

Analytical Methods

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New fluorescent probe for Zn²⁺ imaging in living cells and plants

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Abstract

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3 A new fluorescent probe **L1** based on 8-aminoquinoline has been designed and synthesized,
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5 which exhibits high selectivity and sensitivity toward Zn^{2+} over other common metal ions
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7 especially Cd^{2+} . Under physiological conditions (pH 7.0), the probe displays an 8-fold
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9 fluorescence turn-on response to Zn^{2+} . Furthermore, by laser scanning confocal techniques, the
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11 probe can be used to monitor and distinguish Zn^{2+} from Cd^{2+} effectively in living cells and plant
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13 tissues.

14 15 16 **Keywords**

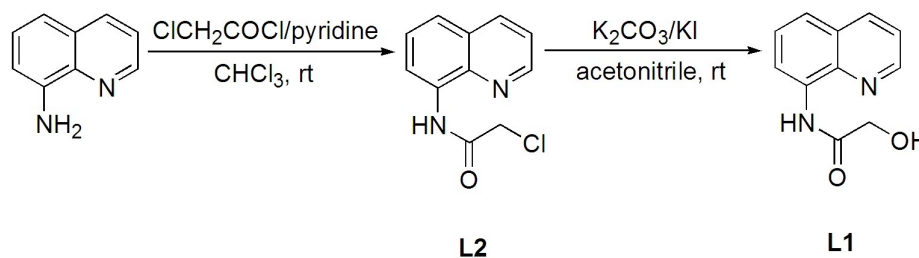
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18 Zn^{2+} probe, high selectivity, plant tissue imaging, cell imaging
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24 **1. Introduction**

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26 Zinc (Zn^{2+}) is an indispensable trace element which plays an important role in organisms
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28 ranging from fungi to mammals [1-3]. Most of the Zn^{2+} in organisms are bound with protein
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30 closely, minority are existing in the form of free distribution. It has been identified that Zn^{2+} exists
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32 in various systems and participates in many chemical processes in vital activities, such as gene
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34 expression, neurotransmission, cells differentiation, metalloenzymes synthesis, auxin regulation
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36 [4-9]. Common diseases, such as immunocompromised, ataxia, Alzheimer, dermatitis and some
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38 others [10-13] are related with the disorder of the Zn^{2+} metabolism. In addition, elevated levels of
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40 Zn^{2+} in environment lead to toxicity effect on plants, reduced protective enzyme activity and
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42 photosynthesis [14-16]. Therefore, detection and monitoring the Zn^{2+} especially in living cells are
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44 of considerable interest.

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46 The research of novel fluorescent probes had obtained a growing attention due to their
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48 applications in various fields. In fact, considerable efforts had been focused on the development of
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50 efficient methods for monitoring Zn^{2+} , especially fluorescent probes [17-24], which are considered
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52 simple and reproducible. However, the application of probe assay in tissue cells to distinguish
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54 Zn^{2+} from various metal cations, especially Cd^{2+} is still less studied up to now. Thus, there is a
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56 great need for designing a sensitive probe, which has high selectivity and permeability, and can
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58 discriminate Zn^{2+} in biological tissues.
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8-aminoquinoline and its derivatives are excellent chromophore which is frequently used as reporters in fluorescent probes [22, 23, 25-27]. Herein, we report a new quinoline-derived probe **L1** (Scheme 1) for Zn^{2+} detection with high sensitivity and selectivity which is fabricated easily and has simple construction. Besides, this probe displays highly selective chelation enhanced fluorescence (CHEF) effect with Zn^{2+} ions which serves as the basis of the probe for Zn^{2+} . Moreover, probe **L1** was also demonstrated in the contribution to image Zn^{2+} in living human cells and plant tissues. "Micro-CT" technology (micro computed tomography) provides a good means for microscopic image of subcellular fraction due to the deficiency of spatial resolution anisotropy in optical microscopy can be overcome. Thus, we incorporated "Micro-CT" technology revealing the imaging position in cells and the well cell permeability of probe **L1**.



< Scheme 1 >

2. Materials and methods

2.1. Materials and instrumentations

All the materials for synthesis were purchased from commercial suppliers and used without further purification. Ethanol for spectra detection was HPLC reagent without fluorescent impurity. 1H NMR and ^{13}C NMR (Fig. S1) were taken on a Varian mercury-400 spectrometer with TMS as an internal standard and $CDCl_3$ as solvent. ESI mass spectra were performed on a Bruker Daltonics Esquire 6000 spectrometer. Fluorescence spectra measurements were performed on a Hitachi F-4500 spectrofluorimeter. Fluorescent pictures were taken on Leica laser scanning confocal microscope. Stock solutions of the metal ions (10 mM) were prepared in deionized water.

2.2. Synthesis of probe L1

2-chloro-N-(quinolin-8-yl) acetamide (**L2**) was prepared from 8-Aminoquinoline. To a

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chloroform (25 mL) solution of 8-Aminoquinoline (0.69 g, 4.8 mmol) and pyridine (1 ml) cooled in an ice bath was added dropwise a chloroform (5 mL) solution of chloroacetyl chloride (0.8 ml, 10.1 mmol) with stirring. After being stirred for 24h at room temperature, the mixture was removed under reduced pressure to obtain a yellow solid, which was purified by silica gel column chromatography using dichloromethane as the eluent to afford **L2** in 90% yield. ESI-MS: (m/z) =221.1.

2-chloro-N-(quinolin-8-yl) acetamide (**L2**, 1.1 g, 5 mmol), KI (42 mg, 0.25 mmol) and K₂CO₃ (1.04 g, 7.5 mmol) were added into the mixture of acetonitrile (40 mL) and distilled water (8 ml). Then stirred and refluxed for 8h at about 80°C. After cooling to room temperature, the mixture was purified by silica gel column chromatography using the dichloromethane as the eluent, and then a white solid was obtained in 57% yield. ¹H NMR (400 MHz, CDCl₃) δ 10.49 (s, 1H), 8.85 (dd, J = 4.3, 1.7 Hz, 1H), 8.83 – 8.73 (m, 1H), 8.22 (dd, J = 8.3, 1.5 Hz, 1H), 7.62 – 7.55 (m, 2H), 7.54 – 7.47 (m, 1H), 4.43 (s, 2H), 2.97 (s, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 169.881, 148.363, 136.587, 128.050, 127.382, 122.206, 121.716, 117.068, 62.915. ESI-MS: (m/z) =203.3.

2.3. Cell incubation and imaging

MDA-231 (breast cancer) cells and HBL-100 (normal human breast) cells were cultured in culture media ((Dulbecco's Modified Eagle's Medium, High Glucose) supplemented with 10% FBS (Fetal Bovine Serum), 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a humidified incubator which was provided with 5% CO₂ and 95 % air. MDA-231 cells were cultured in 12-well plate at a density of 10⁴ cells per well in culture media. After 48 h, they were treated with Zn²⁺ (30 μM) and Cd²⁺ (30 μM) respectively for 30 min at 37°C, and then washed with phosphate-buffered saline (PBS) three times before incubating with probe **L1** (10 μM) for another 10 min and imaged by laser scanning confocal microscopy. The MDA-231 cells only incubated with 10 μM probe **L1** for 10 min at 37 °C under 5% CO₂ was as a control. HBL-100 cells were cultured and imaged as the same process of MDA-231 cells.

2.4. Plants culture and imaging

The seeds of wheat were disinfected by 70% ethanol and 3% sodium hypochlorite solution, washed thoroughly with distilled water five times, then cultured on Petri dishes and covered with sterilized quartz sand under condition of room temperature and 70% relative humidity. The control

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group was supplied only diluted Hoagland, the experimental groups were treated by Hoagland solution with different levels of Zn^{2+} and Cd^{2+} ions respectively after three days. Ten days later, some leaves and roots were collected from different experimental groups. Subsequently, the samples were soaked in the solution containing **L1** (10 μM) for 30 min after washed by distilled water three times, after rinsed with PBS three times again the fluorescent images of the plants samples were obtained by confocal microscopy. The samples only incubated with 10 μM probe **L1** for 30 min was as a control.

3. Results and discussion

3.1. Selectivity and Cation-competitive Experiments

High selectivity is extremely important for an excellent probe. To evaluate the specificity of **L1** toward Zn^{2+} , the selectivity of **L1** toward common metal ions, including Cd^{2+} , K^+ , Na^+ , Fe^{3+} , Cr^{3+} , Ni^{2+} , Mg^{2+} , Ag^+ , Hg^{2+} , Al^{3+} , Co^{2+} , Mn^{2+} , Pb^{2+} , Li^+ , Cu^{2+} and Zn^{2+} , was measured in H_2O /ethanol (8:2, v/v) solutions. As shown in Fig. 1a, only the addition of Zn^{2+} ion could cause a prominent emission enhancement, whereas the other miscellaneous competitive cations tested caused no obvious changes, which demonstrated that **L1** could distinguish Zn^{2+} from other metal ions, especially Cd^{2+} . Correspondingly, the fluorescence color changed from colorless to turquoise (Fig. 1a inset), which could be easily distinguished by the naked eyes under the aid of a normal UV lamp.

To validate the selectivity of **L1** in practice, the cation-competitive experiments were done to further explore the utility of **L1** as an ion-selective probe for Zn^{2+} . As shown in Fig. 1b, although the miscellaneous competitive cations have more or less influence on the fluorescence intensity, significant enhancement in the fluorescence intensity was observed for **L1** when Zn^{2+} ion was added in the presence of the miscellaneous competitive cations. The interference of the other metal ions was relatively limited, indicating that **L1** could be applied to detect Zn^{2+} effectively. Notably, the Cd^{2+} which known as a common competitor for Zn^{2+} did not cause such change either. These results strongly illustrated that **L1** had a high sensitivity and selectivity on Zn^{2+} which was superior to other metal cations.

3.2. Fluorescence Titration

The fluorescence spectrum changes of **L1** were measured with different concentrations of Zn^{2+}

ions (0–20 μM) in $\text{H}_2\text{O}/\text{ethanol}$ (8:2, v/v) solutions. As shown in Fig. 1c, the probe **L1** exhibited a weak emission at 500 nm ($\lambda_{\text{ex}} = 360 \text{ nm}$, $\Phi = 7.6\%$). Upon incremental addition of Zn^{2+} ion (0–2 equiv.), the fluorescence intensity undergoes a ca. 8-fold increase, indicating a turn-on fluorescent response toward Zn^{2+} ion. In addition, the emission intensity increased linearly with the amount of Zn^{2+} ion in the range 0–10 μM (Fig. 1c inset), suggesting the formation of 1/1 complex. From the linear equation (Fig. 1d), the detection limit for Zn^{2+} was calculated to be 0.65 μM .

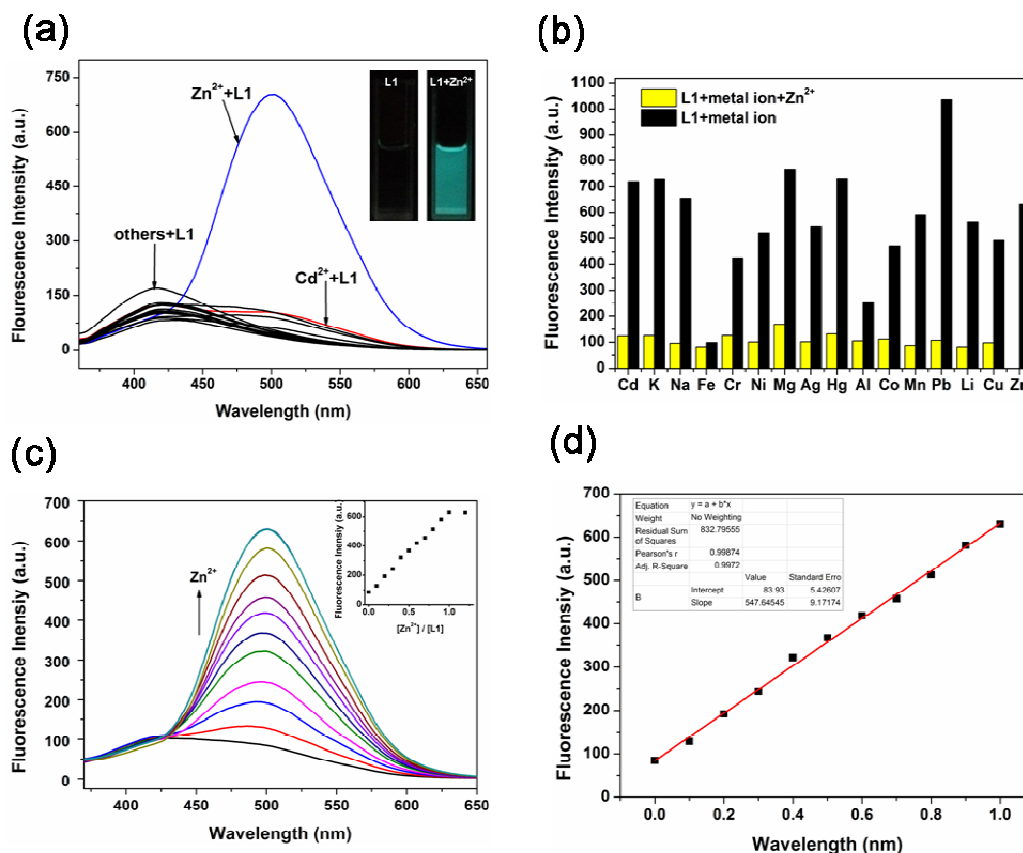


Fig. 1 (a) Fluorescent emission spectra of 100 μM other metal ions and 50 μM Zn^{2+} in the same media. Inset: Photograph of **L1** and **L1** + Zn^{2+} (20 μM). (b) Fluorescence intensities of **L1** (10 μM) upon the addition of various metal ions in $\text{H}_2\text{O}/\text{ethanol}$ (8:2, v/v). Yellow bars represent addition of **L1** (10 μM) to the other miscellaneous competitive cations (20 μM) including Cd^{2+} , K^+ , Na^+ , Fe^{3+} , Cr^{3+} , Ni^{2+} , Mg^{2+} , Ag^+ , Hg^{2+} , Al^{3+} , Co^{2+} , Mn^{2+} , Pb^{2+} , Li^+ , Cu^{2+} and Zn^{2+} . Black bars represent the addition of Zn^{2+} to the solution of **L1** in the presence of different cations. (c) Fluorescence titration spectra of **L1** upon the addition of different concentrations of Zn^{2+} (0–1 equiv.) in $\text{H}_2\text{O}/\text{ethanol}$ (8:2, v/v). (d) Fluorescence intensity at 628 nm of **L1** as a function of Zn^{2+} concentration.

3.3. Mechanism of the Sensing of Zn²⁺

To confirm the binding modes between **L1** and Zn²⁺, the Job's plot was measured to further demonstrate the stoichiometry relationship (Fig. S2). According to Job's plot results, the stoichiometric ratio of **L1** with Zn²⁺ was determined to be 1:1, which was consistent with the results from the fluorescence titration. To get an insight into the molecular structure and the different optical behavior of **L1** before and after the addition of Zn²⁺, density functional theory (DFT) were carried out with the Gaussian 09 suite of programs. The optimized configuration was shown in Fig. S3, which showed that the Zn²⁺ ion bound to **L1** was very well through five coordination sites. Thus, the significant enhancement in emission intensity for **L1** may be attributed to the Zn²⁺-triggered amide tautomerization.

3.4. Application in Bioimaging

In order to exploit the Zn-sensing behavior of probe **L1** in biological systems, we incorporated Laser Scanning Confocal Microscope for imaging the metal ions in living human cells and plants tissues. A significant enhancement of fluorescence in cells was observed after the cells were intervened by metal ions and probe **L1**. The fluorescence of cells treated with Zn²⁺ revealed uniform enhancement in whole cell without the brighter organelles (Fig. 2b), but the cells treated with Cd²⁺ revealed the brightest fluorescence inside of some special organelles (Fig. 2c). The results of HBL-100 cells were similar to the group of MDA-231 cells. Remarkably, very weak intracellular fluorescence was led in the cells in presence of **L1** before the exogenous Zn²⁺ added (Fig. 2d), due to Zn²⁺ was an essential trace element existed normally in human body. Compared Fig. 2a with Fig. 2d, the former showed no fluorescence also conformed with some experimental studies that Zn²⁺ content was abnormal in tumors [11, 28-30]. As shown in Fig. 2f, the outline of some special organelles was more distinct which was treated with Cd²⁺. Then we used the "Micro-CT" scanning the HBL-100 cells layer by layer to confirm the imaging areas (Fig. S4), subcellular distribution of Cd²⁺ differing from Zn²⁺ was illustrated obviously which was needed to study and investigate further.

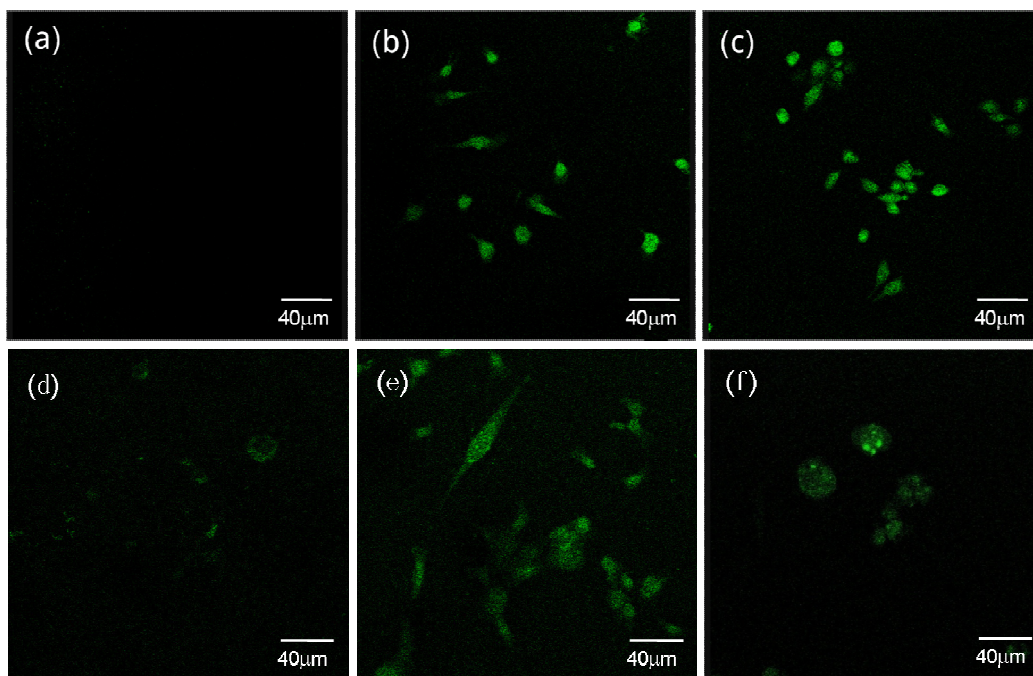


Fig. 2 Fluorescence images of **L1** in MDA-231 (breast cancer) cells (Fig. 4a~c) and HBL-100 (normal human breast) cells (Fig. 4d~f). (a) MDA-231 cells stained with 10 μ M solution of **L1** for 10min. (b) 30 μ M Zn²⁺ supplemented cells and (c) 30 μ M Cd²⁺ supplemented cells respectively for 30min and then incubated with **L1** 10 μ M for 10 min. (d) HBL-100 cells stained with 10 μ M solution of **L1** for 10min. (e) 30 μ M Zn²⁺ supplemented cells and (c) 30 μ M Cd²⁺ supplemented cells respectively for 30min and then incubated with **L1** 10 μ M for 10min. Incubation was performed at 37°C under a humidified atmosphere containing 5% CO₂.

From the plants tissues imaging results, the specimens of leaves and roots displayed an obviously fluorescent enhancement both in the samples treated with Zn²⁺ and Cd²⁺ (Fig. 3c~h). Even more, as the bright and dark fields performed, the Zn²⁺ treated root was brighter in root cap (Fig. 3e) and root hair (Fig. 3f), but the Cd²⁺ treated one was opposite (Fig. 3g~h). The distinguishing images confirmed that Zn²⁺ and Cd²⁺ were existed in different plant tissues structures [31-33]. All these outcomes were also certified that the well cell permeability and histocompatibility of probe **L1** which grant the access to chelate with Zn²⁺ and Cd²⁺ in both human cells and the plant tissues.

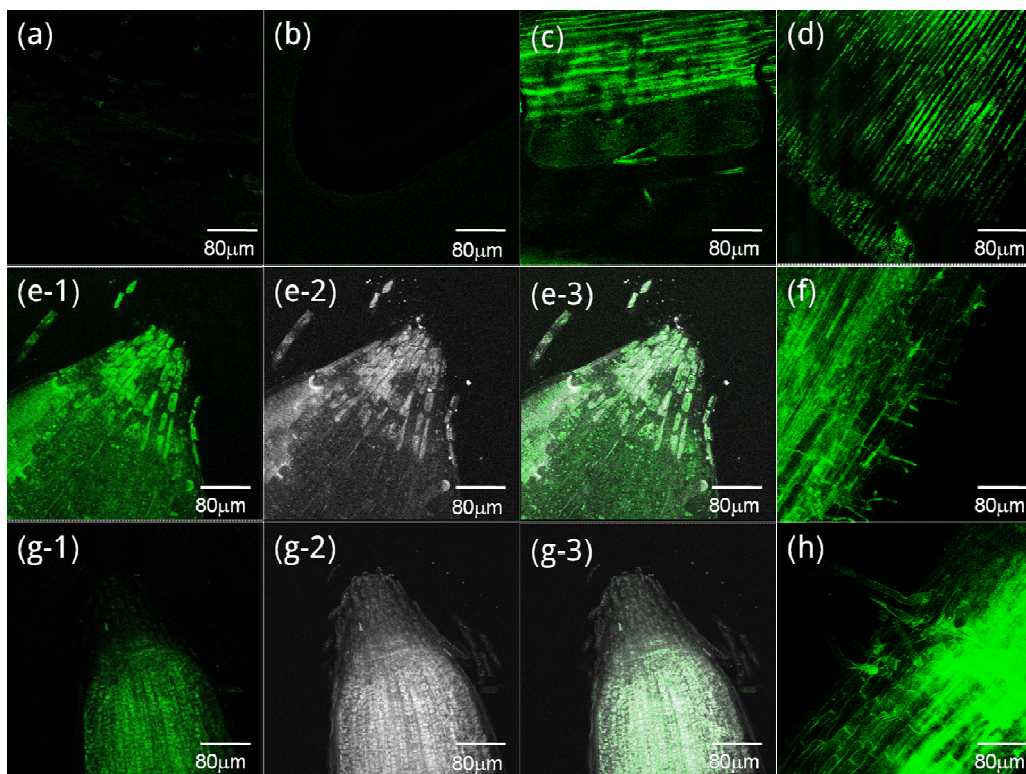


Fig. 3 Fluorescence images of **L1** in wheat leaves and roots. (a) Wheat leaf and (b) root staining with **L1** (10 μM) for 30min at room temperature. (c) Cd^{2+} treated leaf and (d) Zn^{2+} treated leaf for a week and then stained with **L1**(10 μM) for 30min. (e-1) Zn^{2+} treated root supplemented with **L1**, (e-2) brightfield image and (e-3) the overlay image of (e-1) and (e-2). (f) Root hair of Zn^{2+} treated root. (g-1) Cd^{2+} treated root supplemented with **L1**, (g-2) brightfield image and (g-3) overlay image of (g-1) and (g-2). (h) Root hair of Cd^{2+} treated root.

3.5. Measurement of cell viability

Furthermore, in order to evaluate toxic effects of probe **L1**, cytotoxicity test was performed in HBL-100 normal breast cells at different concentrations (5, 10, 25, 50 μM) of probe **L1** respectively. The absorbance of cells displayed that the cells viability was more than 80% (Fig. 4) at 570 nm. This experimental result demonstrates that probe **L1** is of low cytotoxicity to cultivate cells under the concentration from 5 μM to 25 μM which can be used in bioapplication.

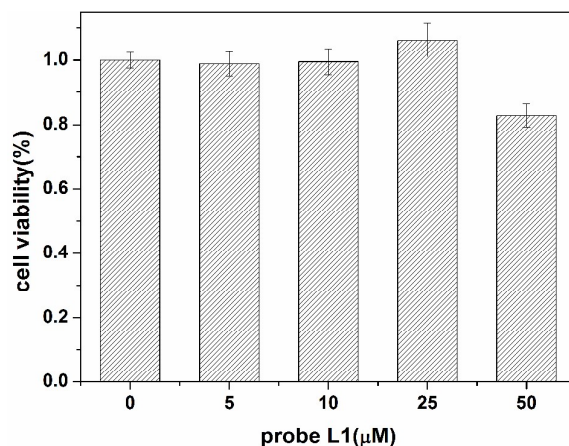


Fig. 4 HBL-100 normal breast cells were cultured in culture media (DMEM, High Glucose) supplemented with 10% FBS (Fetal Bovine Serum) at 37°C in a humidified incubator which was provided with 5% CO₂ and 95% air. The cells were seeded into 96-well plates at a density of 4×10^3 cells per well in culture media, then 0, 5, 10, 25 and 50 μM probe **L1** were added respectively for 24 h. Then, 10 μM cell count kit-8 (CCK-8) was added into each hole and were cultured for 4 h. The absorbance of cells was measured by ELISA ($\lambda_{ex} = 570$ nm).

4. Conclusions

In summary, a new probe **L1** was synthesized and characterized, which exhibited high sensitivity and selectivity for zinc ions over other metal ions, especially Cd²⁺. Moreover, the well permeability and biocompatibility of **L1** made it easy to access the living cells including human cells and plant tissues, meanwhile detect the intracellular Zn²⁺ and Cd²⁺ in different position. We expect that the fluorescent probe **L1** would be a great help for monitoring Zn²⁺ in biological systems.

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Conflict of interest

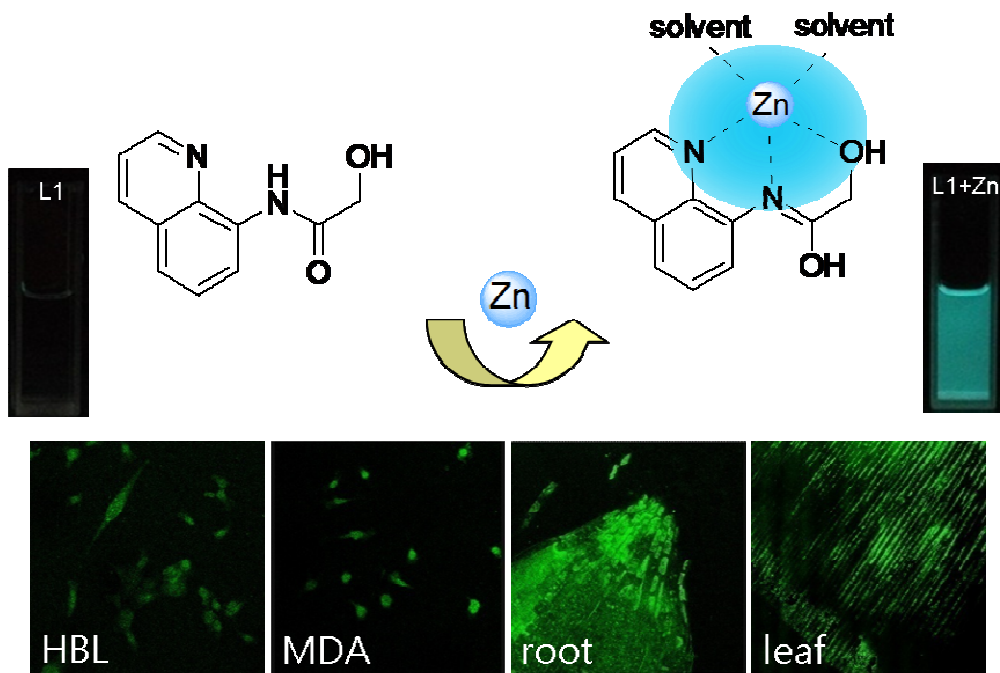
The authors declare that there are no conflicts of interest.

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Graphical abstract



The 8-aminoquinoline based probe exhibited high selectivity and sensitivity toward Zn^{2+} under a physiological pH. Moreover, its high cell permeability grants the access to employ L1 as Zn (II) detector in both human cells and plant tissues.