



## Combined spectral experiment and theoretical calculation to study the chemosensors of copper and their applications in anion bioimaging

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### ABSTRACT

Three salicylaldehyde derivatives, namely 5-nitrosalicylaldehyde fluorescein hydrazone (**1**), 5-diethylaminosalicylaldehyde fluorescein hydrazone (**2**), and di(5-diethylamino salicylaldehyde) hydrazide (**3**), were synthesized and characterized. Their ability to recognize copper ions was investigated by UV-visible and fluorescence spectroscopies, with **2** shown to be an optimal probe for effective detection of Cu<sup>2+</sup>. More importantly, to explore the optical properties of these probes, and their mechanism for recognition of Cu<sup>2+</sup> ions, B3LYP/6-311+G(d,p) density functional calculations were used to investigate the molecular orbitals and electronic excitations of each probe. In addition, because the **2**-Cu<sup>2+</sup> complex exhibits anion-induced fluorescence enhancement, we illustrate its potential application to bioimaging.

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### 1. Introduction

Copper is among the more important of the transition metals in the human body and it is not only important for various physiological aspects, such as bone formation, cellular respiration, and connective tissue development, but also serves as an important catalytic cofactor for several metalloenzymes [1–4]. A deficiency of copper can lead to anemia or pancytopenia, whereas excess copper can lead to the mismetallation of other metal binding sites. Free copper acts as a catalyst for Fenton-type reactions, which produce reactive oxygen species, and misregulation of copper uptake is known to cause Menkes and Wilson's disease [5–7]. On the other hand, copper is the third most abundant essential trace element in the human body (after Fe<sup>2+</sup> and Zn<sup>2+</sup>) and is commonly found as Cu<sup>2+</sup> in natural water [8,9]. The recommended daily allowance of copper, as suggested by National Research Council ranges from 1.5 to 3.0 mg for adults, 1.5 to 2.5 mg for children, and 0.4 to 0.6 mg for infants [10]. According to the guidelines for drinking-water

quality of the World Health Organization, copper is identified as a “chemical of health significance in drinking-water” [11]. Copper is also a widely used industrial metal. Its cation is toxic at high concentrations [12], but in trace amounts it is also involved in brain diseases such as Alzheimer's and Parkinson's, and in prion disease [13,14]. Consequently, effective detection of Cu<sup>2+</sup> in water or physiological samples is of toxicological and environmental concern [15–18].

Copper ions can be detected using several instrumental techniques [19]. However, these methods are time-consuming and require expensive instrumentation. Chemosensors are powerful molecular tools that can be used to detect many different target molecules, including biological markers and environmental pollutants [20–25]. Many of these systems are based on well established and unique molecular frameworks, such as coumarins [26–28], quinolines [29], rhodamines [30–36], BOD-IPY dyes [37–39], calixarenes [40–43], and their detection process and mechanisms are also multifarious [26–46]. In our previous work, we studied and reported on two copper sensors [47,48]. In the present work, we report on the synthesis and characterization of three compounds that contain similar structures to each other: 5-nitrosalicylaldehyde fluorescein hydrazone (**1**), 5-diethylaminosalicylaldehyde fluorescein hydrazone (**2**), and di(5-diethylamino salicylaldehyde) hydrazide (**3**) (Fig. 1). We also investigated the recognition ability of these compounds to

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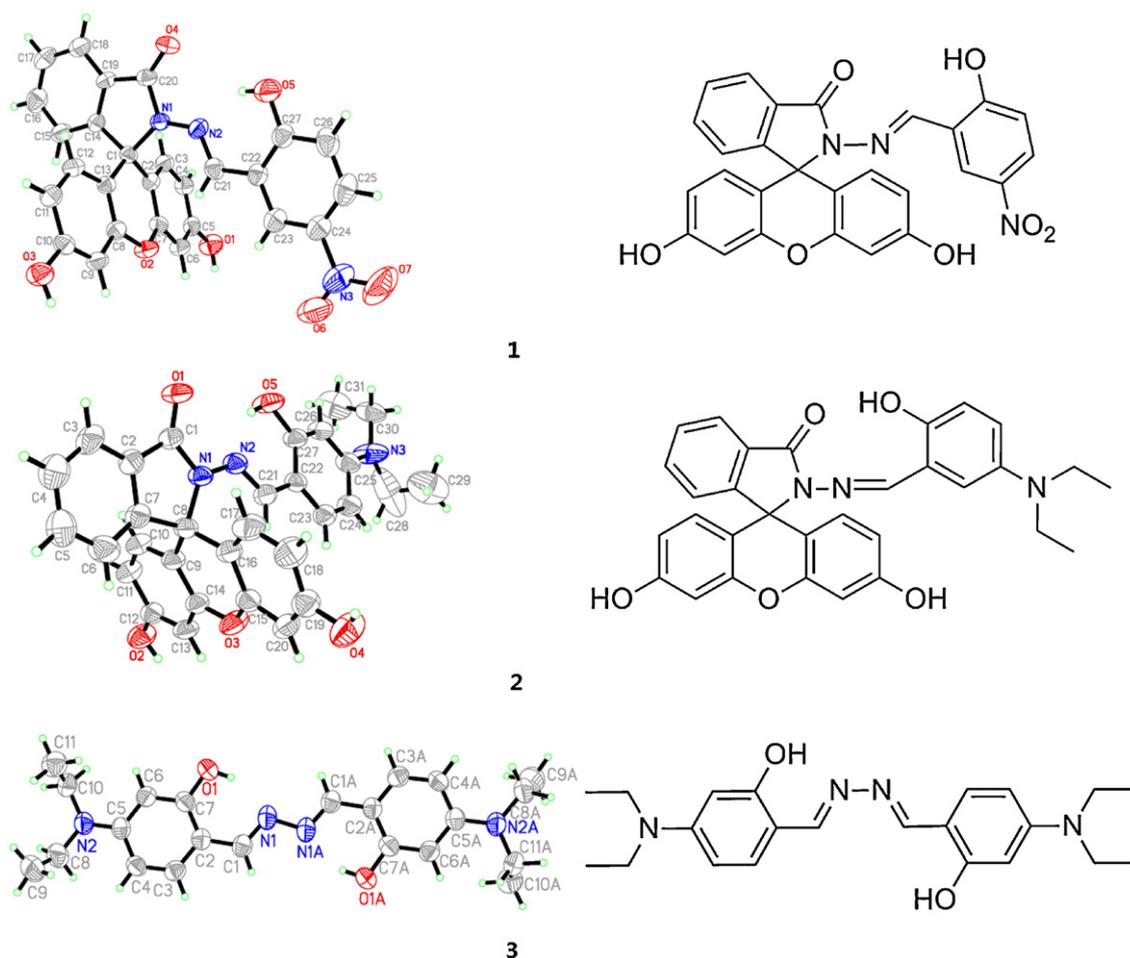


Fig. 1. The structure (top) and thermal ellipsoids of probes are drawn at the 50% probability level.

recognize copper using UV–visible (UV–vis) and fluorescence spectroscopies. Moreover, combinations of the electrospray ionization mass spectrometry (ESI–MS) and theoretical calculation were used to clarify the recognition process involved with these species. On the basis of our studies, it is demonstrated that, as probes for copper, there are significant differences in ability between these three compounds, despite their similar structure. For example, **1** as a UV–vis probe shows markedly different results from the other probes tested, both in terms of UV–vis and fluorescence probes. Both **2** and **3** act as fluorescence on–off probes; however, one distinguishing feature of **3** is that when it is in the *anti* configuration, upon meeting with  $\text{Cu}^{2+}$  it first transforms into the *syn* configuration and then coordinates with  $\text{Cu}^{2+}$ , consequently resulting in fluorescence quenching. In addition, bioimaging was carried out with the **2**– $\text{Cu}^{2+}$  complex because it exhibits anion-induced fluorescence enhancement with a selection of anions.

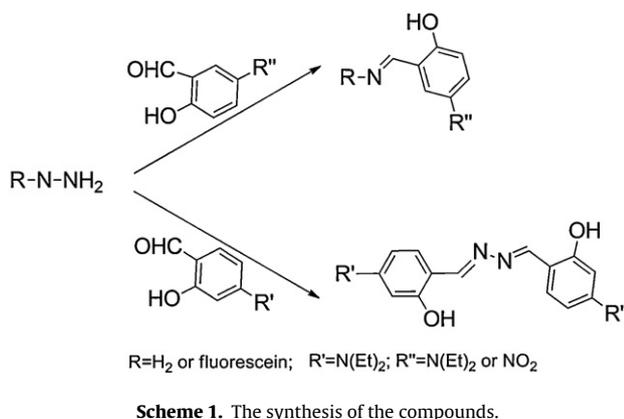
## 2. Materials and methods

### 2.1. Materials

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma–Aldrich (St. Louis, MO). Sodium hydroxide solution (0.1 mol/L) was added to aqueous HEPES (10 mmol/L) to adjust the pH to 7.0. Cationic salts were purchased from Shanghai Experiment Reagent Co., Ltd., China. All other chemicals used were of analytical grade.

### 2.2. Instruments

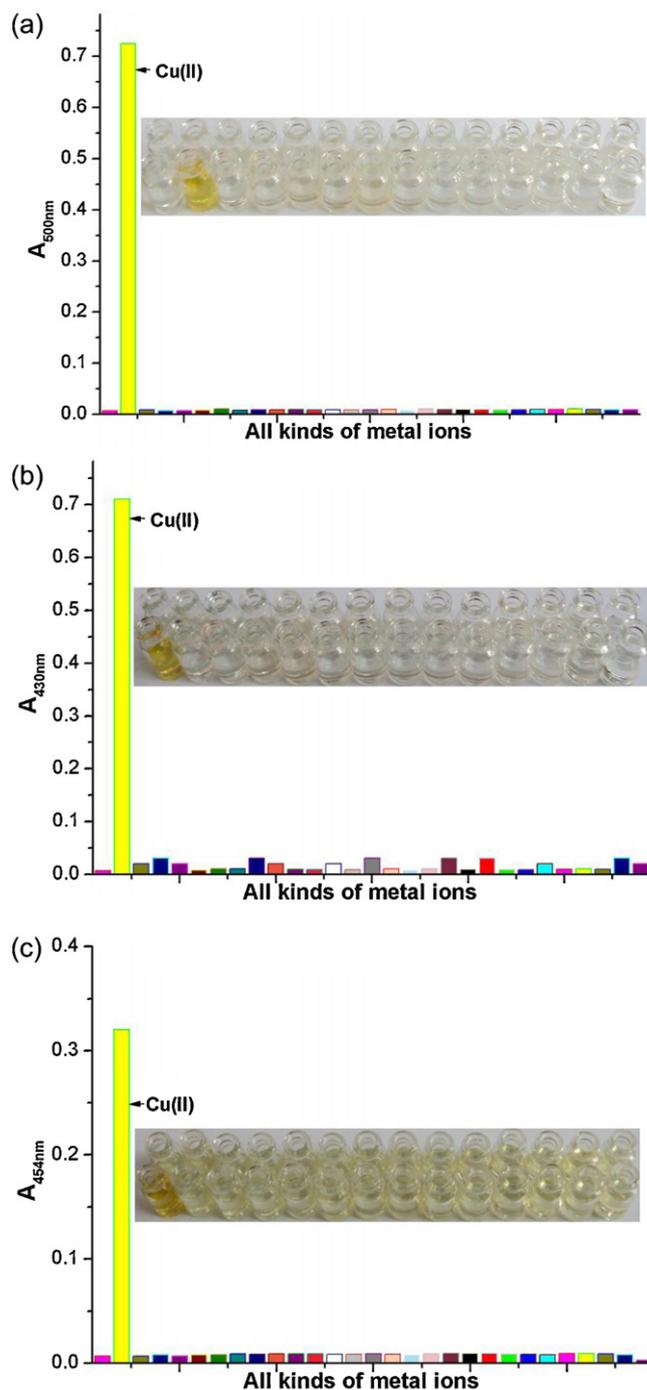
A pH meter (Mettler Toledo, Switzerland) was used to determine the pH. Ultraviolet–visible (UV–vis) spectra were recorded on a Cary 50 Bio UV–Vis spectrophotometer. Fluorescence spectra were measured on Cary Eclipse fluorescence spectrophotometer. A PO-120 quartz cuvette (10 mm) was purchased from Shanghai Huamei Experiment Instrument Plants, China.  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR spectra were recorded on a Bruker AVANCE-300 MHz and 75 MHz NMR spectrometer, respectively (Bruker, Billerica, MA). Electron bombard ionization mass spectrometry (EI–MS) was measured with GCT–MS (Waters) instrument. ESI was measured with an LTQ–MS (Thermo) instrument. The ability of **2**– $\text{Cu}^{2+}$  reacting to anion in the living cells was also evaluated by laser confocal fluorescence imaging using an Leica TCS SP5 laser scanning microscope. The yellow single crystals of **1**, **2**, and **3** were mounted on a glass fiber for data collection. Cell constants and an orientation matrix for data collection were obtained by least-squares refinement of diffraction data from reflections within  $1.00\text{--}25.05^\circ$ ,  $1.00\text{--}25.05^\circ$ , and  $0.99\text{--}27.46^\circ$ , using a Bruker SMART APEX CCD automatic diffractometer. Data were collected at 296 K using Mo  $\text{K}\alpha$  radiation ( $\lambda = 0.710713 \text{ \AA}$ ) and the  $\omega$ -scan technique, and corrected for the Lorentz and polarization effects (SADABS) [49]. The structures were solved by direct methods (SHELX97) [50], and subsequent difference Fourier maps were inspected and then refined in F2 using a full-matrix least-squares procedure and anisotropic displacement parameters.



### 2.3. Preparation of 1, 2, and 3

The synthesis of compounds **1**, **2**, and **3** are summarized in Scheme 1. **1** and **2** were synthesized by a one-step reaction between fluorescein hydrazine and either of 5-nitrosalicylaldehyde (for **1**), 5-diethylaminosalicylaldehyde (for **2**) in methanol containing acetic acid. 0.167 g (1 mmol) 5-nitrosalicylaldehyde (for **1**) or 0.193 g (1 mmol) 5-diethylaminosalicylaldehyde (for **2**) were added to 0.35 g (1 mmol) of fluorescein hydrazine dissolved in 20 ml of methanol and the reaction solution refluxed in an oil bath for 2 h. A white solid appeared which was then filtered from each solution. Each crude product was recrystallized in CH<sub>3</sub>OH and petroleum ether (v/v, 1/1) to give 5-nitrosalicylaldehyde fluorescein hydrazone (**1**) or 5-diethylaminosalicylaldehyde fluorescein hydrazone (**2**) as a yellow powder in 60%, and 65% yields, respectively (Fig. S1). An H<sub>2</sub>O/CH<sub>3</sub>CH<sub>2</sub>OH solution containing the product was allowed to evaporate slowly at room temperature for several days, and the yellow crystals that subsequently formed were suitable for X-ray crystallography formed. **1**: mp 385 °C; FT-IR (KBr, cm<sup>-1</sup>): 3388 (–OH), 3092 (Ar–H), 1696 (C=O), 1630 (C=N), 1262 (C–O); <sup>1</sup>H NMR (300 MHz, 25 °C, DMSO-*d*<sub>6</sub>): δ 11.61 (s, 1H), 9.92 (bs, 2H), 9.05 (s, N=C–H, 1H), 8.30 (d, 1H), 8.06 (d, 1H, *J* = 9.1 Hz), 7.94 (m, 1H, *J* = 6.7 Hz), 7.64 (t, 2H, *J* = 7.2 Hz), 7.13 (d, 1H, *J* = 6.8 Hz), 6.95 (d, 1H, *J* = 9.0 Hz), 6.66 (bs, 2H), 6.49 (d, 4H, *J* = 9.9 Hz); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ 171.48, 169.85, 166.40, 159.80, 158.33, 151.62, 147.63, 142.04, 136.89, 135.57, 134.45, 131.48, 131.20, 130.20, 128.21, 126.55, 124.64, 120.20, 117.09, 110.26, 72.88; EI-MS *m/z* 495 [1]<sup>+</sup>; Elemental analysis (calcd. %) for C<sub>27</sub>H<sub>17</sub>N<sub>3</sub>O<sub>7</sub>: C, 65.45; H, 3.46; N, 8.48; Found: C, 65.48; H, 3.44, N, 8.54; Crystal data for C<sub>27</sub>H<sub>17</sub>N<sub>3</sub>O<sub>7</sub>: crystal size: 0.30 × 0.20 × 0.20, monoclinic, space group P2<sub>1</sub>/n (No. 11). *a* = 11.393(2) Å, *b* = 15.307(3) Å, *c* = 14.354(3) Å, β = 112.338°, *V* = 2315.4(8) Å<sup>3</sup>, *Z* = 4, *T* = 296 K, θ<sub>max</sub> = 25.05°, 12,799 reflections measured, 4097 unique (*R*<sub>int</sub> = 0.0707). Final residual for 338 parameters and 4097 reflections with *I* > 2σ(*I*): *R*<sub>1</sub> = 0.0500, *wR*<sub>2</sub> = 0.0677 and GOF = 1.265 (Fig. S2).

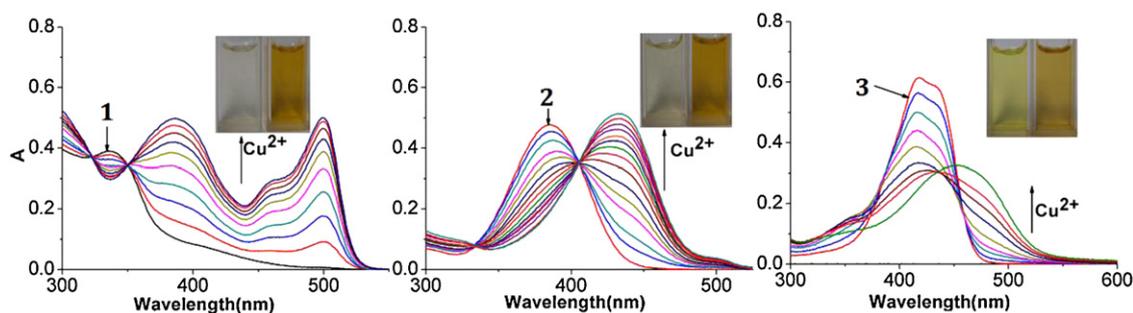
**2**: mp 325 °C; FT-IR (KBr, cm<sup>-1</sup>): 3317 (–OH), 3072 (Ar–H), 2971 (C–H, CH<sub>3</sub>), 2930 (C–H, CH<sub>2</sub>), 1696 (C=O), 1632 (C=N), 1262 (C–O); <sup>1</sup>H NMR (300 MHz, 25 °C, DMSO-*d*<sub>6</sub>): δ 10.38 (s, 1H), 9.87 (bs, 2H), 8.95 (s, N=C–H, 1H), 7.83 (d, 1H), 7.56 (d, 2H), 7.08 (m, 1H), 6.97 (t, 1H, *J* = 8.3 Hz), 6.58 (d, 2H), 6.43 (d, 4H, *J* = 8.2 Hz), 6.13 (bs, 1H), 5.91 (d, 1H), 2.04 (m, 4H), 0.99 (m, 6H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ 159.30, 158.20, 154.43, 151.84, 150.12, 149.66, 133.22, 131.69, 129.14, 128.73, 127.81, 123.38, 122.54, 112.06, 109.23, 105.87, 103.44, 102.03, 96.93, 64.87, 43.44, 12.06; EI-MS *m/z* 521 [2]<sup>+</sup>; Elemental analysis (calcd. %) for C<sub>31</sub>H<sub>27</sub>N<sub>3</sub>O<sub>5</sub>: C, 71.39; H, 5.22; N, 8.06; Found: C, 71.38; H, 5.25, N, 8.04; Crystal data for C<sub>35</sub>H<sub>31</sub>N<sub>5</sub>O<sub>5</sub>: crystal size: 0.30 × 0.20 × 0.20, monoclinic, space group P2<sub>1</sub>/c (No. 14). *a* = 18.555(7) Å, *b* = 10.094(4) Å, *c* = 17.258(7) Å, β = 97.832(7)°, *V* = 3202(2) Å<sup>3</sup>, *Z* = 4, *T* = 296 K, θ<sub>max</sub> = 25.05°, 33,239 reflections



**Fig. 2.** Optical density two-dimensional graph of the three probes (a), (b), and (c) at 500 nm (**1**), 430 nm (**2**), 454 nm (**3**), respectively upon the addition of several metal ions (including Cu<sup>2+</sup>, Cu<sup>+</sup>, Ca<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>, Bi<sup>3+</sup>, Co<sup>2+</sup>, VO<sup>2+</sup>, Mn<sup>2+</sup>, Ru<sup>3+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup>, Ag<sup>+</sup>, La<sup>3+</sup>, Ce<sup>4+</sup>, Yb<sup>3+</sup>, Cr<sup>2+</sup>, Er<sup>3+</sup>, Mg<sup>2+</sup>, Sn<sup>2+</sup>, Al<sup>3+</sup>, Nd<sup>3+</sup>, Zr<sup>4+</sup>, K<sup>+</sup>, Sm<sup>3+</sup>, Fe<sup>3+</sup>, Eu<sup>3+</sup>). Inset: a color change photograph for Cu<sup>2+</sup> and the other metal ions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

measured, 5662 unique (*R*<sub>int</sub> = 0.0904). Final residual for 365 parameters and 5662 reflections with *I* > 2σ(*I*): *R*<sub>1</sub> = 0.0899, *wR*<sub>2</sub> = 0.2902 and GOF = 1.029 (Fig. S3).

**3** was synthesized by a one-step reaction of 5-diethylaminosalicylaldehyde with excess hydrazine hydrate in ethanol, in accordance with the procedure reported in the literature [51]. An excessive hydrazine hydrate (85%, 1.2 mL) was added



**Fig. 3.** Absorption spectral changes of three probes in 10 mM HEPES at pH 7.0 as an aqueous buffer upon the addition of  $\text{Cu}^{2+}$ ;  $\text{Cu}^{2+}$  was added gradually with  $[\text{Cu}^{2+}] = 0\text{--}25$ ,  $0\text{--}16$ , and  $0\text{--}9$   $\mu\text{M}$ , respectively; each spectrum was recorded 30 s after  $\text{Cu}^{2+}$  addition. Inset: a color change photograph for  $\text{Cu}^{2+}$  and three probes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

to a 0.193 g (1 mmol) of di(5-diethylamino salicylaldehyde) that had been dissolved in 20 ml of ethanol. The reaction solution was then refluxed in an oil bath for 8 h and a brown oily product was obtained by evacuating the ethanol under reduced pressure. The solid product was precipitated by adding water and recrystallized from ethanol–water to give di(5-diethylamino salicylaldehyde) hydrazide (**3**) as yellow powder in 80% yield (0.15 g) (Fig. S1). An  $\text{H}_2\text{O}/\text{CH}_3\text{CH}_2\text{OH}$  solution of **3** was allowed to evaporate slowly at room temperature for several days and yellow crystals that were formed were suitable for X-ray crystallography. mp 219–221 °C; FT-IR (KBr,  $\text{cm}^{-1}$ ): 3404 (–OH), 3080 (Ar–H), 2971 (C–H,  $\text{CH}_3$ ), 2930 (C–H,  $\text{CH}_2$ ), 1585 (C=C), 1630 (C=N);  $^1\text{H}$  NMR (300 MHz,  $25^\circ\text{C}$ ,  $\text{DMSO}-d_6$ ):  $\delta$  11.48 (bs, 2H), 8.61 (bs, N=C–H, 2H), 7.28 (d, Ar–H, 2H,  $J=8.7$  Hz), 6.30 (t, Ar–H, 2H,  $J=9.7$  Hz), 6.11 (s, t, 2H), 2.08 (m,  $\text{CH}_2\text{--H}$ , 8H,  $J=6.9$  Hz), 1.10 (t,  $\text{CH}_3\text{--H}$ , 12H,  $J=6.5$  Hz);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  160.34, 150.58, 132.72, 106.06, 103.71, 96.70, 43.55, 38.34, 30.39, 12.23; EI-MS  $m/z$  382 [**3**] $^+$ ; Elemental analysis (calcd. %) for  $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_2$ : C, 69.08; H, 7.91; N, 14.65; Found: C, 69.11; H, 7.94; N, 14.64; Crystal data for  $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_2$ : crystal size:  $0.21 \times 0.18 \times 0.16$ , monoclinic, space group  $\text{P}2(1)/n$  (No. 11).  $a = 11.722(2)$  Å,  $b = 6.5173(13)$  Å,  $c = 13.320(3)$  Å,  $\beta = 96.42^\circ$ ,  $V = 1011.2(3)$  Å $^3$ ,  $Z = 2$ ,  $T = 173$  K,  $\theta_{\text{max}} = 27.46^\circ$ , 4981 reflections measured, 2314 unique ( $R_{\text{int}} = 0.0390$ ). Final residual for 129 parameters and 2314 reflections with  $I > 2\sigma(I)$ :  $R_1 = 0.0589$ ,  $wR_2 = 0.1427$  and  $\text{GOF} = 1.047$  (Fig. S4).

#### 2.4. UV–vis and fluorescence spectroscopies

A  $\text{Cu}^{2+}$  solution was prepared by dissolving copper chloride in deionized water. Probes stock solutions were prepared in ethanol. UV–vis and fluorescence spectra were obtained in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, HEPES aqueous buffer (10 mmol/L, pH 7.0) solutions. Aqueous anion solutions were

also prepared using deionized water. Fluorescence measurements were carried out with a slit width of 5 nm.

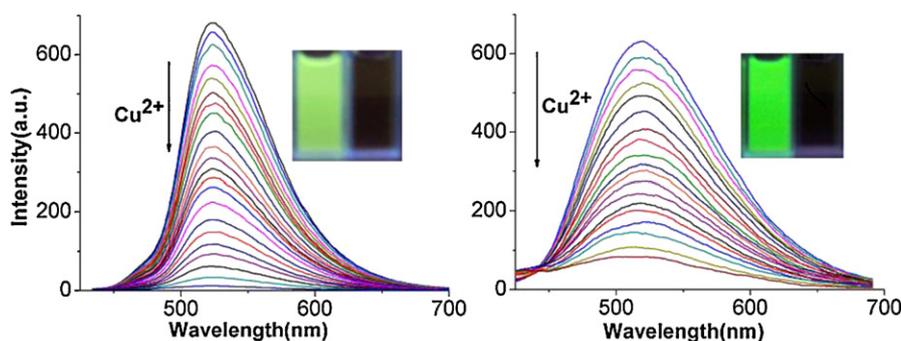
#### 2.5. Computational methods

All calculations reported in this work were performed using Gaussian 09 suite of programs. Ground-state geometries and electron structures of the complexes were optimized by means of the density functional theory (DFT) using the B3LYP/6-311++G (d,p) basis set. Time-dependent DFT (TD-DFT) calculations were used to determine optical properties of the complexes based on their optimized ground-state geometries. Geometrical optimization for the  $\text{Cu}^{2+}$ -addition products were performed using the UB3LYP functional combined with the 6-311+G(d,p) basic set for C, H, N and F atoms and the LANL2DZ basis set for the Cu atoms.

### 3. Results and discussion

#### 3.1. Selectivity over metal ions

The effect of a wide range of environmentally and physiologically active metal ions was investigated for each of compounds **1**, **2**, and **3** using the UV–vis spectra of solutions containing these compounds and the metal ion (100 equiv.) in HEPES aqueous buffer (10 mmol/L, pH 7.0). The results showed that whereas metal ions such as  $\text{Cu}^+$  (sodium ascorbate as stabilizer),  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Bi}^{3+}$ ,  $\text{Co}^{2+}$ ,  $\text{VO}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ru}^{3+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{La}^{3+}$ ,  $\text{Ce}^{4+}$ ,  $\text{Yb}^{3+}$ ,  $\text{Cr}^{2+}$ ,  $\text{Er}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sn}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Nd}^{3+}$ ,  $\text{Zr}^{4+}$ ,  $\text{K}^+$ ,  $\text{Sm}^{3+}$ ,  $\text{Fe}^{3+}$  and  $\text{Eu}^{3+}$  do not result in any apparent changes in absorption peaks, there is notable change when  $\text{Cu}^{2+}$  is involved. Fig. 2 shows that when  $\text{Cu}^{2+}$  is added, strong new absorption peaks appear at 500, 430, or 454 nm for probe **1**, **2**, or **3**, respectively. We also note that the color of the solution changes from a light yellow to an orange yellow.



**Fig. 4.** Fluorescence spectral changes for **2** and **3** probes upon the addition of  $\text{Cu}^{2+}$  in 10 mM HEPES at pH 7.0 as an aqueous buffer with  $[\text{Cu}^{2+}] = 0\text{--}0.18$  and  $0\text{--}0.5$   $\mu\text{M}$ , respectively. Inset: color (left) and visual fluorescence (right) change photographs for probes upon the addition of  $\text{Cu}^{2+}$  in a HEPES (pH 7.0) buffer solution under UV illumination (365 nm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

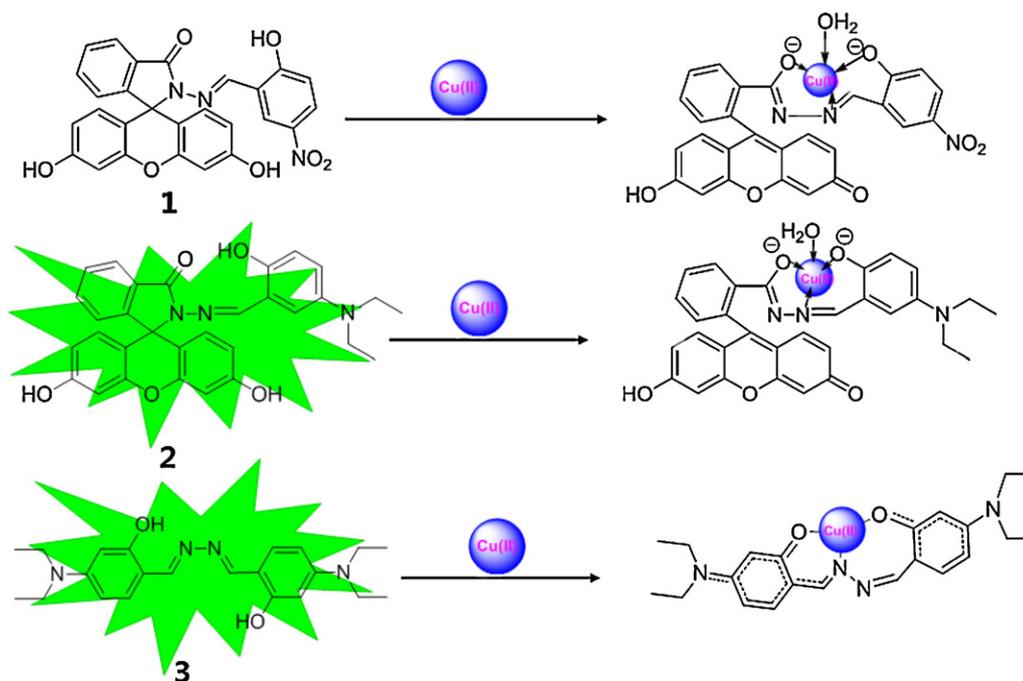


Fig. 5. The proposed structures organized by the  $\text{Cu}^{2+}$  ion when it coordinates with probes to form complexes.

### 3.2. UV–vis spectra of detecting $\text{Cu}^{2+}$

A detailed investigation was carried out into the ability of the three compounds to recognize  $\text{Cu}^{2+}$ . Fig. 3 (left) shows that a regular change in the UV–visible spectrum can be observed when the  $\text{Cu}^{2+}$  solution is added to the HEPES buffer (10 mM, pH 7.0) containing **1** (25  $\mu\text{mol/L}$ ). With an increase in the  $\text{Cu}^{2+}$  concentration, new absorption peaks appear at 386 and 500 nm, and  $A_{386,500\text{nm}}$  gradually increases with isosbestic point at 322 and 350 nm, indicating that there has been formation of a new complex. From the concentration of  $\text{Cu}^{2+}$ , the stoichiometric relationship between the probe and  $\text{Cu}^{2+}$  was found to be 1:1 based on the change in absorbance at 500 nm. Fig. 3 (middle) shows that there is a change in the UV–visible spectrum when the  $\text{Cu}^{2+}$  solution is added to the HEPES buffer (10 mmol/L, pH 7.0) containing **2** (16  $\mu\text{mol/L}$ ). With an increase in the  $\text{Cu}^{2+}$  concentration, a new absorption peak appears at 430 nm and  $A_{430\text{nm}}$  gradually increases with an isosbestic point formed at 404 nm. The stoichiometric relationship between the probe and  $\text{Cu}^{2+}$  was found to be 1:1 based on the change in absorbance at 430 nm. Fig. 3 (right) shows the UV–vis spectra obtained when the solution of  $\text{Cu}^{2+}$  was titrated into the buffer of **3** (9  $\mu\text{mol/L}$ ). Upon the addition of  $\text{Cu}^{2+}$ , the maximum absorption peak gradually shifted from light yellow ( $\lambda_{\text{max}} = 418\text{ nm}$ ) to orange yellow ( $\lambda_{\text{max}} = 454\text{ nm}$ ). The stoichiometric relationship between the probe and  $\text{Cu}^{2+}$  was found to be 1:1. We also studied the ability of each compound to detect  $\text{Cu}^{2+}$  in the presence of several metal ions. Fig. S5 shows that the other ions did not interfere with the determination of  $\text{Cu}^{2+}$ .

### 3.3. pH dependence

The pH range for the determination of  $\text{Cu}^{2+}$  was also studied. For probe **1**, no absorption peak at 500 nm was induced by  $\text{Cu}^{2+}$  when the solution pH was between 2 and 6. However, probe **1** shows a good response to  $\text{Cu}^{2+}$  in the pH range pH 7–12. Therefore, physiological acidity (pH 7.0) was selected for further investigation. Free probe **2** shows absorption at 500 nm for pH 12 and 13, and it also has no fluorescent emission when pH value is 2, 12, or 13. Similar to **1**, probe **2** illustrates a good response to  $\text{Cu}^{2+}$  in the pH range

7–10, with the result that pH 7.0 was also selected for further study with this probe. Using free probe **3**, the absorption peak shifts when the pH is 3, 4, 12, or 13 and there is fluorescence quenching at pH 2, 3, 12, or 13. When the solution pH is 3, 5, or 6,  $\text{Cu}^{2+}$  induced a fluorescence intensity for probe **3** such that there was either no quenching or only partial quenching. Therefore, the pH range of 7–10 is effective for this probe and neutral pH was used for further studies (Fig. S6).

### 3.4. Time-dependence in the detection process of $\text{Cu}^{2+}$

Time-dependent variations in the UV–vis of **1**, **2**, and **3** were monitored in the presence of 10 equiv. of  $\text{Cu}^{2+}$ . The kinetic study showed that the reaction was complete within 15, 5, and 3 s for  $\text{Cu}^{2+}$  with **1**, **2**, and **3**, respectively, thus indicating that these probes react very rapidly with  $\text{Cu}^{2+}$  under the experimental conditions considered (Fig. S7).

### 3.5. Fluorescence spectra

The fluorescence spectra of each of the probes with an increased concentration of  $\text{Cu}^{2+}$  in HEPES buffer are displayed in Fig. 4. The addition of  $\text{Cu}^{2+}$  resulted in no variation to the fluorescence spectra of **1**. However, the addition of  $\text{Cu}^{2+}$  did lead to fluorescence quenching for remaining two probes (**2**:  $\lambda_{\text{ex}} = 382\text{ nm}$  and  $\lambda_{\text{em}} = 520\text{ nm}$ ; **3**:  $\lambda_{\text{ex}} = 425\text{ nm}$  and  $\lambda_{\text{em}} = 524\text{ nm}$ ). Fig. S8 shows the fluorescence changes that the probes undergo upon the addition of various metal ions, including  $\text{Cu}^+$  (sodium ascorbate as stabilizer),  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Bi}^{3+}$ ,  $\text{Co}^{2+}$ ,  $\text{VO}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ru}^{3+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{La}^{3+}$ ,  $\text{Ce}^{4+}$ ,  $\text{Yb}^{3+}$ ,  $\text{Cr}^{2+}$ ,  $\text{Er}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sn}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Nd}^{3+}$ ,  $\text{Zr}^{4+}$ ,  $\text{K}^+$ ,  $\text{Sm}^{3+}$ ,  $\text{Fe}^{3+}$  and  $\text{Eu}^{3+}$  in HEPES (10 mmol/L, pH 7.0). Unlike with  $\text{Cu}^{2+}$ , these other metal ions listed induced no change to the fluorescence emission properties under the same conditions besides.

### 3.6. Proposed mechanism

The proposed mechanisms of detection and the structures of the probes, both with and without the addition of  $\text{Cu}^{2+}$ , are shown in Fig. 5. Probe **1**, which exhibits strong intramolecular charge transfer

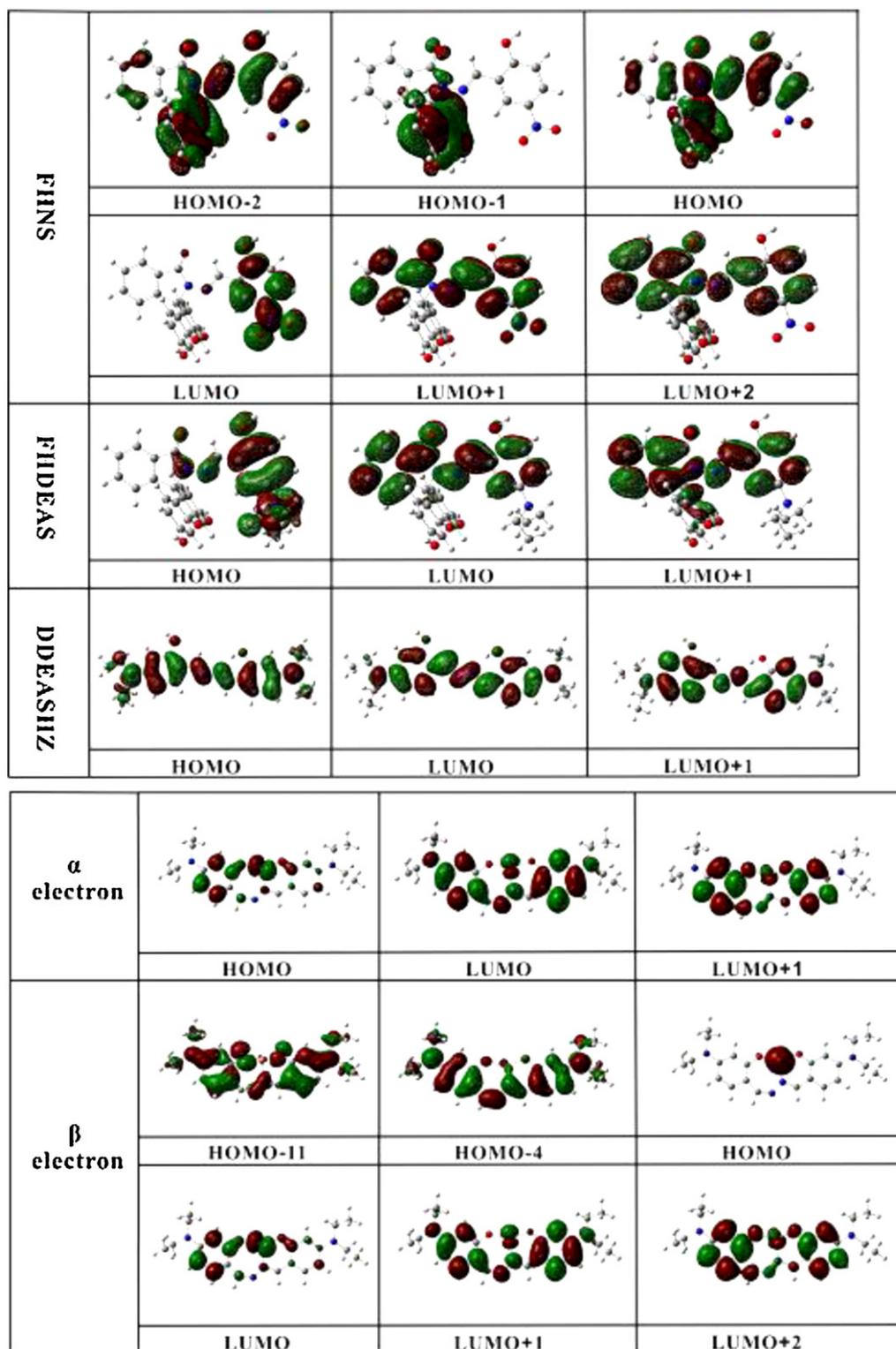


Fig. 6. (a) Molecular surfaces of the three probes; (b) molecular surfaces of DDEASHZ-Cu.

(ICT), is itself nonfluorescent, and the  $1\text{-Cu}^{2+}$  adduct remains non-fluorescent because of the paramagnetism effect from spin-orbit coupling of the  $\text{Cu}^{2+}$  (Fig. S9). Mass spectrometry analysis of a product obtained from the reaction of **1** with  $\text{Cu}^{2+}$  in  $\text{CH}_3\text{OH}$  shows binding between **1** and  $\text{Cu}^{2+}$ , a peak at  $m/z=574.08$ , corresponding to  $[1\text{-Cu}^{2+}\text{-H}_2\text{O}+\text{H}]^+$ , is clearly observed (see Fig. S10). Probe **2** exhibits strong fluorescence in its natural form. However, though the ring is open after coordination with  $\text{Cu}^{2+}$ , the

paramagnetic effect from spin-orbit coupling of the  $\text{Cu}^{2+}$  induces fluorescence quenching (Fig. S11). A peak at  $m/z=623.11$  corresponds to  $[2\text{-Cu(II)-H}_2\text{O}+\text{Na}]^+$  (Fig. S12). The complexes discussed above involving the ligands and copper ion are similar to forms reported previously [52–56]. Free probe **3** is in the *anti* configuration but upon encountering the  $\text{Cu}^{2+}$  it first transforms into the *syn* configuration before coordinating to the  $\text{Cu}^{2+}$ , resulting in fluorescence quenching (Fig. S13). From ESI-MS, a peak at  $m/z=466.14$

**Table 1**  
Calculated TD-DFT excitation properties and experimental  $\lambda$ .

	$\lambda$ (nm) (eV)		Dominant excitations
	Theory	Exp	
FHNS	333	334	H-1 $\rightarrow$ L+1 (0.48822) H-2 $\rightarrow$ L (0.33313) H $\rightarrow$ L+1 (0.31531)
FHNS-Cu	381	386	H-2 $\alpha$ $\rightarrow$ L $\alpha$ (0.52149) H-2 $\beta$ $\rightarrow$ L $\beta$ (0.36026) H-2 $\beta$ $\rightarrow$ L+1 $\beta$ (0.53653)
	503	500	H-2 $\alpha$ $\rightarrow$ L $\alpha$ (0.64491) H-2 $\beta$ $\rightarrow$ L+1 $\beta$ (0.66885)
FHDEAS	392	384	H $\rightarrow$ L (0.70017)
FHDEAS-Cu	424	432	H $\alpha$ $\rightarrow$ L+1 $\alpha$ (0.34363) H-2 $\beta$ $\rightarrow$ L+1 $\beta$ (0.33687) H $\beta$ $\rightarrow$ L+2 $\beta$ (0.42211)
	401	417	H $\rightarrow$ L (0.70317)
DDEASHZ-Cu	444	457	H $\alpha$ $\rightarrow$ L $\alpha$ (0.45366) H-11 $\beta$ $\rightarrow$ L $\beta$ (0.38095) H-4 $\beta$ $\rightarrow$ L $\beta$ (0.41407) H $\beta$ $\rightarrow$ L+1 $\beta$ (0.46609)

H and L denote the HOMO and LUMO, respectively and data in parentheses are coefficient of the wave function for each excitation. And  $\alpha$  and  $\beta$  denote  $\alpha$  and  $\beta$  electrons orbital of the Cu<sup>2+</sup>-products.

clarified the structure that results from coordination between **3** and Cu<sup>2+</sup> (Fig. S14), where the Cu<sup>2+</sup> is coordinated by N and O atoms of **3** [57].

### 3.7. Theoretical predictions

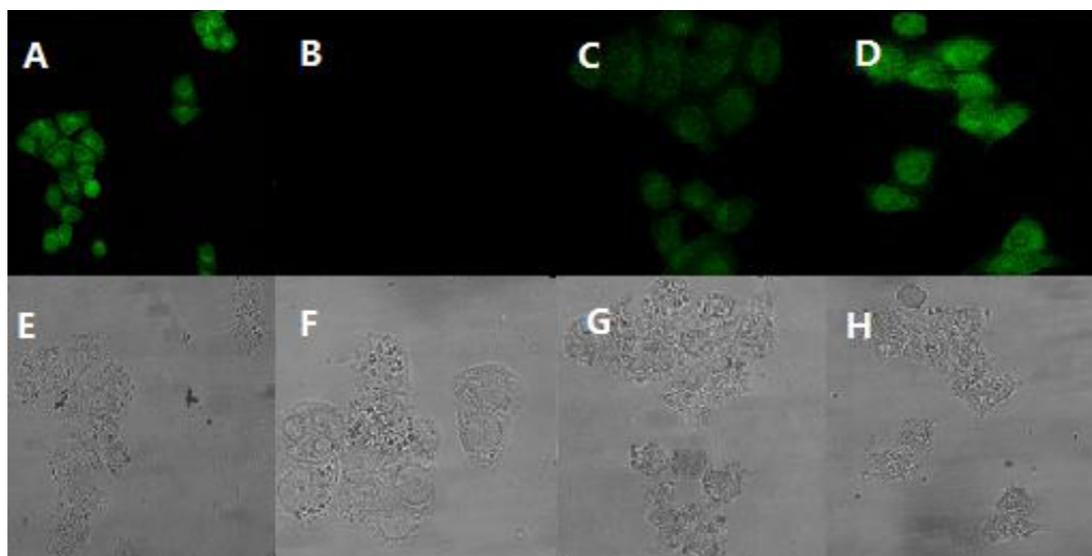
Theoretical calculations were explored to further study the optical properties of the probes and their Cu<sup>2+</sup>-addition products. As shown in Table 1, the TD-DFT calculations reveal that the main adsorption peaks for the three probes are 333 nm (**1**), 392 nm (**2**), and 401 nm (**3**), in close agreement with the experimentally observed adsorption spectra (as shown in Fig. 3). The main absorption bands in **2** (392 nm) and **3** (401 nm) originate from the highest occupied molecular orbital (HOMO)  $\rightarrow$  the lowest unoccupied molecular orbital LUMO transitions. However, the 333 nm

adsorption peak of **1** originates from (HOMO-1)  $\rightarrow$  (LUMO+1), (HOMO-2)  $\rightarrow$  LUMO, and HOMO  $\rightarrow$  (LUMO+1) mono-electronic excitations (see Table 1). On the basis of the molecular orbitals (Fig. 6), the electron density can be said to be mainly located on the same part of the HOMO and LUMO for **2** and **3**, but on different parts of the HOMO and LUMO for **1**. Such different characteristics of the calculated molecular orbitals demonstrate that **1** possesses a stronger ICT than **2** and **3**. It is well known that the fluorogenic process can be facilitated by the ICT mechanism [58–60]. Therefore, the different ICT processes in evidence for the three probes result in strong fluorescence emissions for **2** and **3** and weaker fluorescence emission for **1**, in accordance with the results determined herein by our experiment.

For the Cu<sup>2+</sup>-addition products of the three probes, the adsorption peak predicted by the theoretical methods is red-shifted compared with those of the independent probes (Table 1), which is in good agreement with the experimental observations. For example, upon the addition of Cu<sup>2+</sup> to **3**, the maximum absorption peak was gradually shifted from 401 nm to 444 nm. Based on the calculated molecular orbitals (Fig. 6), the conjugated system is broken with the Cu-products and this change will lead to a strong ICT process in the Cu-product. This can therefore explain the fluorescence quenching of the Cu-addition products that is witnessed experimentally.

### 3.8. Detection range

To investigate the detection limit of the probes for Cu<sup>2+</sup>, **1** (25  $\mu$ mol/L) was treated with various concentrations of Cu<sup>2+</sup> (0–25  $\mu$ mol/L) and the absorbance intensity at 500 nm was plotted as a function of Cu<sup>2+</sup> concentration (Fig. S15). The absorbance intensity of **1** is linearly proportional to the Cu<sup>2+</sup> concentration, and a concentration of Cu<sup>2+</sup> as low as 1.89  $\mu$ mol/L can be detected using **1**. In a similar procedure, **2** (0.1875  $\mu$ mol/L) was treated with various concentrations of Cu<sup>2+</sup> (0–9.375  $\mu$ mol/L) and the emission intensity at 520 nm plotted as a function of the Cu<sup>2+</sup> concentration. From this it can be seen that a concentration of Cu<sup>2+</sup> as low as 0.11  $\mu$ mol/L can be detected using **2**. Similarly, the detection limit was estimated to be less than 0.011  $\mu$ mol/L for **3**. These detection limits indicate that fluorescence probes **2** and **3** show a high



**Fig. 7.** Confocal fluorescence images in HepG2 cells. (A) Fluorescence image of HepG2 cells with adding **2** (10  $\mu$ M). (B) Fluorescence image of HepG2 cells incubated with 10  $\mu$ M **2** for 30 min at 37  $^{\circ}$ C and then incubated with 10  $\mu$ M CuCl<sub>2</sub> for 30 min at 37  $^{\circ}$ C. (C) after (B), then incubated with Na<sub>2</sub>S (10  $\mu$ M) for 30 min at 37  $^{\circ}$ C. (D) after (B), then incubated with Na<sub>2</sub>S (20  $\mu$ M).

sensitivity toward  $\text{Cu}^{2+}$  that is comparable to the UV–vis probe **1** chemosensor.

### 3.9. Their application in anion biomaging

It is well known that chemosensor reversibility is required for reuse. Addition of  $\text{P}_2\text{O}_7^{4-}$  or  $\text{C}_2\text{O}_4^{2-}$  to the **1**- $\text{Cu}^{2+}$  complex in HEPES buffer (10 mmol/L, pH 7.0) results in the spectrum of **1** reverting to that of its original pre-complex state. Furthermore, when the concentrations of  $\text{P}_2\text{O}_7^{4-}$  or  $\text{C}_2\text{O}_4^{2-}$  are increased, the spectra renew gradually the spectra of free **1**. Simultaneously, the solution changes color from orange yellow to a light shade of yellow. For **2**, in addition to  $\text{P}_2\text{O}_7^{4-}$  and  $\text{C}_2\text{O}_4^{2-}$ , Cys,  $\text{S}_2\text{O}_3^{2-}$ ,  $\text{S}^{2-}$ , and citrate can play a similar role to that described. However, the addition of  $\text{P}_2\text{O}_7^{4-}$  and  $\text{C}_2\text{O}_4^{2-}$  results in a partial release of **3** from its complex (Fig. S16). Anions with a physiological function, including  $\text{F}^-$ ,  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ , acetate ( $\text{AcO}^-$ ),  $\text{SCN}^-$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{CO}_3^{2-}$ ,  $\text{C}_2\text{O}_4^{2-}$ ,  $\text{PO}_4^{3-}$ , and  $\text{CN}^-$ , were also investigated but their effect on the UV–vis spectra was not as evident as for  $\text{P}_2\text{O}_7^{4-}/\text{C}_2\text{O}_4^{2-}$ . These results indicate that **2** is an optimal probe for  $\text{Cu}^{2+}$  because it is easily regenerated.

The **2**- $\text{Cu}^{2+}$  complex exhibits an anion-induced increase in fluorescence, which thus allows for the possibility of using it in bioimaging. The ability of **2**- $\text{Cu}^{2+}$  to react with anions in living cells was also evaluated. Under selective excitation at 405 nm, HepG2 cells showed green fluorescence after they were incubated with 20  $\mu\text{mol/L}$  of **2** for 30 min at 37 °C, and with 10  $\mu\text{mol/L}$  of  $\text{S}^{2-}$  (as a delegate) added for the final 30 min (Fig. 7C). HepG2 cells incubated with probe **2** for 30 min at 37 °C with 20  $\mu\text{mol/L}$  of  $\text{S}^{2-}$  added for the final 30 min showed stronger green fluorescence (Fig. 7D). These cell experiments show that **2** can permeate through cell membranes.

## 4. Conclusions

Three compounds, namely 5-nitrosalicylaldehyde fluorescein hydrazone (**1**), 5-diethylaminosalicylaldehyde fluorescein hydrazone (**2**), and di(5-diethylamino salicylaldehyde) hydrazide (**3**), were successfully synthesized and characterized. Their abilities to recognize copper were investigated using UV–vis and fluorescence spectroscopies. The results showed that the recognition processes for each of the probes have some differences despite their similar structure, with the conclusion that probe **2** is an optimal probe for  $\text{Cu}^{2+}$ . More importantly, the optical properties of the probes and their  $\text{Cu}^{2+}$ -addition products were studied using DFT calculations and the fluorescence quenching mechanism revealed. Application in bioimaging was illustrated and anions can induce **2**- $\text{Cu}^{2+}$  to show strong fluorescence in living cells. This work will therefore prove useful for the future design and application of copper chemosensors.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.snb.2012.12.043>.

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