⁺ in the "dip-stick" method with an evident color gradation, and



confocal fluorescence microscopy imaging results reveal that R6G-FC can be used to visualize Cu2+ in living HeLa cells with low cytotoxicity.

INTRODUCTION

Copper, which ranks third in abundance among the essential trace metal ions (after iron and zinc) in the human body, plays an important role in various environmental, chemical, and physiological systems.¹ There are many reactions in biological processes that involve electron transfer that are catalyzed by copper-containing enzymes.² Disturbing the concentration balance of intracellular Cu2+ may cause several neurological diseases, such as Menkes syndrome,³ Wilson's disease,^{3b,e,4} amyotrophic lateral sclerosis,^{3e,5} Alzheimer's disease,^{5b,d,6} and prion disorder.^{5c,d,6c,d,7} Therefore, it is of great importance to accurately detect copper ions in the real environment, especially to strictly monitor and control the distribution of intracellular Cu²⁺.

In the last few decades, many advanced analytical methods have been developed to both qualitatively and quantitatively determine Cu²⁺, such as spectrophotometry,⁸ atomic absorption spectroscopy,9 inductively coupled plasma mass spectroscopy (ICP-MS),¹⁰ inductively coupled plasma atomic emission spectrometry (ICP-AES),¹¹ and voltammetry.¹² Among them, owing to the high sensitivity, excellent selectivity, nondestructiveness, and low cost, novel fluorescent chemosensors have drawn a lot of attention in the area of analytical chemistry, especially in biological analysis. Cu²⁺, with the 3d⁹ outermost electron structure, is known as a fluorescence quencher because of its paramagnetic property.¹³ As a result, many of the

reported fluorescent chemosensors detect Cu²⁺ through a fluorescence quenching process that undergoes an energy or electron transfer mechanism.¹⁴ In the consideration of sensitivity, fluorescence enhancement (turn-on) signals are much easier to detect than fluorescence quenching (turn-off) ones. The rhodamine fluorophore, known for its excellent spectroscopic properties and good water solubility, is an ideal moiety to construct "turn-on"-type fluorescent chemosensors in terms of metal ions triggering the spirolactam ring-opening mechanism along with changes in chromaticity and fluorescence at the same time.^{15,16} Considering the high affinity of Cu²⁺ to N and O atoms, rhodamine-hydrazone is an excellent platform to design novel fluorescent chemosensors for specifically sensing Cu^{2+} with high sensitivity in an aqueous solvent, ^{15h,m,16} even *in vivo*, ¹⁶ which has drawn a great deal of researchers' attention in recent years. On the other hand, as a traditional electrochemical signal donor, ferrocene has been widely applied in designing chemosensors for various cations and anions due to its good solubility and high biocompatibility.¹⁷ When interacting with an analyte, ferrocene-based chemosensors usually express a significant potential shift of the Fe^{III}/Fe^{II} redox couple, which could be in combination with other signals to obtain a multichannel sensing event.^{18,19} In

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comparison with traditional single-signal chemosensors, smallmolecule scaffolds with the ability to generate multichannel signals (chromogenic, fluorogenic, and electrochemical) provide better selectivity, higher sensitivity, and self-calibration ability through variable detecting methods with low background in complex systems. The development of multichannel Cu²⁺selective chemosensors based on rhodamine-ferrocene, in particular, is a relatively unexplored area with only a very few examples being reported including one of our group's recent work (Table S1, Supporting Information).²⁰ Therefore, it is still in demand and meaningful to keep modifying the molecular structures and examining the sensing behavior between the new rhodamine-ferrocene multichannel chemosensors and Cu2+. Inspired by the work of Tong,²¹ we additionally introduced a ferrocene unit into a salicylaldehyde Rhodamine 6G hydrazone platform through an alkynyl to construct a novel multisignaling chemosensor, R6G-FC (Scheme 1), for selectively sensing Cu²





with colorimetric, fluormetric, and electrometric readouts (Table S2, Supporting Information). Moreover, with low cytotoxicity and good solubility, **R6G-FC** could be used as a fluorescent chemosensor to image Cu^{2+} in living HeLa cells.

EXPERIMENTAL SECTION

Instruments and Reagents. Chromatographic separation was done by column chromatography using 300-400 mesh silica gel. ¹H NMR and ¹³C NMR were recorded on BrukerAV-500 and BrukerAV-400 spectrometers with chemical shifts reported in ppm (in CDCl₃; TMS as internal standard). Electrospray ionization mass spectra (ESI-MS) were analyzed on a Micromass LCTTM system and Thermo Finnigan LCQ Advantage spectrometer. Elemental analyses (C, H, and N) were carried out on a PerkinElmer 240C elemental analyzer. Melting points were determined on an XT4-100A hot-plate melting point apparatus and are uncorrected. All the pH measurements were monitored with a Sartorius PB-10 basic pH meter. UV-vis spectra and fluorescence spectra were recorded on a PerkinElmer Lambda 35 spectrometer and a PerkinElmer LS-50B fluorescence spectrophotometer at room temperature, respectively. Electrochemical measurements were conducted with a Shanghai CHI 660D electrochemical workstation. Fluorescence imaging was done with an Olympus BX51. The cytotoxicity assay was determined using an SLT Spectra Shell microplate reader spectrophotometer. Unless otherwise specified, materials were obtained from commercial suppliers and were used as received. All the solvents were of analytic grade, and water was redistilled. The salts used in stock solutions of metal ions were NaNO₃, KNO₃, Mg(NO₃)₂·6H₂O, Ca(NO₃)₂·4H₂O, Al(NO₃)·9H₂O, $Cr(NO_3)_3 \cdot 9H_2O$, $AgNO_3$, $Ba(NO_3)_2$, $Hg(NO_3)_2 \cdot H_2O$, $Mn(NO_3)_2 \cdot H_2O$, Mn($4H_2O$, $Pb(NO_3)_2$, $Cd(NO_3)_2 \cdot 4H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 - 6H_2O$ 6H₂O, Co(NO₃)₂·6H₂O, Fe(NO₃)₃·9H₂O, and Cu(NO₃)₂·3H₂O. Rhodamine 6G, ethynylferrocene, 5-bromosalicylaldehyde, Pd-(PPh₃)₂Cl₂, and CuI were purchased from Alfa Aesar. The other reagents were purchased from Taiyuan RHF Reagents Co. Ltd.

Property Test Procedures. A stock solution $(1.0 \times 10^{-3} \text{ M})$ of **R6G-FC** was prepared in DMSO. Stock solutions $(1.0 \times 10^{-2} \text{ M})$ of the nitrate salts of Na⁺, K⁺, Mg²⁺, Ca²⁺, Al³⁺, Cr³⁺, Ag⁺, Ba²⁺, Hg²⁺,

Mn²⁺, Pb²⁺, Cd²⁺, Ni²⁺, Zn²⁺, Co²⁺, Fe³⁺, and Cu²⁺ in water were prepared as well. Before spectral measurements, R6G-FC with different concentrations of Cu²⁺ was freshly prepared by diluting the high-concentration stock solution with EtOH/H₂O (1:1, v/v, pH = (7.0) to the corresponding solution and shaken for 10 s. 5 and 20 min latency times were needed before UV-vis and fluorescence detection, respectively. Fluorescence measurements were carried out with a 500 nm excitation wavelength and 10 nm slit width. All electrochemical experiments were measured under a nitrogen atmosphere at room temperature in a one-compartment container equipped with a glassycarbon working electrode, a platinum-wire auxiliary electrode, and an SCE reference electrode. The supported electrolyte was 50 mM tetrabutyl ammonium hexafluorophosphate $((n-Bu)_4NPF_6)$ in a solution of CH_3CN/H_2O (7:3, v/v, pH = 7.0). In selectivity experiments, one batch of sample was prepared by separately placing different kinds of metal ions into a stock solution of R6G-FC and then diluted with EtOH/H₂O (1:1, v/v, pH = 7.0) to the target concentration of R6G-FC (10 μ M). The other batch of sample was additionally prepared by adding a proper amount of Cu²⁺ into each of the latter samples. Ethylenediamine tetraacetic acid disodium salt (EDTA) was chosen as the chelating agent with Cu²⁺ to confirm the reversibility of the chemosensor R6G-FC. The wide pH range solutions were prepared by adjustment of pH buffer solutions with HCl or NaOH. In the "dip-stick" method, TLC plates were first immersed into an ethanol solution of R6G-FC $(1 \times 10^{-4} \text{ M})$ and then the solvent was evaporated to dryness. The modified TLC plates were separately dropped into different concentrations of Cu²⁺ (0, 1, 10, and 100 ppm) in distilled water and then dried in the dark in the air.

Cell Culture and Fluorescence Imaging. HeLa cells were cultured on a 24-well plate with a density of 2×10^3 cells per well in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin (100 μ g·mL⁻¹), and streptomycin (100 μ g·mL⁻¹) at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air for 24 h prior to staining. After washing with PBS buffer (phosphate-buffered saline, pH = 7.2, Gibco), HeLa cells were then incubated with chemosensor R6G-FC (10 μ M) and Hoechst 33342 (10 μ M) in the culture media containing ethanol/PBS (1:49, v/v) for 30 min at 37 °C. To remove the remaining R6G-FC, cells were additionally washed with PBS buffer three times. Experiments to assess Cu(II) uptake were performed in the same culture media supplemented with 100 μ M Cu(NO₃)₂ for 1 h. The treated HeLa cells were rinsed with PBS three times, and the fluorescence images were acquired through fluorescence microscopy (BX51, Olympus, Japan).

Cytotoxicity Assay. To investigate the cytotoxicity of the chemosensor R6G-FC in the cells over a 24 h period, a typical MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was performed. Before the treatment of cells with R6G-FC, HeLa cells under log phase growth were plated in 96-well plates $(5 \times 10^4$ cells per well) overnight. Chemosensor R6G-FC with final concentrations of 1, 5, 25, 50, and 100 μ M, which were diluted from the stock solution, was separately added to HeLa cells, and the treated cells were incubated at 37 °C under 5% CO2 and 95% air for 24 h. After removing the medium and adding 20 μ L of MTT solution (5 mg/mL, PBS), the HeLa cells were incubated for another 4 h at 37 °C under 5% CO₂ and 95% air. Then the solution was removed, and 150 μ L of DMSO was added to the cells. After agitating on an orbital shaker for 10 min, the absorbance of each well at 570 nm was recorded. The other batch of cells was treated according to the above steps and incubated for 48 h before recording the absorbance. The cell viability (%) was calculated according to the following equation: cell viability (%) = $OD_{570(sample)}/OD_{570(control)} \times 100$, where $OD_{570(sample)}$ represents the optical densities of the wells treated with various concentrations of chemosensor R6G-FC and OD_{570(control)} represents that of the wells treated with DMEM plus 10% FBS. The percentage cell survival values are relative to untreated control cells.

Synthesis of Rhodamine 6G Hydrazide (1). Rhodamine 6G hydrazide (1) was synthesized according to the reported method.^{19c} ¹H NMR (500 MHz, CDCl₃): δ = 7.95 (d, *J* = 3.5 Hz, 1H, Ar–H), 7.45 (s, 2H, Ar–H), 7.06 (d, *J* = 3.5 Hz, 1H, Ar–H), 6.39 (s, 2H,

Scheme 2. Synthetic Route of Chemosensor R6G-FC



Figure 1. (a) UV–vis absorption spectra of **R6G-FC** (10 μ M) upon addition of different amounts of Cu²⁺ (0–20 μ M) in EtOH/H₂O (1:1, v/v, pH = 7.0). Inset: The value of UV–vis absorption at 530 nm as a function of Cu²⁺ concentrations. (b) Job's plot of chemosensor **R6G-FC** with Cu²⁺ ([**R6G-FC**] + [Cu²⁺] = 20 μ M). The detection wavelength was 530 nm.

xanthene–H), 6.26 (s, 2H, xanthene–H), 3.58 (s, 2H, $-NH_2$), 3.48 (br s, 2H, -NH-), 3.22 (q, J = 6.8 Hz, 4H, $-CH_2-$), 1.92 (s, 6H, Ar–CH₃), 1.32 (t, J = 6.6 Hz, 6H, $-CH_3$).

Synthesis of 5-Ethynylferrocene-salicylaldehyde (2). In a 250 mL flask, ethynylferrocene (7.5 mmol, 1.58 g), 5-bromosalicylaldehyde (5 mmol, 1.01 g), Pd(PPh₃)₂Cl₂ (0.5 mmol, 0.35 g), and CuI (0.12 mmol, 0.03 g) were all dissolved in a mixed solvent (160 mL) of THF/Et₃N (1:1, v/v), and the reaction mixture was refluxed at 80 °C overnight under a N2 atmosphere. At the end of the reaction, the resulting ammonium salt was filtered off, and the amine solution was evaporated under reduced pressure. The crude product was purified by flash chromatography first with petroleum ether/ CH_2Cl_2 (6:1, v/v) to remove low-polar impurities followed by an elution of petroleum ether/CH₂Cl₂ (3:1, v/v, $R_f = 0.25$). The fourth band was collected, and an orange solid 2 (0.67 g, yield: 40%) was afforded after removal of the solvent.²² Mp: 164.9–165.3 °C. ¹H NMR (400 MHz, CDCl₃): $\delta = 11.07$ (s, 1H, -OH), 9.89 (s, 1H, -CHO), 7.70 (d, J = 2.1 Hz, 1H, Ar-H), 7.62 (dd, J = 8.6, 2.1 Hz, 1H, Ar-H), 6.96 (d, J = 8.6 Hz, 1H, Ar-H), 4.49 (t, J = 1.8 Hz, 2H, Fc-H), 4.26 (s, 7H, Fc-H). ¹³C NMR (101 MHz, CDCl₃): δ = 196.18, 160.98, 139.75, 136.57, 120.50, 118.02, 115.97, 87.88, 83.84, 71.38, 69.99, 68.92, 64.87. Anal. Calcd for C19H14FeO2: C, 69.12; H, 4.27. Found: C, 69.05; H, 4.33. TOF-MS (negative ion mode): m/z 329.1 $[M - H]^+$, 330.1 $[M]^+$.

Synthesis of Chemosensor **R6G-FC** (3). Rhodamine 6G hydrazide (1) (1.0 mmol, 0.43 g) and 5-ethynylferrocene-salicylaldehyde (2) (1.5 mmol, 0.49 g) were dissolved in anhydrous ethanol (10 mL), and the mixture was heated at 60 °C for 12 h under a N₂ atmosphere. The resulting wine red mixture was filtered and washed with cold ethanol to afford an earthy red solid, which was then recrystallized from anhydrous ethanol to provide the pure final product **R6G-FC** (3) (0.43 g, 58%).²¹ Mp: 235.2–236.0 °C. ¹H NMR (400 MHz, CDCl₃): $\delta = 11.22$ (s, 1H, –OH,), 8.83 (s, 1H, N=C–H), 8.01 (d, J = 8.0 Hz, 1H, Ar–H), 7.52 (p, J = 7.6 Hz, 2H, Ar–H), 7.29 (d, J = 1.4 Hz, 1H, Ar–H), 7.16 (d, J = 1.3 Hz, 1H, Ar–H), 7.11 (dd, J = 4.0 Hz, 1H, Ar–

H), 6.81 (d, J = 8.5 Hz, 1H, Ar–H), 6.44 (s, 2H, xanthene–H), 6.30 (s, 2H, xanthene–H), 4.45 (s, 2H, Fc–H), 4.21 (d, J = 4.8 Hz, 7H, Fc–H), 3.55–3.47 (m, 2H, –NH–), 3.22 (d, J = 6.7 Hz, 4H, –CH₂–), 1.89 (s, 6H, Ar–CH₃), 1.32 (t, J = 7.1 Hz, 6H, –CH₃). ¹³C NMR (101 MHz, CDCl₃): δ 164.45, 158.32, 151.64, 151.55, 150.18, 147.80, 134.22, 133.74, 129.01, 128.57, 127.67, 124.02, 123.46, 118.55, 118.20, 117.35, 114.37, 107.70, 105.35, 96.82, 86.47, 85.07, 71.24, 69.91, 68.63, 65.58, 38.34, 16.76, 14.74. Anal. Calcd for C₄₅H₄₀FeN₄O₃: C, 72.97; H, 5.44; N, 7.56. Found: C, 73.05; H, 5.50; N, 7.49. TOF-MS: m/z 741.9 [M + H]⁺.

RESULTS AND DISCUSSION

Synthesis and Characterization. Chemosensor R6G-FC is a Schiff base synthesized according to Scheme 2. Ethynylferrocene first reacted with 5-bromosalicylaldehyde through a Sonogashira coupling reaction and then was connected with Rhodamine 6G hydrazone to afford the target product R6G-FC as an earthy red solid with a yield of 58%. In the structure of R6G-FC, the Rhodamine 6G fluorophore acts as a colorimetric and fluorescent signal donor, and the ferrocene moiety provides an electrochemical signal with salicylaldeheyde and the Schiff base as the spacer and sensing part. **R6G-FC** is stable in its DMSO stock solution (1.0×10^{-3}) M) for over 2 weeks. The sensing behavior of R6G-FC with Cu²⁺ was preliminarily tested in several common solvents (ethanol, water, acetonitrile, methanol, dimethyl sulfoxide), which were used for spectral detection. As depicted in Figure S8 (Supporting Information), except for dimethyl sulfoxide quenching the fluorescence of the ring-opened form of Rhodamine 6G, R6G-FC in the five different solvents could all give signal responses to Cu²⁺ in varying degrees. Ethanol

solution of **R6G-FC** gave the best signal response to Cu^{2+} in both UV–vis absorption and fluorescence emission spectra compared to the other four solvents. Considering the intensity of the signal and the toxicity of the solvent, the ethanol–water mixed solvent (1:1, v/v) was chosen as the spectral test solution of **R6G-FC** for sensing Cu^{2+} . The time dependence experiment of **R6G-FC** after adding Cu^{2+} was investigated with both the UV–vis absorption and fluorescence emission method, and the signals remained stable after 5 and 20 min, respectively (Figure S9, Supporting Information).

UV-Visible Absorption Studies. The UV-vis spectral properties of R6G-FC were examined through a titration experiment (Figure 1a). The initial state of R6G-FC exhibited almost no absorption from 450 to 650 nm, indicating that most of the R6G-FC molecules stayed in the ring-closed spirolactam form. With the addition of Cu2+, a new absorption peak emerged at 530 nm, which was in accordance with the typical absorption wavelength of ring-open Rhodamine 6G. The intensity of absorption increased along with the increasing concentration of Cu^{2+} (0-0.55 equiv) in a good linear relationship ($R^2 = 0.9990$, Figure S10, Supporting Information). After titrating approximately 1 equiv of Cu^{2+} into the system of R6G-FC, the intensity of absorption at 530 nm no longer increased to afford the $R6G-FC/Cu^{2+}$ complex with the mole absorption coefficient ε of 8.16 \times 10⁴ L·mol⁻¹·cm⁻¹, suggesting the completed formation of spirolactam ring-opened R6G-FC. Additionally, the ratio relationship between R6G-FC and Cu²⁺ was estimated with a Job's plot experiment. From Figure 1b, it could be seen that the maximum value of absorption appeared at 0.5, which indicated that the stoichiometry of the R6G-FC/Cu²⁺ complex was 1:1. According to the UV-vis titration experiment, the value of $1/(A - A_0)$ of R6G-FC at 530 nm versus $1/[Cu^{2+}]$ also had a good linear relationship with $R^2 = 0.9960$, which was consistent with the Job's plot result (Figure S11, Supporting Information). Hence, the association constant K_{a} of R6G-FC/Cu²⁺ could be calculated based on the revised Benesi-Hildebrand equation as 1.52×10^{6} M⁻¹ (error <10%) (eq 1, Supporting Information).²³

Fluorescence Emission Studies. In the investigation of the fluorescence property of R6G-FC (Figure 2), only weak fluorescence of the individual R6G-FC system was observed due to the main existing form of the spirolactam structure in R6G-FC. When Cu^{2+} was added to the system, the fluorescence



Figure 2. (a) Fluorescence emission spectra of **R6G-FC** (10 μ M) in the presence of different concentrations of Cu²⁺ (0–32 μ M) in EtOH/ H₂O (1:1, v/v, pH = 7.0). Inset: The value of fluorescent intensity at 554 nm as a function of Cu²⁺ concentrations. Excitation wavelength was 500 nm. (b) Image of fluorescence change of **R6G-NA** (10 μ M) before (left) and after (right) interaction with Cu²⁺ (30 μ M).

emission centered at 554 nm gradually increased along with the increasing amount of Cu2+, indicating that the ring-opened form of R6G-FC became the predominant species to release the fluorescence of Rhodamine 6G. Furthermore, the fluorescent color of the solution of R6G-FC changed significantly after interaction with Cu2+ from dark to bright yellow by naked-eye observations under UV light (365 nm) (Figure 2b). The values of fluorescence intensity were proportional to the concentration of Cu^{2+} within two ranges, 0.5-8.0 and 8.0-15 μ M, respectively, and with a good linear relationship (Figure S12, Supporting Information). Also, the detection limit (S/N = 3) of **R6G-FC** to Cu²⁺ was determined to be 6.97×10^{-7} M (Figure S13, Supporting Information).²⁴ The fluorescence turn-on response of R6G-FC was in good agreement with the results of the UV-vis titration studies, which demonstrated that R6G-FC was a qualitative and quantitative chemosensor for Cu²⁺ in aqueous solution.

Selectivity Studies. The signal response of R6G-FC toward various metal ions, such as Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Al^{3+} , Cr^{3+} , Ag^+ , Ba^{2+} , Hg^{2+} , Mn^{2+} , Pb^{2+} , Cd^{2+} , Ni^{2+} , Zn^{2+} , Co^{2+} , and Fe²⁺, was also studied with both UV-vis absorption and fluorescence emission spectroscopy under the identical conditions of Cu^{2+} (Figure 3). Compared to the remarkable enhancement of absorption intensity of R6G-FC induced by Cu²⁺, the influence caused by other metal ions was negligible even at the millimolar level (alkali and alkali-earth metal ions) in addition to the slight absorption enhancement brought by Fe²⁺.²¹ Consistent with the absorption enhancement, the color of the R6G-FC system obviously changed from colorless to pink after adding Cu²⁺, which could be observed by the nakedeye (Figure 3a). To further explore the selectivity of R6G-FC to Cu^{2+} , a competition experiment was conducted by additionally mixing Cu^{2+} with an R6G-FC solution in the presence of other metal ions (Figure 3b). The bar graph of the fluorescence emission intensity revealed that the Cu²⁺-triggered fluorescence enhancement of R6G-FC almost remained unaffected in the presence of higher concentrations of other metal ions. Hence, the above results of the optical experiments all indicated that R6G-FC had a high sensitivity and selectivity toward Cu²⁺ with no interference from other metal ions even at higher concentrations, thus revealing that R6G-FC could be assumed to be a Cu²⁺-selective chemosensor.

Electrochemical Studies. The electrochemical signal, which can be easily read out on-site, is another essential parameter for ferrocene-containing chemosensors in the recognition process.²⁵ After introducing Cu^{2+} into the chemosensor system, the triggered ring-opening process of the spirolactam in R6G-FC would induce a change in the molecular configuration and then influence the electron density within the ferrocene group to express distinct electrochemical signals. The cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiment of the Cu^{2+} -recognition properties of R6G-FC were conducted in an aqueous CH₃CN solution with (n-Bu)₄NPF₆ (50 mM) as supporting electrolyte. Free R6G-FC displayed a reversible one-electron oxidation process at 0.508 V $(\Delta E_{1/2} = 62 \text{ mV})$ in the cyclic voltammogram, corresponding to the ferrocene/ferrocenium redox couple (Figure 4a).²⁶ Upon stepwise addition of Cu^{2+} (0-2.8 equiv), the original peak gradually evolved to 0.495 V ($\Delta E_{1/2}$ = 140 mV), associated with the formation of the Cu²⁺/R6G-FC complexed species. Meanwhile, the value of the current intensity of the oxidation peak had a good linear relationship with the concentration of Cu^{2+} added (inset in Figure 4a), which could be used to



Figure 3. (a) UV–vis absorption spectra of **R6G-FC** (10 μ M) in EtOH/H₂O (1:1, v/v, pH = 7.0) in the presence of different metal ions (1 mM for alkali and alkali-earth metal ions and 20 μ M for transition metal ions): Na⁺, K⁺, Mg²⁺, Ca²⁺, Al³⁺, Cr³⁺, Ag⁺, Ba²⁺, Hg²⁺, Mn²⁺, Pb²⁺, Cd²⁺, Ni²⁺, Zn²⁺, Co²⁺, Fe²⁺, Cu²⁺, and blank. Inset: Color changes of **R6G-FC** (10 μ M) before (left) and after (right) addition of Cu²⁺ (2 equiv). (b) Fluorescence responses of 10 μ M **R6G-FC** to various 20 μ M transition metal ions (1 mM for alkali and alkali-earth metal ions). Spectra were acquired in EtOH/H₂O (1:1, v/v, pH = 7.0) with a 500 nm excitation wavelength. Bars represent the final fluorescent intensity at 554 nm. The black bars represent the fluorescence emission of a 10 μ M **R6G-FC** solution with different competing metal ions. The red bars represent the change of the emission intensity that occurred on subsequent addition of 20 μ M Cu²⁺ to the above solutions.



Figure 4. (a) CV of free **R6G-FC** (black) (50 μ M) in CH₃CN/H₂O (7:3, v/v, pH = 7.0) and after adding different amounts of Cu²⁺ (0–140 μ M). Initial electric potential was 0.2 V. Inset: Plot of the currents of the oxidation peak in the CV (scan rate is 100 mV/s) of free **R6G-FC** (50 μ M) upon the addition of Cu²⁺ (0–140 μ M). (b) DPV (200 ms pulse width) of free **R6G-FC** (black) (50 μ M) upon the addition of Cu²⁺ (0–140 μ M) in CH₃CN/H₂O (7:3, v/v, pH = 7.0), showing the changes of the peak potentials. (c) The family of CV curves of the **R6G-FC**-Cu²⁺ complexation species showing the changes of the current with the scan rate. Inset: Plots of anodic and cathodic peak currents versus the square root of the scan rates, $\nu^{1/2}$. (*n*-Bu)₄NPF₆ (50 mM) was used as supporting electrolyte at 25 °C.

quantitatively detect Cu^{2+} at the micromole level with a detection limit of approximately 3 μ M (Figure S14, Supporting Information), whereas almost no deviation of the CV curves of **R6G-FC** was observed in the presence of other metal cations, such as Na⁺, K⁺, Mg²⁺, Ca²⁺, Al³⁺, Cr³⁺, Ag⁺, Ba²⁺, Pb²⁺, Cd²⁺, Ni²⁺, Zn²⁺, Co²⁺, and Fe²⁺ as their nitrate salts. The DPV curve of **R6G-FC** further illustrated the similarity of the results to those obtained from CV with a slight anodic shift and the decrease of the current intensity upon increasing the concentration of Cu²⁺ in aqueous solution, which might be caused by the slow diffusion of **R6G-FC** to the electrode after

the interaction with Cu^{2+} (Figure 4b).²⁷ The reversibility of the Fc/Fc⁺ redox couple of the chemosensor **R6G-FC** in the presence of 2.8 equiv of Cu^{2+} was confirmed by the linear relation between the peak currents and the square root of the scan rates (inset in Figure 4c), the small difference between anodic and cathodic peak currents, and the small peak-to-peak potential separations.^{19c,d,20a}

Mechanism Studies of R6G-FC/Cu²⁺ Complexation. The reversibility of the sensing progress between **R6G-FC** and Cu^{2+} was examined with the EDTA-adding experiment (Figure S15, Supporting Information).^{21,28} The spectral data revealed Scheme 3. Proposed Coordination Mechanism of R6G-FC with Cu²⁺



that adding 1 equiv of EDTA to the R6G-FC/Cu²⁺ system could restore the "on state" back to the "off state" in both absorption and fluorescence signals. Moreover, adding extra Cu²⁺ to the system could once again turn on the signals, which proved the sensing progress was reversible. Considering the spectral changes investigated above and the 1:1 binding stoichiometry confirmed by the Job's plot, the sensing mechanism between R6G-FC and Cu2+ could be speculated as in Scheme 3. Cu²⁺ first entered into the cave in the structure of R6G-FC and then coordinated with carbonyl O, imino N, and phenol O atoms, which would trigger the spirolactam of R6G-FC to open accompanied with a color change from colorless to pink and the fluorescence of Rhodamine 6G turning on as well. After the complexation, the electronic density within the ferrocene moiety was influenced by the Cu²⁺-binding-induced electron portion of the Rhodamine 6G moiety, which would consequently cause a significant shift of the redox potential of the ferrocenyl group. In addition, the proposed sensing mechanism between R6G-FC and Cu²⁺ was then confirmed with the TOF-MS results with the appearance of a new ion peak of m/z 802.9, which indicated the formation of [R6G-FC+Cu(II)-H]⁺ (calcd 803.2) (Figure S16, Supporting Information).

R6G-FC Performance in Pure Water and Dip-Stick Studies. In order to demonstrate practical applications of this new chemosensor, **R6G-FC** was used to judge the existence of Cu^{2+} in a pure water system (Figure 5a). The color of the **R6G-**FC aqueous solution (10 μ M) changed from colorless to light pink even when only 0.5 ppm of Cu^{2+} was present, which identified that **R6G-FC** could serve as a diagnostic kit for trace



Figure 5. (a) Color changes of **R6G-FC** (10 μ M) in aqueous solutions in the presence of Cu²⁺ with concentrations of 0, 0.5, and 5 ppm. (b) Thin-layer chromatography of **R6G-FC** before and after immersing in 1, 10, and 100 ppm of Cu²⁺ in distilled water, respectively.

amounts of Cu²⁺ in water. In addition, an easy "dip-stick" experiment with TLC plates bearing **R6G-FC** was performed to sense Cu²⁺ in pure water as well. Figure 5b presents the distinct color changes of TLC plates before and after being immersed into Cu(II)-containing water with different concentrations. Through this real-time monitoring method, it was possible to *in situ* detect Cu²⁺ at ppm levels in water without any help from analytical instruments. According to the U.S. Environmental Protection Agency (EPA), the limit of Cu²⁺ in drinking water is 1.3 ppm (~20 μ M), which could be adequately detected with the chemosensor **R6G-FC** by the naked-eye inspection in both solution and TLC plates.²⁹

Fluorescence Imaging Studies. The pH dependence experiment revealed that the fluorescence intensity of both **R6G-FC** and **R6G-FC**/ Cu^{2+} could remain stable in a wide pH range from 5.0 to 10.5 (Figure S17, Supporting Information), inferring that **R6G-FC** could be applied to monitor Cu²⁺ under physiological conditions. Therefore, HeLa cells were chosen as the living model to investigate the capability of R6G-FC to fluorescence bioimaging Cu²⁺. Initially, HeLa cells were cultured with only R6G-FC (10 µM) at 37 °C for 30 min, and almost no fluorescence was captured intracellularly by confocal fluorescence microscopy (Figure 6a). However, after adding 100 μ M Cu²⁺ to the HeLa cells and then incubating for one more hour, remarkable red fluorescence was detected, which was consistent with the fluorescence turn-on mechanism of **R6G-FC** in the presence of Cu^{2+} in the solution (Figure 6d). Moreover, Hoechst 33342, a well-known cell nucleus staining reagent, was additionally used to show that R6G-FC had low cytotoxicity to the cells (Figure 6b,e). The overlay image also shows that the areas of red fluorescence and blue fluorescence complement each other and revealed that Cu²⁺ predominantly exists in the cytoplasmic part of the HeLa cells (Figure 6f). The results established that R6G-FC had good cell-membrane permeability and could be used in visualizing Cu²⁺ in living cells and potentially in vivo.

MTT Assay Studies. The MTT assay was conducted to further inspect the cytotoxicity of **R6G-FC** to the HeLa cell line when applied in bioapplications (Figure S18, Supporting Information). The HeLa cells were treated with 1, 5, 25, 50, and 100 μ M **R6G-FC** and cultured for 24 and 48 h, respectively. Over the entire concentration range of **R6G-FC** employed in the experiment, after a 24 h incubation time, HeLa cell viability of the control remained above 80%. As for the low concentrations of **R6G-FC** (1–25 μ M), a decrease of less than 15% in cell viability could still be observed, contrasting with the control group, even after 48 h of incubation time. Therefore, the concentration of **R6G-FC** (10 μ M) used to acquire the confocal fluorescence images in Figure 6 was in the low concentration range and confirmed the low cytoxicity to HeLa



Figure 6. Confocal fluorescence images of **R6G-FC** in living HeLa cells. (a) Cells incubated with **R6G-FC** (10 μ M) in ethanol/PBS (1:49, v/v) buffer for 30 min at 37 °C from the red channel. (b) Cell nucleus labeled with Hoechst 33342 (10 μ M) for 30 min from the blue channel. (c) Overlay image of (a) and (b). (d) Cells pretreated with **R6G-FC** (10 μ M) for 30 min, then incubated with Cu²⁺ (100 μ M) for 1 h from the red channel. (e) Cells pretreated with **R6G-FC** (10 μ M) and Hoechst 33342 (10 μ M) for 30 min, then incubated with Cu²⁺ (100 μ M) for 1 h from the blue channel. (f) Overlay image of (d) and (e).

cells, indicating that it would be suitable for this concentration of **R6G-FC** to be applied in cellular imaging studies.

CONCLUSION

In summary, a novel multisignaling chemosensor, R6G-FC, was designed and synthesized through the classic Sonogashira coupling reaction to introduce a ferrocenvl moiety into a modified Rhodamine 6G fluorophore. The addition of Cu²⁺ to an R6G-FC aqueous solution would result in a new UV-vis absorption peak and fluorescence emission peak in the optical spectrum, which was due to the binding-induced spirolactam ring opening of R6G-FC. Due to the high affinity of Cu^{2+} with N and O atoms, R6G-FC had good selectivity and high sensitivity to Cu²⁺ without interference from other metal ions. The detection limit obtained from fluorescence titration was down to 6.96 \times 10⁻⁷ M, and the association constant was calculated from absorbance titration as $1.52 \times 10^6 \text{ M}^{-1}$. In addition, the molecular configuration change of R6G-FC after interaction with Cu2+ influenced the electronic density within the ferrocene group to express perturbation of the electrochemical parameters, and the value of the current intensity of the oxidation peak had a good linear relationship with the added concentration of Cu^{2+} , which could be another quantitative basis for detecting Cu²⁺ with a detection limit of approximately 3 μ M. The response of R6G-FC to Cu²⁺ could be accomplished within a short time, with a good reversibility, in a wide pH range. The 1:1 binding mechanism between R6G-FC and Cu²⁺ was confirmed by the Job's plot experiment and TOF-MS result. The "dip-stick" experiment showed an evident color gradation, which illustrated that the chemosensor R6G-FC could act as a naked-eye detection approach to sense Cu²⁺. Moreover, due to the good solubility and biocompatibility, **R6G-FC** could be used for the fluorescence imaging of Cu^{2+} in living HeLa cells with low cytotoxicity.

ASSOCIATED CONTENT

Supporting Information

Reported structures of multisignal chemosensors for sensing Cu²⁺. Comparison of rhodamine-based systems with or without a neighboring ferrocene moiety around the phenol group. ¹H NMR, ¹³C NMR, and mass spectra as characterization data of intermediates, final product **R6G-FC**, and **R6G-FC**/Cu²⁺ complex. Solvent, reversibility, time, and pH dependence curve. Response of UV–vis absorption, fluorescence emission, and oxidation peak currents to the increasing amounts of Cu²⁺. Calculation of association constant and detection limit. Cytotoxicity assay by the MTT test. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.organomet.5b00285.

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Notes

The authors declare no competing financial interest.

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