Inorganic Chemistry Communications 43 (2014) 173–178

Contents lists available at ScienceDirect

Inorganic Chemistry Communications

journal homepage: www.elsevier.com/locate/inoche

4-Amino-1,8-naphthalimide-based fluorescent sensor with high selectivity and sensitivity for Zn^{2+} imaging in living cells

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ARTICLE INFO

Article history:
Received 19 December 2013
Accepted 24 February 2014
Available online 28 February 2014

Keywords:
Fluorescent sensor
Naphthalimide
Iminodiacetic acid
Zinc

ABSTRACT

A new 4-amino-1,8-naphthalimide-based fluorescent sensor with iminodiacetic acid as receptor, was synthesized and characterized. Under physiological pH conditions, it demonstrates high selectivity and sensitivity for sensing Zn^{2+} with about 50-fold enhancement in fluorescence intensity. The fluorescent sensor exhibited a characteristic emission band of 4-amino-1,8-naphthalimide with a green color centered at ~550 nm and was successfully applied to image Zn^{2+} in living cells. Upon sensing of Zn^{2+} the fluorescence emission spectrum is "switched on" demonstrating the suppression of PET from the receptor to the fluorophore.

Zinc plays an important role in many biological and environmental processes. It is the second most abundant transition metal ions found in physiology, where it has multiple roles in both extra- and intracellular functions [1]. It is an essential element needed by human body and is commonly found in nutritional supplements. It is believed that disorder of zinc homeostasis is implicated in a number of diseases, such as Alzheimer's disease, cerebral ischemia, and epilepsy [2]. However, taking too much zinc into the body can affect your health. Therefore, there is a great need for methods of detecting and monitoring zinc levels in medicine and biology as well as in environment. Currently, there is great interest in the development of fluorescent sensors for quantifying and exploring the role of Zn^{2+} in various aspects because of their simplicity, high sensitivity, excellent selectivity and real-time detection [3]. However, improvements are needed to overcome several limitations when they were applied to detect zinc in biological samples. First, most of reported sensors need to be excited by UV light, which can cause damage to living cells [4]. Second, some of reported sensors need to be measured in organic solvent or mixed organic solvent [5]. Third, a few reported sensors have small Stokes shifts. Furthermore, these sensors often involve lengthy and cumbersome synthesis [3]. So far, there is no 4-amino-1,8-naphthalimide-based fluorescent sensor available to be applied in living cells.

Herein, we report new, simple and practical 4-amino-1,8-naphthalimide-PET-based fluorescent sensors with iminodiacetic acid as a receptor, which is able to sense Zn^{2+} with high selectivity and sensitivity under physiological pH conditions. And it was successfully applied to image Zn^{2+} in living cells.

Photo-induced electron transfer (PET) is an electron transfer which occurs when certain photoactive materials interact with light. The general design of a PET-type fluoroionophore is the "fluorophore–spacer–receptor (ionophore)" format. A fluorescent moiety (fluorophore) is covalently linked to an ion receptor by means of a non-π-electron-conjugating spacer group, e.g. arkyl group with one to four carbons. Typically, the ionophore will contain a tertiary amine; the electrons of which can ligate the cation. In the absence of a bound cation, the HOMO (highest occupied molecular orbital) of the unbound receptor has a higher energy than the half-filled HOMO of the excited fluorophore. This energy difference drives rapid electron transfer from the receptor to the excited-state fluorophore, thus the fluorescence is quenched, or "switched off". However, when the ionophore is bound to a cation, the energy level of the receptor's electron pair is lower than that of the HOMO of the excited fluorophore. As a result, the ionophore is stabilized energetically, the electron transfer is not favored, and thus, fluorescence is "switched on" [6].

Therefore, we chose to use 4-amino-1,8-naphthalimide as the fluorophore reporter in designing Sensor Zn, as it absorbs in the visible region (λ ~ 470 nm), emits in the green (λ ~ 550 nm), with Stokes shifts of ca. 80 nm, and possesses high fluorescence quantum yield and excellent fluorescence enhancement based on photo-induced electron transfer (PET) [6,7], as well as being photo-stable, in comparison with those

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http://dx.doi.org/10.1016/j.inoche.2014.02.035
1387-7003/

conventional fluorophores such as fluorescein, rhodamine, coumarins and BODIPY, which had only survived for several weeks, stored in the pH 7.4 HEPES at 31 °C [8] and had importantly, low sensitivity to pH because of the absence of ionizable functional groups within the physiological pH range. Thanks to the powerful electron-withdrawing property of the diimide moiety, the pKa of the amino group in the 4-amino-1,8-naphthalimide was found to be around 2.5, much lower than 4.5 for the unsubstituted naphthylamine. This makes the fluorophore very insensitive to the pH near the range of physiological pH, whose pH value varies typically between 7.34 and 7.45 [9]. Based on above-mentioned considerations, 4-amino-1,8-naphthalimide was selected rationally as the fluorophore.

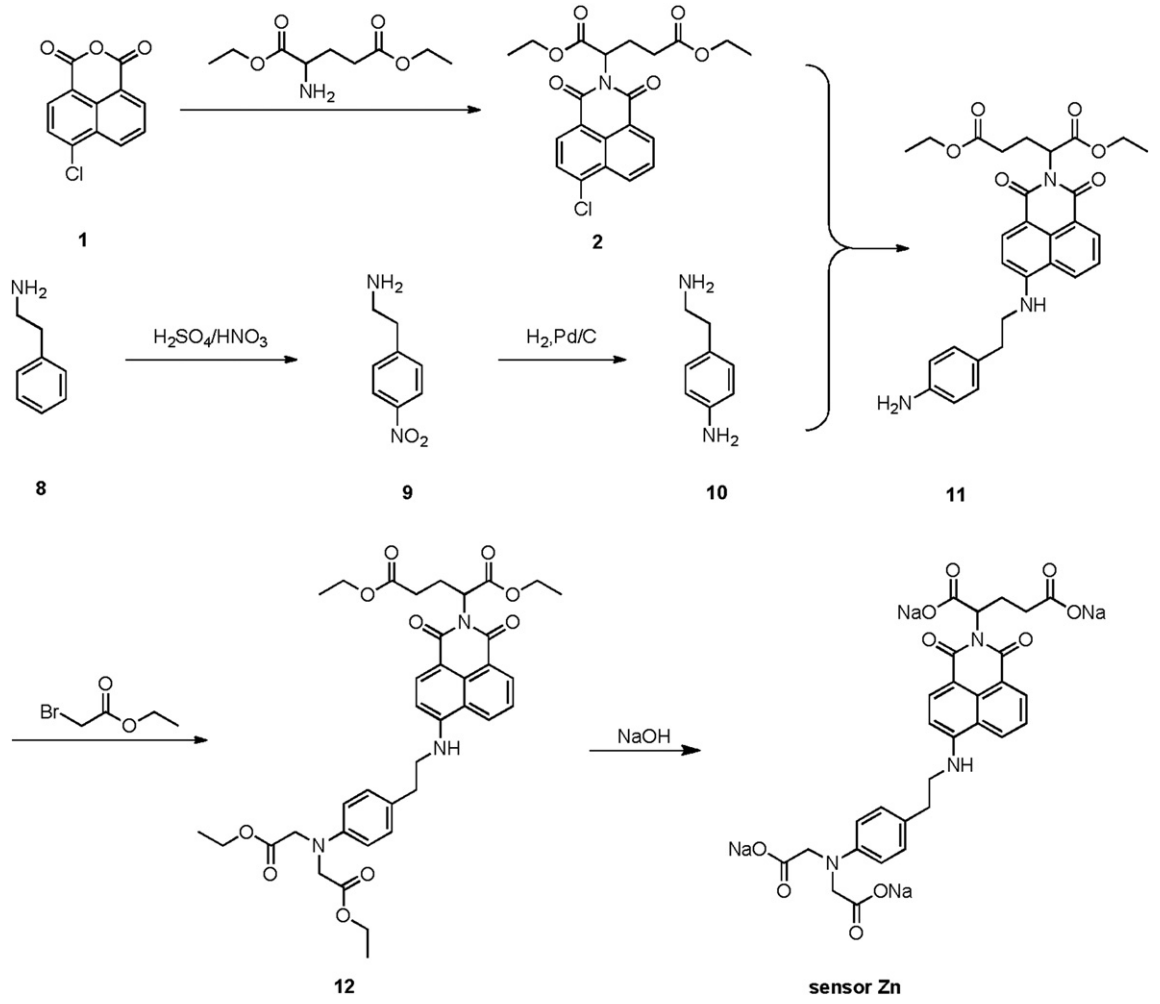
The introduction of glutamate greatly increased the solubility of the sensor in water and helped retain the sensor inside of the cell. Before staining the cell, the glutamate is kept as diester form, which is very lipid soluble and diffuses readily across the lipophilic cell membranes. Two ester groups are hydrolyzed inside of the cell by esterase, and the resulting glutamate anions can dissolve easily in water under physiological pH conditions, and retain inside of the stained cell for a long time.

The selection of iminodiacetic ionophore was driven by several design criteria: (A) must contain tertiary nitrogen that can act as an electron donor and will also interact with a bound zinc cation; (B) binding properties should be insensitive to pH changes in the physiological pH range of 7.34–7.45 so as to minimize undesirable pH interference to the measurement of Zn^{2+}; (C) must possess an adequate chemostability during the wet storage at room temperature; (D) should preferentially bind zinc with a dissociation constant (Kd) in the aqueous medium near the desired measuring range of 1 fM in *E. coli* to almost 0.5 mM in mammalian cells [10]; and (E) must have short and convenient synthesis. Among the available ionophore groups, iminodiacetic acid moiety is selected naturally as the ionophore [11].

Now, we present our design and synthesis of a new 4-amino-1,8-naphthalimide-based fluorescent sensor with iminodiacetic acid as receptor. Scheme 1 explains the synthetic route of Sensor Zn. The detailed procedures and characterization of the new compounds are described in the Supporting Information.

To obtain an insight into the binding properties of Sensor Zn towards metal ions, we investigated absorption and fluorescence changes upon the addition of a wide range of metal ions including Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, Ni^{2+}, La^{3+}, Eu^{3+}, and Er^{3+} in HEPES buffer solution (20 mM, pH = 7.4). The fluorescence changes of Sensor Zn are depicted in Fig. 1.

The addition of Zn^{2+} to aqueous solution of Sensor Zn bearing iminodiacetic acid as a metal chelating group caused a remarkable fluorescence enhancement, about 50-fold enhancement. By contrast, minor fluorescence enhancements were also observed upon the addition of Cd^{2+} and Ni^{2+}. No fluorescence spectral changes were observed with other metal ions. Although the addition of Cd^{2+} and Ni^{2+} also induced an emission enhancement to a certain extent, Cd^{2+} and Ni^{2+} which are the highly toxic metal ions appearing in vivo are very low concentrations



Scheme 1. Synthesis of Sensor Zn.

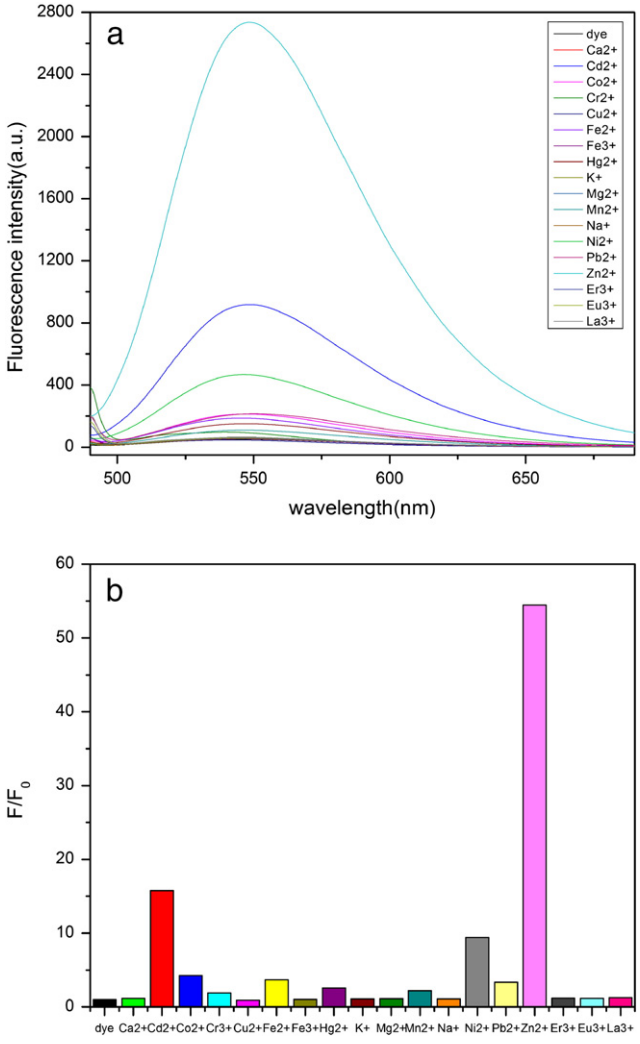


Fig. 1. (a) Fluorescence spectra of Sensor Zn (25 μM, $\lambda_{ex} = 470$ nm) in the presence of various metal ions in HEPES buffer (20 mM, pH = 7.4). (b) The relative fluorescence intensity of Sensor Zn (25 μM) in the presence of various metal ions: Na^+, K^+, Mg^{2+}, Ca^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+},Ni^{2+} and La^{3+}, Eu^{3+}, Er^{3+} in HEPES buffer (20 mM, pH = 7.4).

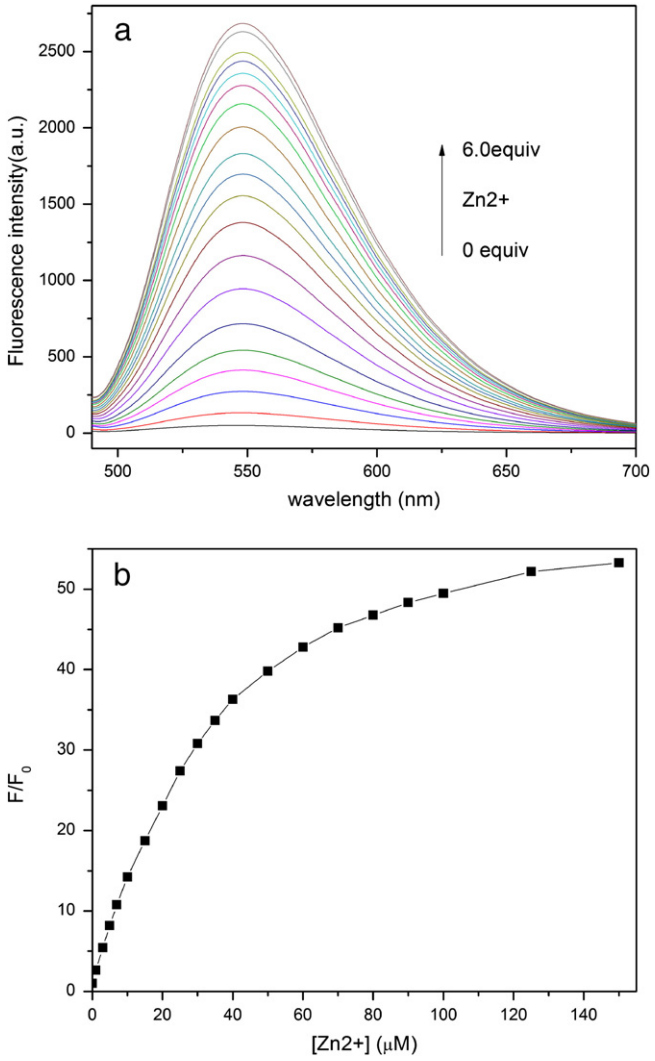


Fig. 2. (a) Fluorescence spectra of Sensor Zn (25 μM, $\lambda_{ex} = 470$ nm) in HEPES buffer (20 mM, pH = 7.4) in the presence of different concentration of Zn^{2+}. (b) The corresponding Zn^{2+} titration profile of the emission at 550 nm.

[5(c), 12]. This small interference does not affect the application of this sensor in living cell.

The fluorescence changes of Sensor Zn (25 μM) in response to increasing Zn^{2+} concentration were measured in 20 mM HEPES buffer (Fig. 2). The fluorescence emission intensity of Sensor Zn gradually increased and became saturated when 6.0 equiv. of Zn^{2+} was added to Sensor Zn. The dissociation constant (Kd) between Sensor Zn and Zn^{2+} was measured to be 2.4×10^{-5} M by fluorescence titration curve fitting. These results clearly demonstrate that the Sensor Zn has excellent affinity for Zn^{2+} over other metal ions.

In order to further clarify the binding property of Sensor Zn–Zn^{2+} in solution, ^{1}H NMR titrations were carried out and the results are shown in Fig. 3. Upon the addition of Zn^{2+} (from 0.0 equiv. to 2.0 equiv.) to the DMSO-d6 solution of Sensor Zn, the peak assigned to the proton of the H_a moiety was gradually broadened and gotten lower, shifted downfield from $\delta = 3.909$ to 7.001 ppm in the end. And moreover, the peak assigned to the proton of the H_b moiety was also shifted downfield from $\delta = 6.361$ to 7.001 ppm.

To further illustrate the binding property of Sensor Zn–Zn^{2+} in solution, MS of Sensor Zn–Zn^{2+} was carried out and the results are exhibited in Fig. 5. MS (+ESI): Calc. for $M_1(-c)$, 577.17, Found, 576.2; Calc. for $M_2(-c)$, 675.10, Found, 674.0 (chemical structures of M_1 and M_2 are shown below, in Fig. 4).

Therefore, based the above ^{1}H NMR titrations and MS data, we show the proposed binding mechanism of the Sensor Zn with Zn^{2+} (see Fig. 6).

Photo-induced electron transfer (PET) can be explained more clearly by using this real example shown in Fig. 7. In the absence of Zn^{2+}, the HOMO of the unbound iminodiacetic acid moiety has a higher energy than the half-filled HOMO of the excited 4-amino-1,8-naphthalimide. This energy difference drives rapid electron transfer from the iminodiacetic acid moiety to the excited-state 4-amino-1,8-naphthalimide, thus the fluorescence is quenched. Consequently, it shows weak fluorescence. However, when the iminodiacetic acid moiety is bound to Zn^{2+}, the energy level of the iminodiacetic acid moiety is lower than that of the HOMO of the excited 4-amino-1,8-naphthalimide, and the electron transfer is not energetically favored. The fluorescence is “switched on”.

To further demonstrate the practical biological application of Sensor Zn, fluorescence imaging experiments were carried out in living cells. Firstly, we had incorporated the MTT method to test the cytotoxicity of Sensor Zn, and we found that Sensor Zn doesn't have cytotoxicity under the concentration of 40 μM. As shown in Fig. 8, after being

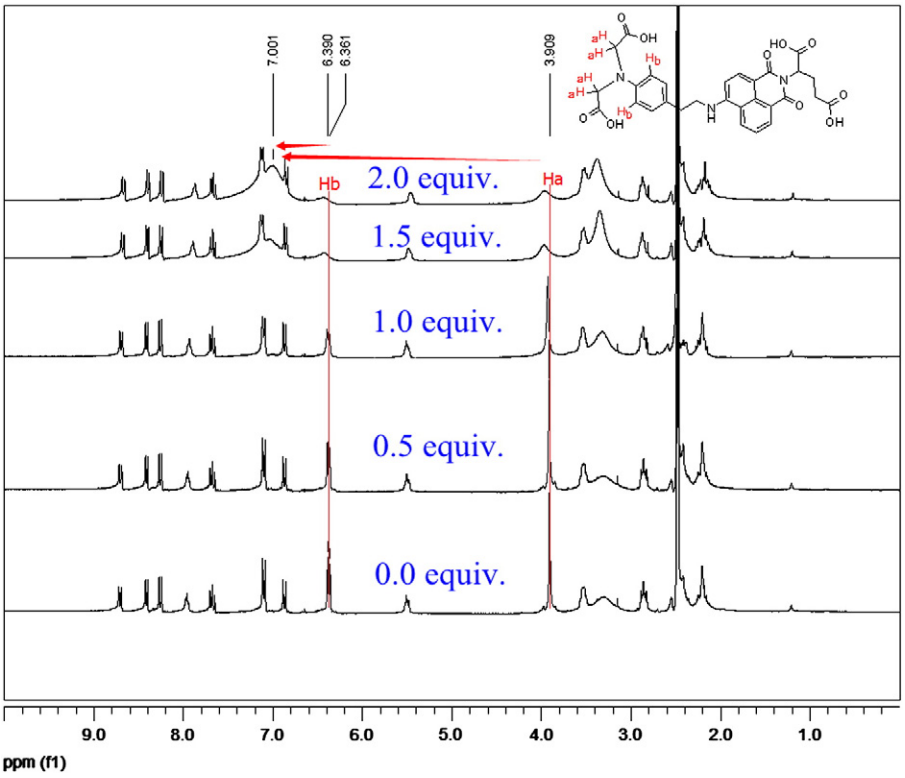


Fig. 3. ^{1}H NMR (300 MHz) spectra of Sensor Zn in DMSO-d6 with the addition of $ZnCl_2$.

incubated with Sensor Zn (0.2 mM) in the growth medium (1 mL) for 8 h at 37 °C and washed with phosphate buffered saline to remove excess Sensor Zn, HeLa cells displayed strong fluorescence under irradiation with blue light. The bright-field transmission and fluorescence images revealed that the fluorescence signal resulted from the intracellular region. The green fluorescence of the cells is similar to that of Sensor Zn in solution, which indicates that Sensor Zn is membrane permeable. These results suggest that Sensor Zn has good membrane permeability and can be used as a sensor for detecting Zn^{2+} in living cells.

In conclusion, a new fluorescent sensor for Zn^{2+} based on 4-amino-1,8-naphthalimide has been developed. The new fluorescent sensor demonstrated highly selective and sensitive binding affinity towards Zn^{2+} in HEPES buffer solution, and was applied successfully to image Zn^{2+} in living cells.

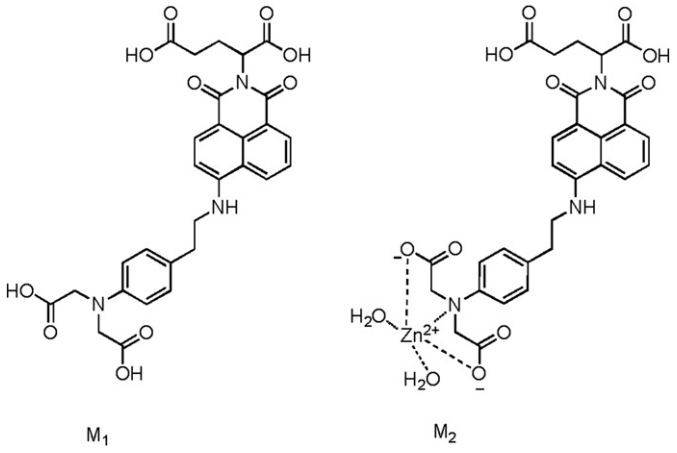


Fig. 4. Chemical structures of M_1 and M_2.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 20941004, 21071084 and 90922032), the MOE (IRT-0927), and the Tianjin Key Laboratory of Metal and Molecule Based Material Chemistry and Tianjin Natural Science Foundation (No. 11JCYBJC03500). The authors acknowledge the helpful discussions and collaboration from co-workers within Heowns BioChem Technologies LLC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.inoche.2014.02.035.

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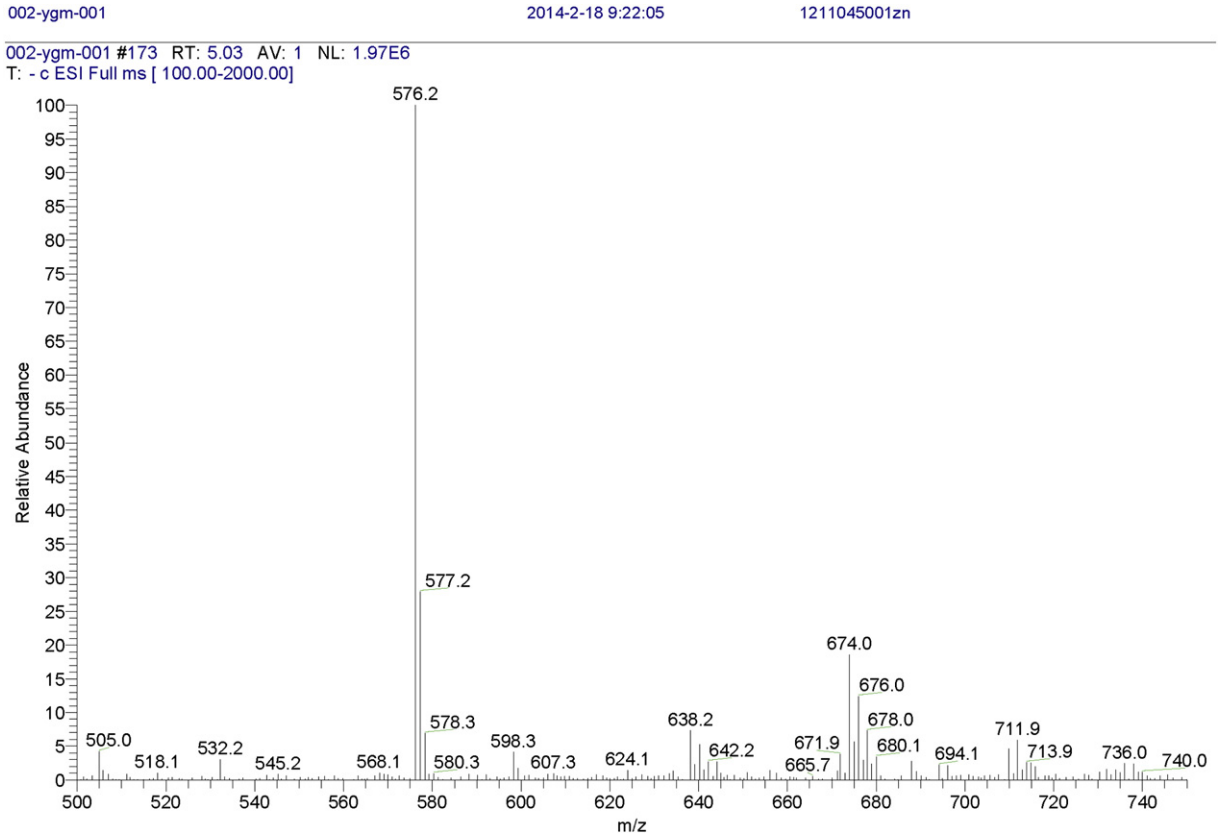

Fig. 5. MS of Sensor Zn–Zn^{2+}.

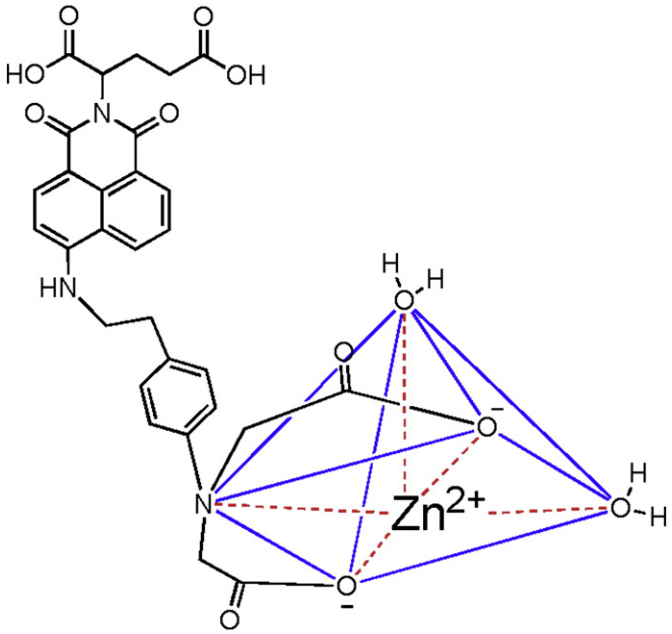

Fig. 6. Proposed binding mechanism of the Sensor Zn with Zn^{2+}.

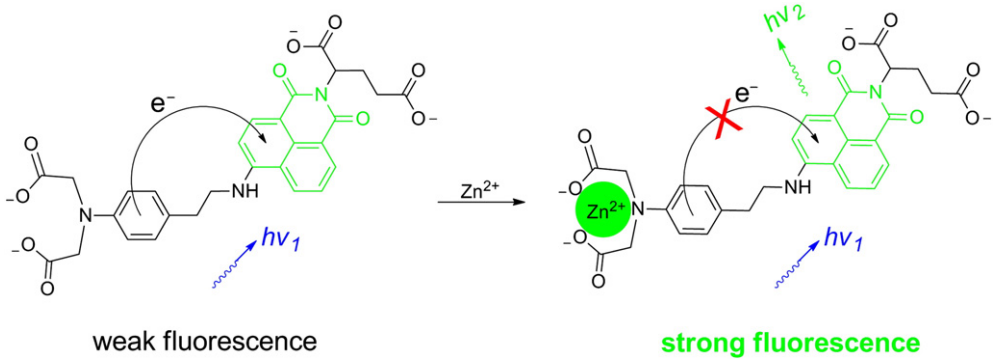

Fig. 7. PET mechanism of the Sensor Zn with Zn^{2+}.

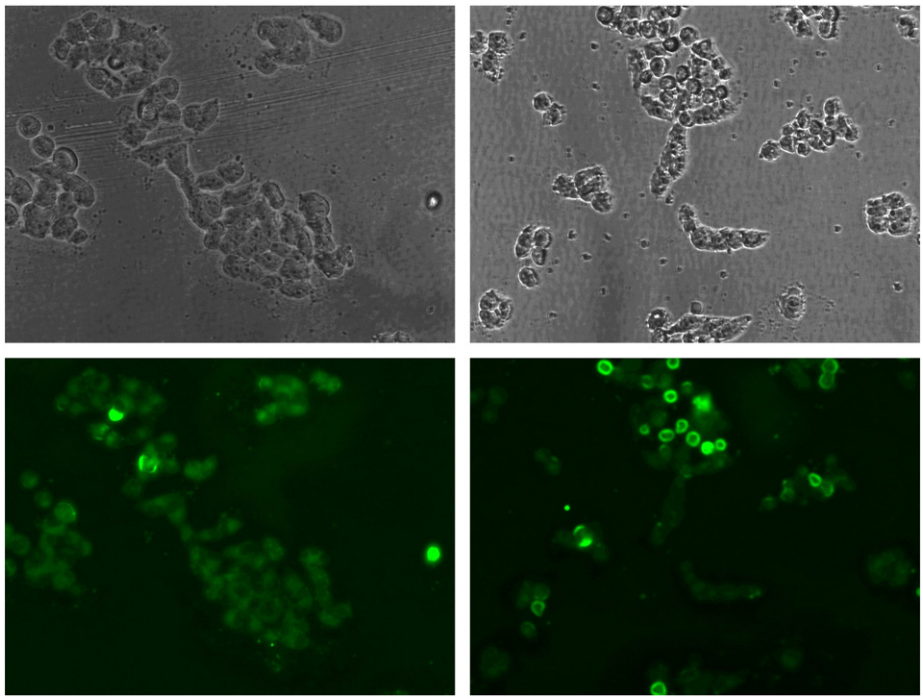

Fig. 8. Fluorescence images and their corresponding bright-field transmission images: HeLa cells were incubated with Sensor Zn (2 μM) in a fresh serum free medium for 8 h, then, incubated with TBAF (10 mM) in a fresh serum free medium for 0.5 h.

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