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# A novel biphenyl-derived salicylhydrazone Schiff base fluorescent probes for identification of $Cu^{2+}$ and application in living cells

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ABSTRACT

A novel biphenyl-derived salicylhydrazone Schiff base (**BSS**) fluorescent probes for highly sensitive and selective identification of $Cu^{2+}$ has been synthesized. In addition, the recognition has been proved experimentally. The results indicated that the complex forms a 1:1 complex with $Cu^{2+}$ shows fluorescent quenching. Furthermore, the detection limit of $1.54 \times 10^{-8}$ M. More interesting, the probe **BSS** not only have a good biocompatibility in living cells, but also the sense behavior of $Cu^{2+}$ in the cell nucleus.



## 1. Introduction

Copper followed by iron and zinc is the third most abundant transition elements in human body [1]. The World Health Organization (WHO) report suggests that under normal conditions the average concentration of copper in the blood should not exceed 100–150 g/dl (15.7–23.6 μM) [2]. If people intake of copper exceeds the normal concentration of $Cu^{2+}$ in body causes imbalance in cellular processes resulting in pathogenesis such as Wilson's disease [3], amyotrophic sclerosis [4], and Alzheimer's disease [5]. More than this, it plays an important role in many physiological process of the foundation in organisms [6,7], such as cellular metabolism [8] neurotransmitter transfer [9], enzyme catalysis [10]. Apart from biological importance copper is the most useful material for making machine parts, fertilizers, alloys, batteries and electrical wires because of its relatively high malleability, low-cost, availability, electrical and thermal conductivity [11]. Copper toxicity is also a widely has attracted considerable attention due to its widespread utility in biology, industry, and agriculture [12]. Consequently, much attention to the development of selective sensing and monitoring of $Cu^{2+}$ is highly important [13,14].

Currently a precise analytical technique for the determination of $Cu^{2+}$ has been established, such as atomic absorption spectrometry [15] inductively coupled plasma mass spectroscopy [16] electrothermal atomic absorption spectrometry [17] cold vapor atomic absorption spectrometry [18] and others. These analytical techniques require expensive instruments, involve time consuming, well controlled experimental conditions, multistep and complicated sample preparation processes.

Therefore, the briefness, convenience, good sensitive, highly selective and easily synthesized colorimetric sensors are needed [19]. Simple Schiff base originated imine (HC═N—) group ligands are known for their metal binding affinity [20,21] some Schiff base derivatives will change the initial state of the target, the metal ion concentration different effects on the fluorescence intensity or peak position are different, so it can be use of metal ions by fluorescence, spectrometer [22,23]. Specially, the biphenyl-derived salicylhydrazone is a kind of Schiff base, it not only has general advantage, but also its strong coordination ability and various coordination forms, metal complexes can be formed with many metal ions [24]. Recently, particular attention has been paid to the synthesis and study of metal complexes of Schiff bases [25,26]. Some of the Schiff base metal complexes possess antitumor properties [27], attractive electronic and photophysical properties [28]. However, very few reports were used for the construction and design of probe for $Cu^{2+}$ sensors and application in cells. Therefore, as an ideal fluorescent analysis reagent, the determination of $Cu^{2+}$ has shown many advantages.

Based on the above mentioned, herein we report the synthesis and photophysical properties of a novel biphenyl-derived salicylhydrazone Schiff base (**BSS**) fluorescent probes for $Cu^{2+}$, which shows highly sensitive and selective colorimetric response to $Cu^{2+}$ in EtOH/$H_2O$ mixed

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solution. Furthermore, the probe **BSS** not only have a good biocompatibility in living cells, but also the sense behavior of $Cu^{2+}$ in the cell's nucleus.

## 2. Experimental

### 2.1. Materials

Deionized water was used throughout the experiment. All the reagents were purchased from commercial suppliers and used without further purification. The aqueous solutions of metal ions were prepared from $KNO_3$, $NaNO_3$, $Ca(NO_3)_2$, $Mg(NO_3)_2$, $Fe(NO_3)_3$, $AgNO_3$, $Al(NO_3)_3$, $Ba(NO_3)_2$, $Mn(NO_3)_2$, $Co(NO_3)_2$, $Ni(NO_3)_2$, $CdCl_2$, $Pb(NO_3)_2$, $Cu(NO_3)_2$, $Zn(NO_3)_2$, $LiNO_3$ and $HgCl_2$, respectively. All samples were prepared at room temperature, shaken for 10 s and stood for 18 h before UV–vis and fluorescence determination. Ethyl alcohol was purchased from Sinopharm chemical reagent co., Ltd.

Thin-layer chromatography (TLC) was conducted on silica gel 60F254 plates (Merck KGaA). The UV–vis spectra were recorded on UV-2700 spectrophotometer. Fluorescent measurements were recorded on a Hitachi F-7000 fluorescence spectrophotometer. $^1H$ NMR and $^{13}C$ NMR spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer, using DMSO *d*6 as solvent and tetramethylsilane (TMS) as internal standard. HRMS spectra were recorded on the Bruker Daltonics Esquire 6000. FT-IR spectra were recorded on the Fourier Transform Infrared Spectrometer from Nicolet, USA.

The cells were imaged under Leica TCS SP8MP multiphoton microscopy, Hela cells were provided by the Lanzhou veterinary research institute, Chinese academy of agricultural sciences.

### 2.2. Synthesis and Characterization

The probe **BSS** synthesis as follows: compound (a) and (d) procured from commercial suppliers. Compound (b) [29] and Compound (c) [30], were prepared according to the literature procedures. The probe **BSS** synthesis was performed according to literature and the condensation of the corresponding amines with salicylaldehyde according to the standard procedures as previously reported [31] As follows: 1,1′-(4,4′-dihydroxy-[1,1′-biphenyl]-3,3′-diyl) diethanon (27 mg;0.1 nmol) and 2-hydroxy benzohydrazide (30.4 mg;0.2 nmol) were dissolved in 30 ml of methanol solution stirring to dissolve completely, then add 2 ml of glacial acetic acid to the mixture solution, 80 °C reflux for 8 h, with a light yellow solid generation, cooling, filtration. Pure substance is obtained after 2–3 times of recrystallization of anhydrous methanol and vacuum drying. Its yield was 76.3% (yellow powder) and slightly soluble in other organic solvents, easily soluble in DMSO. The analysis data is as follows: $^1H$ NMR (DMSO $d_6$, 400 MHz): $\delta$ (ppm) = 13.20 (s, 2H), 11.64 (s, 2H), 7.97 (d, 2H), 7.83 (s, 2H), 7.62 (d, 2H), 7.43 (d, 2H), 7.01 (m, 8H), 2.53 (s,6H)· $^{13}C$ NMR (DMSO $d_6$, 400 MHz): $\delta$(ppm) = 162.76, 158.13, 157.17, 156.54, 134.19, 131.02, 130.98, 129.87, 126.61, 120.16, 119.91, 118.23, 117.69, 117.37, 31.13. Elemental analysis (%) for $C_{30}H_{26}N_4O_6$ (538.55) C, 66.91; H, 4.87; N, 10.40; found: C, 66.87; H, 4.84; N, 10.03. HRMS ([BSS + H]$^+$, MW = 539.5507; calcd for $C_{30}H_{26}N_4O_6$, 538.55; [BSS + $Cu^{2+}$–2H]$^+$, MW = 600.1088; calcd for $C_{30}H_{24}CuN_4O_6$, 600.08).

## 3. Results and Discussion

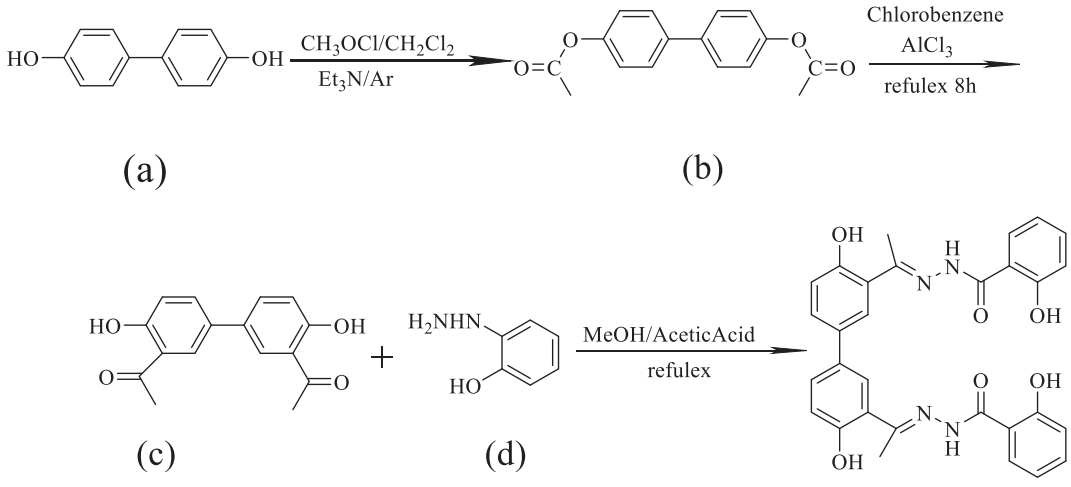
### 3.1. Design and Synthesis

The synthetic route of proposed compound **BSS** is shown in Scheme 1. 1,1′-(4,4′-dihydroxy-[1,1′-biphenyl]-3,3′-diyl) diethanon (27 mg; 0.1 nmol) and 2-hydroxy benzohydrazide (30.4 mg; 0.2 nmol) were dissolved in 30 ml of methanol solution stirring to dissolve completely, then add 2 ml of glacial acetic acid to the mixture solution, 80 °C reflux for 8 h, with a light yellow solid generation, cooling, filtration. Pure substance is obtained after 2–3 times of recrystallization of anhydrous methanol and vacuum drying (Scheme 1). The probe **BSS** was characterized by $^1H$ NMR, $^{13}C$ NMR, HRMS was shown Support information.

### 3.2. Selective Identification of Metal Ions by Probe BSS

In order to exploration the practical applications in biological environment, the absorption spectral property of probe **BSS** toward various metal ions was investigated in the mixture solvent EtOH/Deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature.

The UV–visible absorption spectrum of the probe **BSS** is shown Fig. 1, the spectrum of **BSS** is characterized by two bands in 300 nm and 365 nm The former can be assigned to n-π* transition involving molecular orbitals particularly localized on the C═N group and benzene ring, the low intensity absorption around 365 nm is assigned to n-π* transition involving molecular orbitals of the C═N chromophore and benzene ring [32]. Upon adding $Cu^{2+}$ 2 eq and other equivalent metal ions ($Li^+$, $K^+$, $Mg^{2+}$, $Ag^+$, $Hg^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Ba^{2+}$, $Cd^{2+}$, $Al^{3+}$, $Na^+$, $Fe^{3+}$, $Mn^{2+}$, $Pb^{2+}$, $Ni^{2+}$), We found that BSS showed no change in the absorption intensity relative to the free ligand except for $Fe^{3+}$, $Hg^{2+}$ and $Ag^+$ (Fig. 1a). There was no change in the solution color of **BSS** with addition of these metal ions. In the presence of $Hg^{2+}$ and $Ag^+$ the absorption band was slightly blue shifted, $Fe^{3+}$ have influential but the main peak shape did not change. However, there was no appreciable change in the solution color except for $Cu^{2+}$, under the UV–vis lamp, but the




**Scheme 1.** Synthetic route to sensing probe BSS.

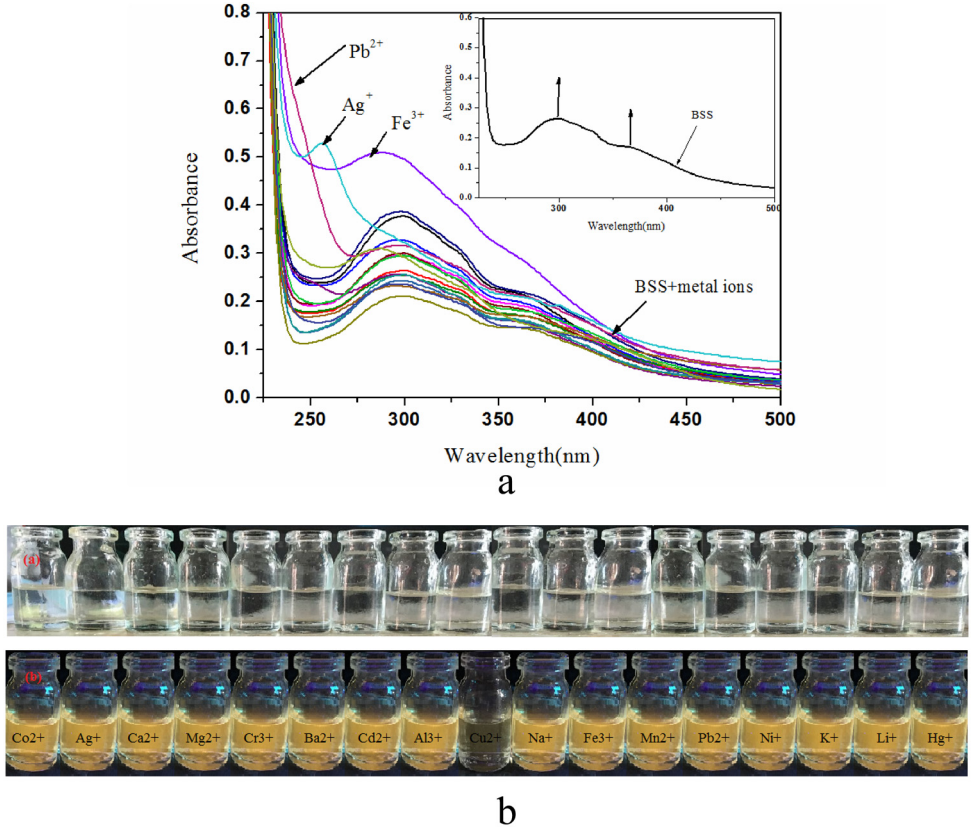



**Fig. 1.** a UV–vis spectral changes of probe BSS upon additions of various metal ions in the mixture solvent EtOH/deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature. b The color change of the solution under normal light (a) and UV-light (b).

color of the solution no change under normal light (Fig. 1b). In order to systematically investigated spectral property of the probe **BSS**, the fluorescence spectra could be mention.

### 3.3. Fluorescence Spectra Elective of the Prob. BSS

The fluorescence spectrum of the property of probe **BSS** ($10^{-5}$ M) toward various metal ions ($Li^{+}$, $K^{+}$, $Mg^{2+}$, $Ag^{+}$, $Hg^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Ba^{2+}$, $Cd^{2+}$, $Al^{3+}$, $Na^{+}$, $Fe^{3+}$, $Mn^{2+}$, $Pb^{2+}$, $Ni^{2+}$, $Cu^{2+}$), were investigated in the mixture solvent EtOH/deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature is shown in Fig. 2, interesting, when the excitation wavelength is 400 nm, the probe **BSS** showed a fine fluorescence peak at 566 nm. When adding 2 eq all kinds of metal ions only $Cu^{2+}$ and $Zn^{2+}$ had a clear change on the fluorescence peak as shown, we are clearly seed that the $Cu^{2+}$ shows a strong quenching characteristic, an obviously decreased in fluorescence intensity was observed accompanying a distinct color change from orange color to colorless under the UV–vis lamp but the $Zn^{2+}$ is merely a change in displacement (blue shift) and the shape of the peak has not changed too much, other remaining ions could not cause the change of fluorescence spectrum. All those indict that the probe **BSS** has good selectivity for $Cu^{2+}$.

### 3.4. Fluorescence Spectra of Probe BSS for Anti-interference Detection

To further examine its selectivity, fluorescence competition experiments were subsequently carried out as shown in Fig. 3a, in the absence of $Cu^{2+}$, the fluorescence intensity did not significantly quench in the presence of the selected potential competitive metal, whereas it was dramatically quenched when other metal ions are added to the equivalent. These results demonstrate that the coexisting metal ion has no significant interference on $Cu^{2+}$ recognition.

Additionally, in order to further explore the probe **BSS** has strong anti-interference recognition. Fluorescence phenomenon of probe **BSS** to sulfate, carbonate, and nitrate salts of copper, as shown in Fig. 3b, there were no obvious changes in the fluorescence responses of probe **BSS** to $CuSO_4$, $Cu(CO_3)_2$, and $Cu(NO_3)_2$. The results indicated that the probe **BSS** has strong anti-interference for anion.

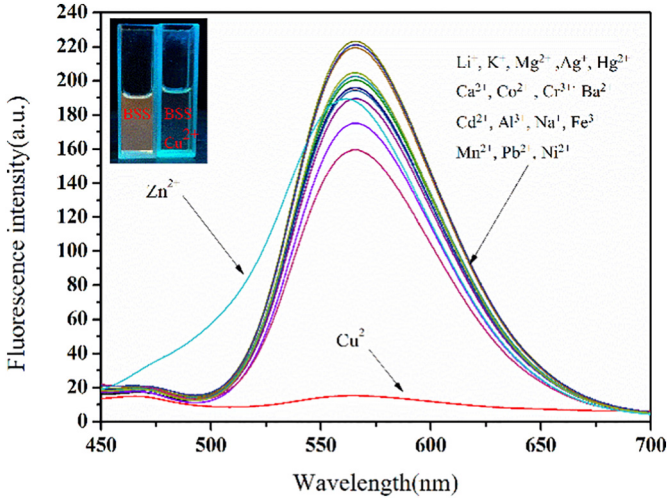



**Fig. 2.** Fluorescence emission spectra of **BSS** in the mixture solvent EtOH/Deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature.

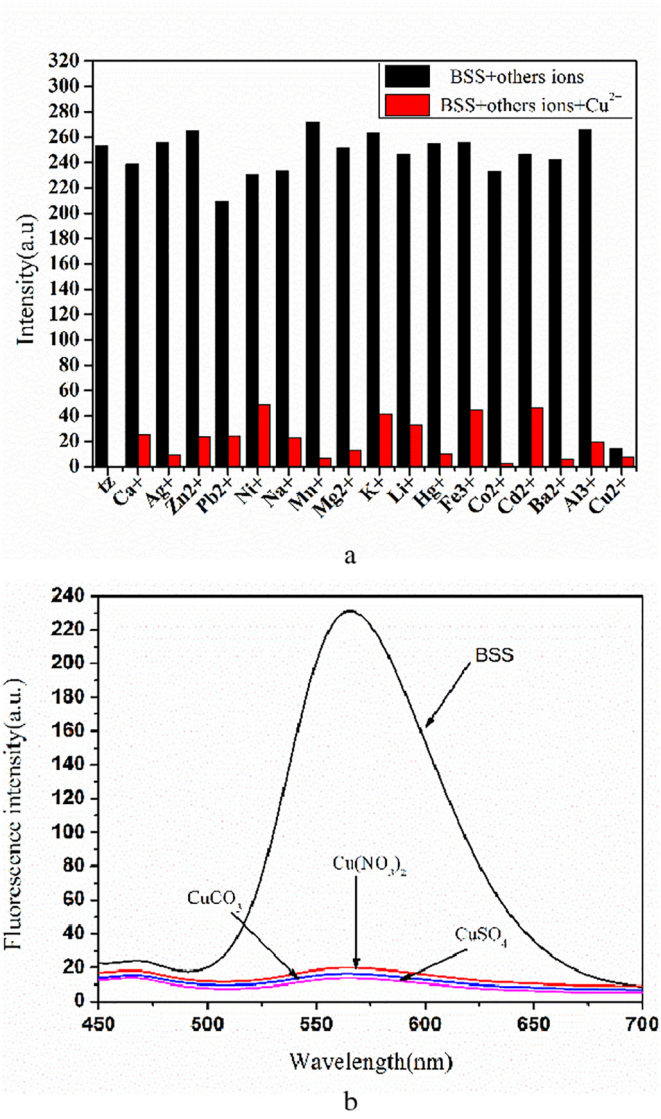



**Fig. 3.** a Fluorescence response of **BSS** in the presence of $Cu^{2+}$ and various additional metal ions in the mixture solvent EtOH/deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature. The black bars represent the addition of other metal ions solution of **BSS**. There bars represent the subsequent addition of the equal amount of $Cu^{2+}$ in mixture solution. b Fluorescence spectra of probe **BSS** in the mixture solvent EtOH/deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature and different copper salts.

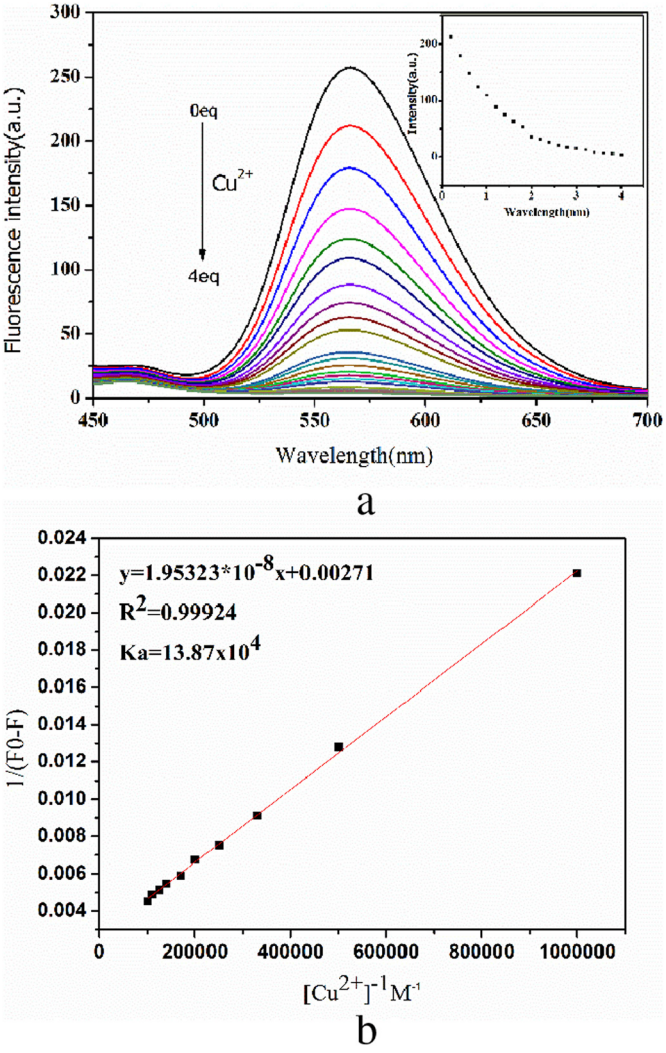



**Fig. 4.** a Fluorescence emission spectra of probe **BSS** was following addition of $Cu^{2+}$ (0–4 eq) in the mixture solvent EtOH/deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature. b Benesi-Hildebrand linear analysis plots of **BSS** at different $Cu^{2+}$ concentration in the mixture solvent EtOH/deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature.

### 3.5. Linear Relationship between the Probe BSS and the Target Ion

To further understand the sensing behavior of **BSS** to $Cu^{2+}$, fluorescence titration experiments were conducted (Fig. 4a) Upon incremental addition of $Cu^{2+}$ (0–4.0 equiv.) in the mixture solvent (2 ml) EtOH/Deionized water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4) at room temperature, the fluorescence emission was gradually quenched and reached the saturation state when 4.0 equiv. of $Cu^{2+}$ ions the fluorescence of weakening trend showed negligible changes and the curve remained relatively constant The effective fluorescence quenching of sensor **BSS** was attributed to the coordination of a paramagnetic $Cu^{2+}$ center. The fluorescence quantum yield decreases from 0.049 for probe **BSS** to 0.0169 [33] for the complex of probe **BSS**-$Cu^{2+}$, correspondingly. In addition, with a certain amount of $Cu^{2+}$ addition, the probe **BSS** show good linear relationship with $Cu^{2+}$ and equation of linear regression was showed in Fig. 4b ($y = a + bx$, $Ka = a/b$, $X = 1/[Cu^{2+}]$, $Y = 1/(F_0 - F)$; Ka: the association constant, $F_0$: the fluorescent intensity of **BSS**. F: the fluorescent intensity of BSS–$Cu^{2+}$ complex; $a = 0.00271$, $b = 1.95323 \times 10^{-8}$, $R^2 = 0.99924$, $Ka = a/b = 13.87 \times 10^4$). It showed a linear fitting and confirms the 1:1 stoichiometry of binding of $Cu^{2+}$ to probe **BSS**. Furthermore, through probe **BSS** ten times the relative standard deviation fluorescence intensity is calculated the calculated detection limit is $1.54 \times 10^{-8}$ M.

### 3.6. Binding Stoichiometry

To further explore the linear relationship between the probe **BSS** and the target ion, the fluorescence titration of probe **BSS** in the presence of different $Cu^{2+}$ concentrations was then performed in aqueous solution EtOH/water (1:1, v/v) containing HEPES buffer (10 mM, pH = 7.4). Keeping the total concentration of probe **BSS** and $Cu^{2+}$ for $1.0 \times 10^{-5}$ M, and the concentration ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 between probe **BSS** and $Cu^{2+}$ was titrated. By measuring the fluorescence intensity at 566 nm made complex curve, a maximum emission was observed when the molar fraction of $Cu^{2+}$ reached 0.5 (Fig. 5), the result further indicated that $Cu^{2+}$ ions form a 1:1 complex with the sensing compound in the media.

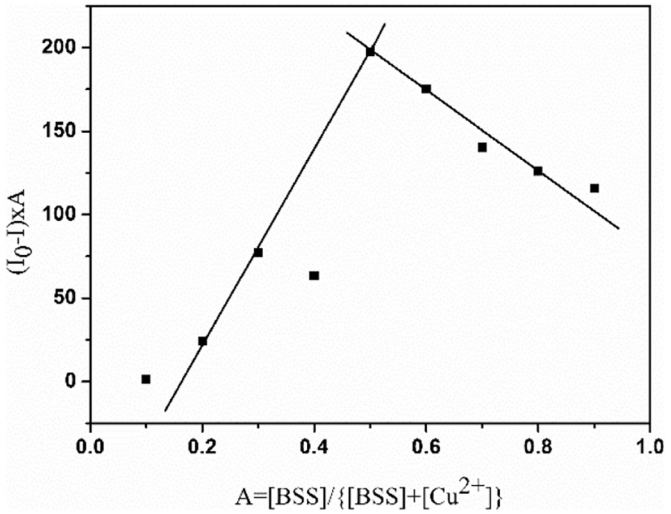



**Fig. 5.** Job's plot for determining the stoichiometry for probe **BSS** and $Cu^{2+}$. $I_0$: the fluorescent intensity of **BSS**. I: the fluorescent intensity of **BSS**–$Cu^{2+}$ complex.

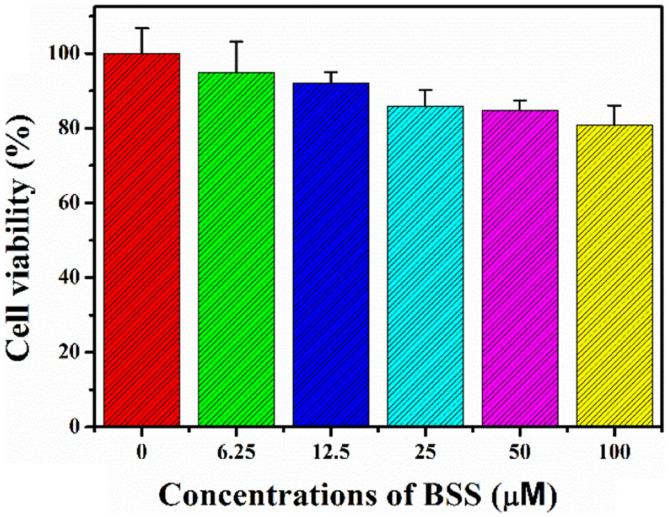



**Fig. 7.** Cytotoxicity of the Probe **BSS** in Hela cells.

### 3.7. Imaging of Intracellular $Cu^{2+}$

Based on the previous study, we try to investigate whether **BSS** could spy intracellular $Cu^{2+}$ in live cells. The intracellular $Cu^{2+}$ imaging behavior of probe **BSS**. 2 ml of Hela cells were incubated with 60 μM of probe **BSS** in DMEM for 180 min at 37 °C in Fig. 6a. Then, three times washing with PBS buffer the probe **BSS** was cell permeable and Hela cells. The probe **BSS** in the intracellular area showed very strong yellow fluorescence in cell nucleus by a fluorescence microscope [34], after incubation with these living cells with $Cu^{2+}$ (100 μM) for 180 min at 37 °C. The same treatment with probe **BSS** showed in cell nucleus fluorescence decreased significantly (Fig. 6b). Meanwhile, fluorescence imaging figure corresponding bright-field image has good coincidence degree. The overlay of fluorescent and bright-field images confirmed that the fluorescent signals were localized in the perinuclear area of the nucleus. The result indicated that **BSS** was able to penetrate the cell membrane and the probes are able to sense changes of the concentration of $Cu^{2+}$ in living cells.

### 3.8. Cytotoxicity of the Probe BSS

The results obtained from the above experiments were encouraging and we sought to investigate its cytotoxic potential in Fig. 7. To that end, an MTT assay was performed. When the concentration was increased from 0 μl to 100 μl, a indistinctive decrease in cell viability was observed. It was showed that **BSS** did not negatively affect Hela cells viability over the full range of concentrations measured and cell viability was around 80%. The data indicate that probe **BSS** has low cytotoxicity for intracellular.

### 3.9. Speculate Mechanism of Fluorescence

To investigate the mechanism of the fluorescence quenching for **BSS**, according to the study, the fluorescence of many probes changed, which

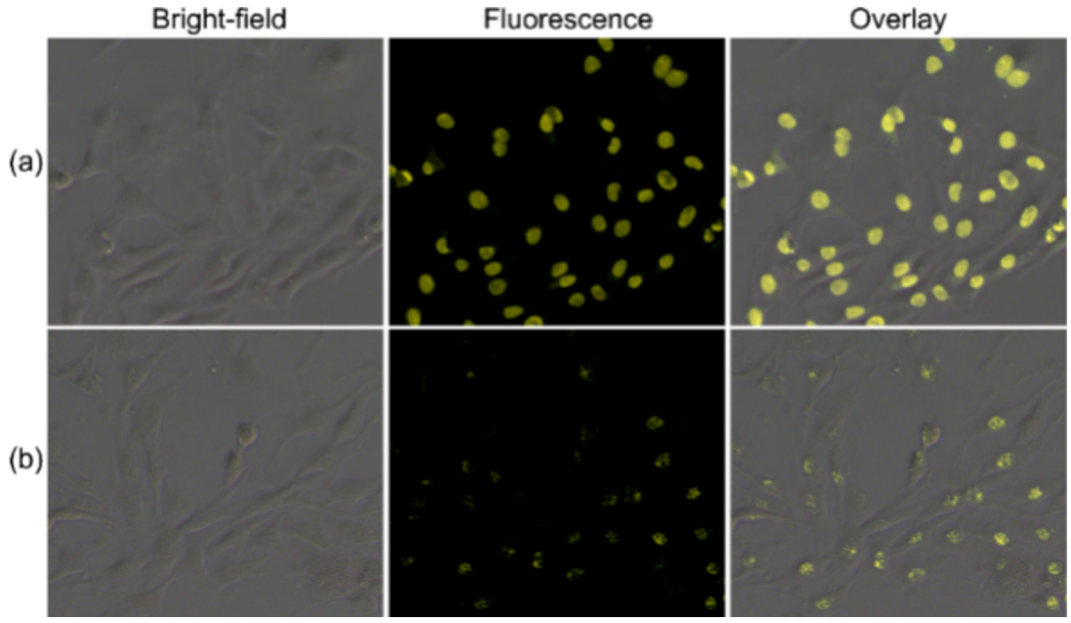
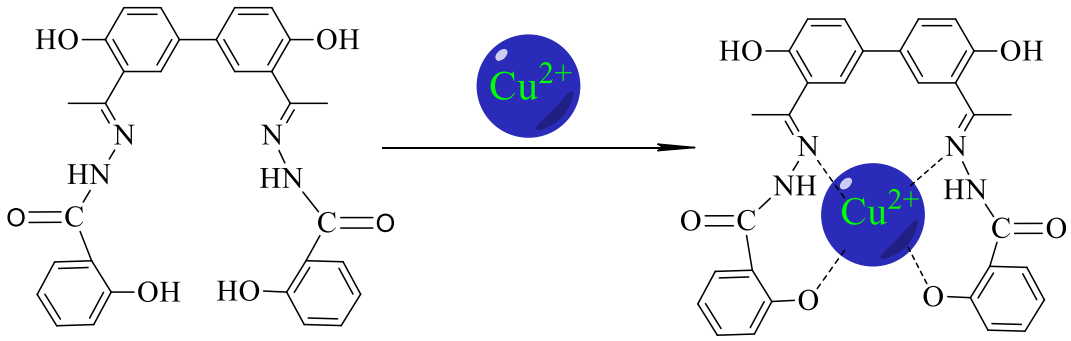



**Fig. 6.** Fluorescence images of probe **BSS** as a function of concentration of $Cu^{2+}$ in Hela cell nucleus: (a) fluorescence images of Hela cell nucleus treated with probe **BSS**; (b) fluorescence images after presence of $Cu^{2+}$ (100 μM).




**Scheme 2.** Synthetic route to sensing probe **BSS.**

based on the different functional groups, their common characteristic is that hydroxyl, and C═N on the molecular group all play important role in the complexation of Cu [35]. Therefore, we are hypothesized that In the complex, the metal ion possibly coordinates with the 2 oxygen of hydroxyl and 2 nitrogen atoms of C═N, as shown Scheme 2, the capture of $Cu^{2+}$ resulted in the electron or energy transfer from excited state of probe **BSS** to $Cu^{2+}$ lead to the fluorescence quenching.

### *3.10. Characterization of BSS-$Cu^{2+}$ Complexes*

In infrared spectra of the Biphenyl-derived salicylhydrazone Schiff-base present a strong absorption at 1678 $cm^{-1}$ which attributed to the C═N vibrations characteristic of imines [36,37]. The characteristic absorption peak of —OH appears at 3463 $cm^{-1}$. Whereas, the IR spectra of the BSS-$Cu^{2+}$ showed that the νC═N in the ligand, and νC—O of the phenolic hydroxyl group were red-shifted to 1652 and 3437 $cm^{-1}$ (supporting formation), which is due to decrease in electron density by the coordination of —OH, and C═N groups with $Cu^{2+}$. The results indicate that the N in the C═N and the O of the hydroxyl group coordinate with the metal ion [38].

## 4. Conclusions

In summary, we have successfully designed and synthesized a novel biphenyl-derived salicylhydrazone Schiff base fluorescent probe **BSS** as a promising analytical tool for detecting $Cu^{2+}$. It showed outstanding cell permeation and low toxicity, leading to the possibility of applying it as a highly selective chemosensor for biological systems, which laid the foundation for practical application of fluorescence detection method. Moreover, this work will inspire the development of biphenyl-derived salicylhydrazone Schiff base fluorescent probe for sensing the target metal ions in environmental and biological systems.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2018.03.060.

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