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"Turn-on" fluorescent probe detection of Ca^{2+} ions and applications to bioimaging

Huifang zhang[a,1], Caixia Yin[a,*], Tao Liu[a,1], Yongbin Zhang[b], Fangjun Huo[b,**]

[a]Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Key Laboratory of Materials for Energy Conversion and Storage of Shanxi Province, Institute of Molecular Science, Shanxi University, Taiyuan, 030006, China;[b]Research Institute of Applied Chemistry, Shanxi University, Taiyuan, 030006, China.

**Corresponding author: F.J. Huo, Email: huofj@sxu.edu.cn.

*Corresponding author: C.X. Yin, E-mail: yincx@sxu.edu.cn;

[1]Huifang zhang and Tao Liu contributed equally

Abstract: Ca^{2+} is intracellular divalent cation with the largest concentration variations and involved in many biological phenomena and often acted as a second messenger in signaling pathway. Therefore, the development of probes for specific Ca^{2+} detection is of great importance. Herein, a novel turn-on fluorescent probe for the detection of Ca^{2+} in MeCN-aqueous medium was designed and synthesized. The probe displayed responses to Ca^{2+} with a fluorescence enhancement at 525 nm, accompanying with a distinct fluorescence change from nearly colorless to bright yellow-green. Besides, the probe exhibited a rapid signal response time (within 25 s), a good linearity range and a lower detection limit (2.70×10^{-7} M). In addition, the ability of the probe to detect Ca^{2+} in living cells (HeLa cells) via an enhancement of the fluorescence has also been demonstrated.

Keywords: Ca^{2+}; Detection; Fluorescent probe; Bioimaging.

1. Introduction

Ca^{2+} is the intracellular divalent cation with the largest concentration variations and involved in many biological phenomena and often acted as a second messenger in signaling pathway.[1-3] For example, changes in the intracellular Ca^{2+} concentration are related to physiological responses in obesity, as well as immune responses and pathological responses in Alzheimer's disease.[4-9]

There are a few fluorescent probes for measuring the free intracellular Ca^{2+} concentration since the first introduction of small-molecule fluorescent indicators by Tsien and colleagues in the mid-eighties.[4,10-17] Draw from the data collection and analysis, fluorescent Ca^{2+} probes can fall into two main categories: as genetically encoded fluorescent proteins for fulfilling the roles of physiological mechanisms including cardiac function, cell signaling and neuronal network activity[18-19] and as fluorescent small organic molecules for bulk-loading into live cells with no need for transfection and non-disruptive loading at the same time.[20-23]

Most currently used small-molecular fluorescent probes for Ca^{2+} include fluorescein derivatives, such as Fluo-3, Fluo-4, Calcium Green-1, Oregon Green 488 BAPTA-1 and rhodamine derivative, such as Rhod-2.[23-25] In this contribution, probe (NR) was successfully synthesized starting from the reported 1,8-naphthalimide-based R1[26] and rhodamine derivative as a novel fluorescent probe for Ca^{2+}.

Herein, we have presented the synthesis and spectral properties of probe NR. The probe exhibited obvious changes in both UV-vis spectra and fluorescence emission spectra. Moreover, the sensitivity and selectivity of NR for the detection of Ca^{2+} in the

presence of other competing species were established. The compatibility and fluorescence characteristics of NR in physiologically relevant conditions were exploited for the visualization of living cells exposed to Ca^{2+} ions.

2. Materials and methods

2.1. Instruments and materials

All solvents and reagents for synthesis and analyses were of analytical grade and bought from Sigma-Aldrich (St. Louis, MO) without further purification. The solutions of cation were prepared from their chloride salts. Fresh double distilled water was used throughout the experiment.

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-Visible spectrophotometer and fluorescence spectra were measured on F-7000 FL fluorescence spectrophotometer. A PO-120 quartz cuvette (10 mm) was purchased from Shanghai Huamei Experiment Instrument Plants, China. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III-600 MHz and 150 MHz NMR spectrometer (Bruker, Billerica, MA) respectively and chemical shifts are reported in ppm relative to the internal standard TMS. ESI-MS was carried out on AB Triple TOF 5600plus System (AB SCIEX, Framingham, USA). The ability of probe reacting to Ca^{2+} in the living cells was also evaluated using Leica DMi8 Microsystems.

2.2. Preparation and characterization of the probe NR

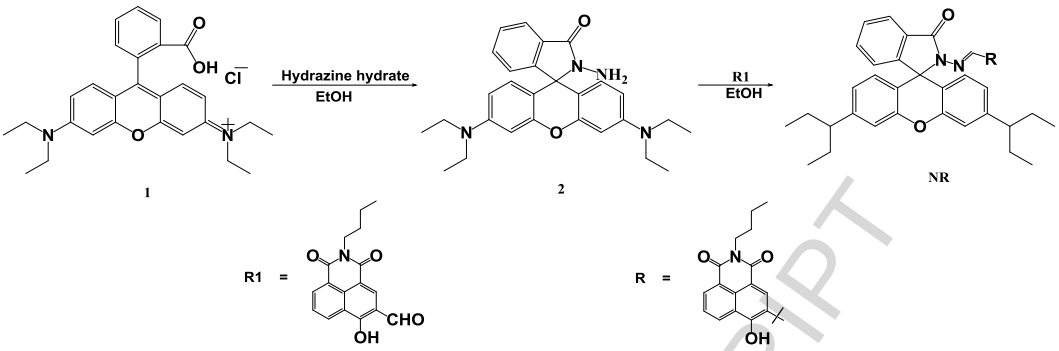
2.2.1. Preparation and characterization of compound 2

The synthesis approach of probe is summarized in Scheme 1. To a solution of rhodamine B (0.96 g, 2 mmol) in ethanol, $N_2H_4 \cdot H_2O$ (80%, 15mmol) was dropwise added with refluxing at 70°C for 5h. When cooled to room temperature, the mixture was filtered, washed and recrystallized with ethanol. The pale pink solid thus obtained and dried under vacuum to give compound 2 (0.85 g) in 93% yield. ^{1}H NMR ($CDCl_3$-d_1, 600 MHz): δ (ppm): 7.96 (d, J = 7.6 Hz, 1H), 7.46 (d, J = 3.9 Hz, 2H), 7.12 (d, J = 6.0 Hz, 1H), 6.52 – 6.38 (m, 4H), 6.31 (d, J = 8.5 Hz, 2H), 3.63 (s, 2H), 3.36 (q, J = 6.9 Hz, 8H), 1.19 (t, J = 6.9 Hz, 12H). ^{13}C NMR ($CDCl_3$-d_1, 151 MHz): δ (ppm): 166.2, 153.9, 151.6, 148.9, 132.5, 130.1, 128.1, 123.9, 123.0, 108.0, 104.6, 98.0, 65.9, 44.44, 12.6 (Fig. S1).

2.2.2. Preparation and characterization of the probe NR

A mixture of R1 (0.28 g, 1 mmol) and compound 2 (0.46 g, 1 mmol) in ethanol (30 mL) was refluxed for 5 h. After the reaction was completed, the reaction mixture was filtered, washed with ethanol and dried to obtain a yellow solid product (0.72 g) in 98% yield. ^{1}H NMR ($CDCl_3$-d_1, 600 MHz) : δ (ppm): 13.55 (s, 1H), 8.72 – 8.63 (m, 2H), 8.58 (d, J = 7.2 Hz, 1H), 8.26 (s, 1H), 8.05 (d, J = 7.4 Hz, 1H), 7.68 (t, J = 7.8 Hz, 1H), 7.55 (d, t, J = 22.8, 7.1 Hz, 2H), 7.18 (d, J = 7.4 Hz, 1H), 6.63 – 6.48 (m, 4H), 6.29 (d, J = 8.3 Hz, 2H), 4.15 (t, J = 7.4 Hz, 2H), 3.36 (d, J = 6.7 Hz, 8H), 1.69 (d, t, J = 14.8, 7.5 Hz, 2H), 1.43 (d, t, J = 14.3, 7.2 Hz, 2H), 1.19 (t, J = 6.8 Hz, 12H), 0.97 (t, J = 7.3 Hz, 3H). ^{13}C NMR ($CDCl_3$-d_1, 151 MHz): δ (ppm): 164.6, 164.4, 161.7, 153.0, 149.3, 147.8, 134.2, 134.0, 132.5, 130.0, 129.3, 128.6, 127.9, 127.8, 125.9, 124.0, 123.7, 123.3, 122.3, 113.4, 108.3, 104.2, 98.2, 65.8, 44.4, 40.1, 30.2, 20.4, 13.9, 12.6

(Fig. S2). ESI–MS m/z: [Probe + H]$^+$ Calcd. for $C_{45}H_{45}N_5O_5$ 736.34, Found 736.36 (Fig. S3).



Scheme 1 The synthesis of the probe NR.

2.3. Solutions preparation and optical measurements

The optical spectra of the probe NR were measured in a mixture of HEPES: MeCN = 1:9 (v/v, pH =7.4) solution at room temperature. The stock solutions of probe was prepared in acetonitrile in the concentration of 0.4 mM, stock solution of various analytes metal ions, Fe^{3+}, Na^+, Zn^{2+}, K^+, Pb^{2+}, Al^{3+}, Hg^{2+}, Cd^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ag^+, Cr^{3+}, Co^{2+}, Ba^{2+}, Ni^{2+}, and Ca^{2+} are prepared in deionized water in the concentration of 20 mM. UV-vis or fluorescence spectra were recorded upon the addition of various analytes. And any changes in the fluorescence intensity are monitored using a fluorescence spectrometer (λ_{em} = 525 nm, λ_{ex} = 404 nm, slit: 5 nm/5 nm). Statistical analysis of the data was carried out using Origin 8.6.

2.4. Cell imaging experiments

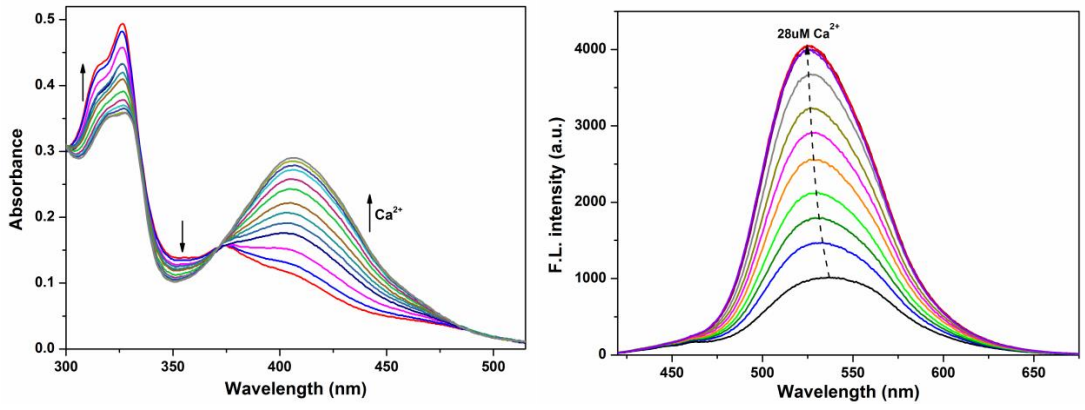
HeLa cells were cultured in 1× SPP medium (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA ferricsodium salt) at 37 °C. The cells were plated on 6-well plates and allowed to adhere for 12 h. Probe dissolved in MeCN (50 μL, 10 μM) was added to the cell medium (2 mL) at 10 μM final concentration.

After incubating for 30 min, excess probe was gently washed with phosphate buffered saline (PBS, pH=7.4) three times. Meanwhile, another portion of HeLa cells pre-treated with probe was treated with Ca^{2+} (30 μM) and incubated for further 30 min at 37 °C. Cell imaging was then carried out after washing cells with PBS buffer three times. Besides, fluorescence images were recorded at blue channel.

3. Results and discussion

3.1. Optical properties of the probe

To investigate the optical properties of the probe, the UV-vis absorption spectra and fluorescence spectra were both carried out. Fig. 1 (a) showed that the probe (10 μM) exhibited one absorption at 375 nm in the absence of Ca^{2+}, and with the addition of Ca^{2+} (0-28 μM), the absorption at 406 nm increased gradually. Accordingly, as shown in Fig. 1 (b), upon excitation at 404 nm, the probe (10 μM) displays a weak emission band centered at 537 nm. Addition of Ca^{2+} (0-28 μM) significantly increases the fluorescence intensity with a blue-shift centered at 525 nm—a 4.5-fold increase in fluorescence was observed.



(a) (b)

Fig. 1 (a) UV-vis spectra of the probe (10 μM) on the addition of Ca^{2+} (0-28 μM) to the HEPES: MeCN = 1:9 (v/v, pH =7.4) solution. (b) Fluorescence spectral change of the probe (10 μM) upon addition of Ca^{2+} (0-28 μM) in HEPES: MeCN = 1:9 (v/v, pH =7.4) solution (λ_{ex} = 404 nm, λ_{em} =525 nm, slit: 5 nm/5 nm).

From the changes in Ca^{2+}-dependent fluorescence intensity, the detection limit is estimated to be 2.70×10^{-7} M when the fluorescence emission intensity was plotted as a function of the Ca^{2+} concentration (Fig. 2).[27-33] The nonlinear curve fitness based on 1:1 complex expression, where F and F_0 are the fluorescence intensity of NR in the presence and absence of Ca^{2+}, F_{max} is the final fluorescence intensity in titration experiment, K_s is the stability constant (Equation (1)). The stability constant K_s is calculated as 2.05×10^5 M^{-1} (R^2 = 0.995) for NR-Ca^{2+} by using a nonlinear least-square analysis (Fig. 3).[34-35] The stability constant K for NR-Ca^{2+} calculated from the plots of $A_0/(A-A_0)$ versus NR-Ca^{2+} based on the standard Benesi-Hildebrand method is 1.30×10^5 M^{-1} (R^2 = 0.976) (Fig. S4), and this value is similar to above Ks from fluorescence titration experiments.[36]

$$F = F_0 + \frac{F_{max} - F_0}{1 + exp\,(-lnK_s - ln[Ca^{2+}])} \qquad \text{(Equation (1))}$$

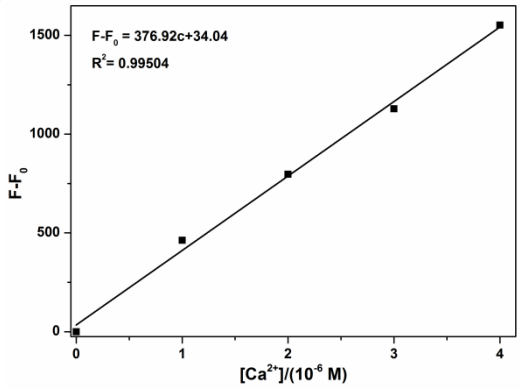


Fig. 2 Plot of emission intensity at 525 nm of probe as a function of Ca^{2+} concentration in HEPES: MeCN = 1:9 (v/v, pH =7.4) solution.

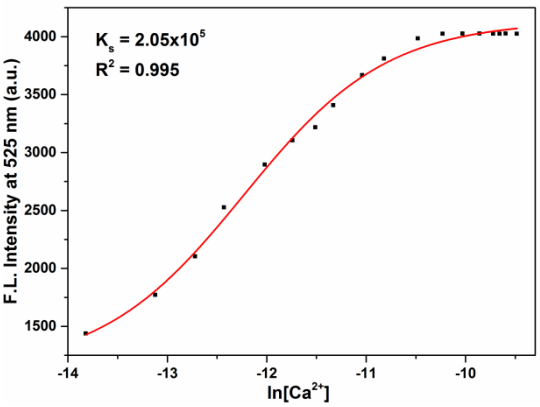


Fig.3 A nonlinear least-square analysis of a 1:1 complex of NR and Ca^{2+}.

It is notable that a 1: 1 complexation stoichiometry for NR-Ca^{2+} was established through a Job's plot analysis at 525 nm, where the products between molar fractions and the intensity of the fluorescence emission were plotted against molar fractions of NR under the condition of a constant total concentration. When the molar fraction of NR/(NR+[Ca^{2+}]) was 0.50, the emission intensity of NR-Ca^{2+} reached maximum (Fig. 4).[37-39]

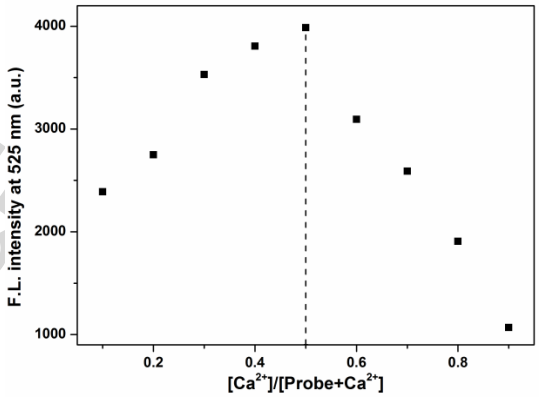


Fig.4 Job's plot for NR-Ca^{2+} complex at 525 nm in HEPES: MeCN = 1:9 (v/v, pH =7.4) solution. The total concentration of NR and Ca^{2+} is 40 μM.

3.2. The Selective response of probes to Ca^{2+}

The research of NR to the selective recognition of other cations (Fe^{3+}, Na^{+}, Zn^{2+}, K^{+}, Pb^{2+}, Al^{3+}, Hg^{2+}, Cd^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}, Cr^{3+},Co^{2+}, Ba^{2+},Ni^{2+} and Ca^{2+}) by

fluorescent spectra under HEPES: MeCN = 1:9 (v/v, pH = 7.4) was carried out (Fig. 5). The free probe exhibited a weak emission peak at 525 nm (λ_{ex}= 404 nm, λ_{em} =525 nm, slit: 5 nm/5 nm), and upon the addition of 20.0 equiv. of other cations, some fluorescence emission of the probe did not have obvious increase, while some cations turn off the fluorescence of the probe, such as (Fe^{3+}, Zn^{2+}, Al^{3+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+}). Whereas upon the addition of the same amount of Ca^{2+}, the emission peak at 525 nm enhanced remarkably (the intensity being about 4 times compared with that of free probe).

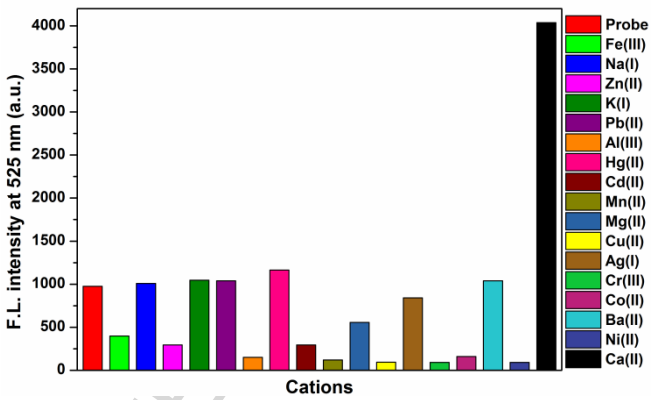


Fig. 5 Optical density two-dimensional graph of emission at 525 nm of the probe (10 μM) in the presence of 20.0 equiv. various metal ions for 5 min in the HEPES: MeCN = 1:9 (v/v, pH =7.4) solution (λ_{ex} = 404 nm, λ_{em} =525 nm, slit: 5 nm/5 nm) (From left to right: Fe^{3+}, Na^{+}, Zn^{2+}, K^{+}, Pb^{2+}, Al^{3+}, Hg^{2+}, Cd^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ag^{+}, Cr^{3+},Co^{2+}, Ba^{2+},Ni^{2+}, Ca^{2+}).

3.3. pH dependent of the probes

The effect of pH on the fluorescence intensity of probe in the absence and presence of Ca^{2+} was also investigated as the pH value of system is often considered as a significant influencing factor on interactions (Figure 6). Under pH range from 4 to 11, the emission intensity of probe showed a weak fluorescence and the addition of Ca^{2+} could induce the enhancement of the fluorescence, which showed that the fluorescence probe to Ca^{2+} works well during this pH range. The weaker response at

pH＜4 was presumably attributed to diminished concentration of the probe, because of the ring-open of the rhodamine. There is no response at pH＞11, which may results from the hydrolysis of C＝N. Thus, the physiological pH could be selected for the further research.

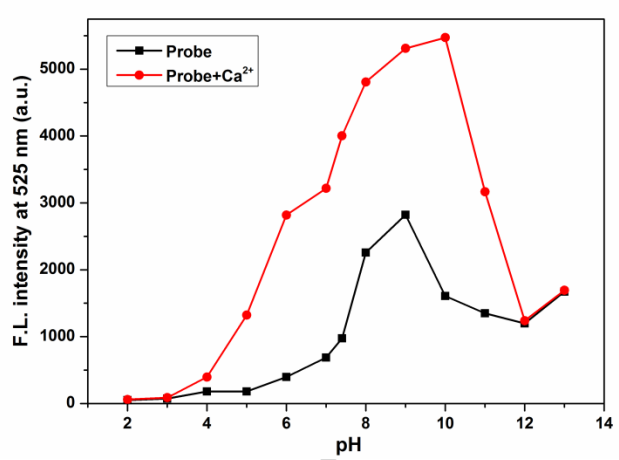


Fig. 6 Fluorescence emission at 525 nm of probe at different pH values in the absence or presence of Ca^{2+}.

3.4. The kinetic study in the detection process of Ca^{2+}

Time-dependent modulation in the fluorescence spectra of probe was monitored in the presence of 10 equiv. of Ca^{2+}. The kinetic study showed that the fluorescence emission at 525 nm was immediately initiated and leveled off within 25 s, displaying a rapid response between probe and Ca^{2+} under the selected experimental conditions (Fig. 7).

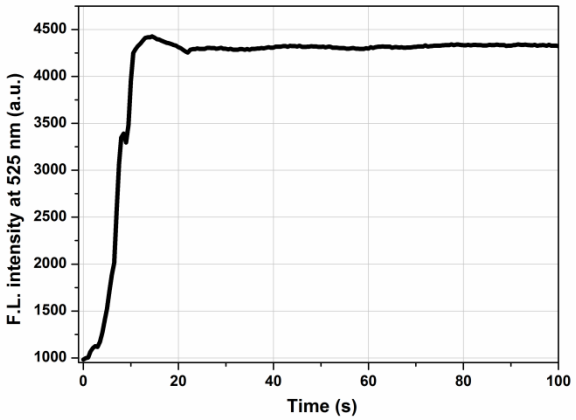
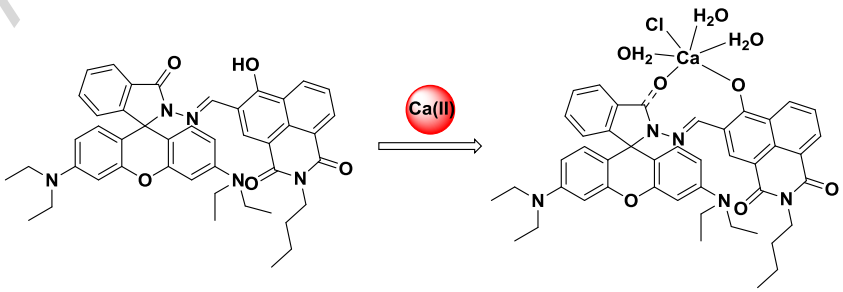


Fig. 7 Reaction time profiles of probe in the presence of Ca^{2+} in HEPES: MeCN = 1:9 (v/v, pH =7.4) solution.

3.5. Proposed mechanism

The reaction mechanism of the system was also studied. It was presumed that the turn-on fluorescence could be attributed to the coordination of probe with Ca^{2+}. The measured intensity F at 525 nm varied as a function of ln[Ca^{2+}] in a nonlinear relationship (R^2 =0.995), which indicated that the formation of 1:1 stoichiometry between Ca^{2+} and probe and the association constant of probe with Ca^{2+} was calculated to be 2.05×10^5 M^{-1} (Fig. 3). Moreover, according to a Job's plot analysis at 525 nm, when the molar fraction of NR/(NR+[Ca^{2+}]) was 0.50, the emission intensity of NR-Ca^{2+} reached maximum, which further demonstrated a 1: 1 complexation stoichiometry for NR-Ca^{2+} was established (Fig. 4). In addition, the identification of the coordination product in the ESI-MS analysis made it possible to propose the signaling mechanism: a peak at m/z = 863.24, corresponding to [probe-Ca + H_2O + Cl], is clearly observed (Fig. S5). ^{1}H NMR titration were carried out, results were showed in fig. S6. The addition of Ca^{2+} into probe will induce some protons to shift, such as H^b, H^c.[40-43] The proposed detection mechanism of Ca^{2+} was shown in Scheme 2.



Scheme 2 Proposed sensing process of the probe for Ca^{2+}

3.6. Imaging of living cell

It is known that cell imaging experiments could prove further application for fluorescent probes.[44-45] In order to evaluate the cell permeability and reaction of probe NR to Ca^{2+}, cellular imaging studies were also carried out (Fig. 8). As shown in Fig. 8b, HeLa cells incubated with 10 μM of NR for 30 min at 37 ℃ showed nearly no fluorescence. In a further experiment it was found that Hela cells displayed bright yellow-green fluorescence when the cells were first incubated with 10μM of NR for 30 min and then incubated with 30 μM Ca^{2+} for 30 min at 37 ℃ (Fig. 8e). These imaging experiments indicated that probe can be used to detect Ca^{2+} in imaging modality.

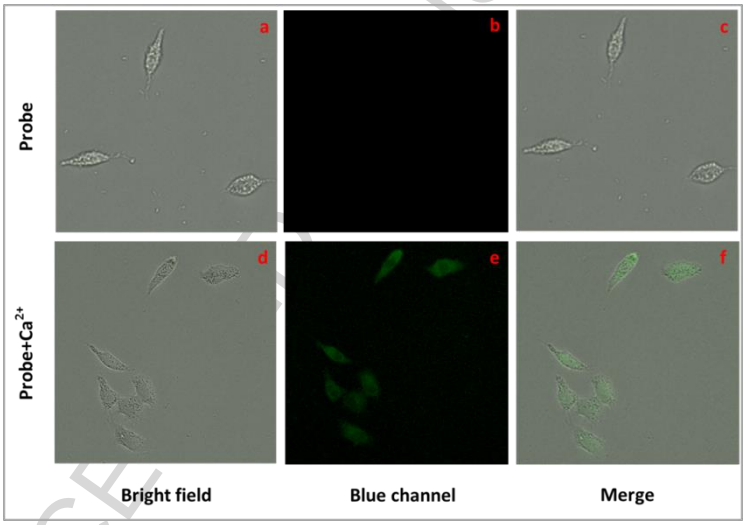


Fig. 8 Fluorescence images in HeLa cells. Fluorescence image of HeLa cells incubated with probe (10 μM) for 30 min at 37 °C (a-c); Cells pretreated with probe and then incubated with 30 μM Ca^{2+} for 30 min at 37 °C (d-f).

4. Conclusions

In this study, a novel fluorescent probe based on 1, 8-naphthalimide and rhodamine derivative for the detection of Ca^{2+} has been developed, which displayed highly selective and sensitive fluorescence enhancement and color change upon the addition of Ca^{2+} over other metal ions. The experiment also showed response

mechanism towards Ca^{2+} as a 1: 1 binding mode at MeCN-aqueous media. Moreover, the probe showed a rapid detection process (within 25 s), a good linearity range and a low detection limit of Ca^{2+} (2.70×10^{-7} M). The fluorescence scanning microscopic experiments demonstrated that the probe can be used to detect intracellular Ca^{2+} in living HeLa cells.

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Abstract Graphic

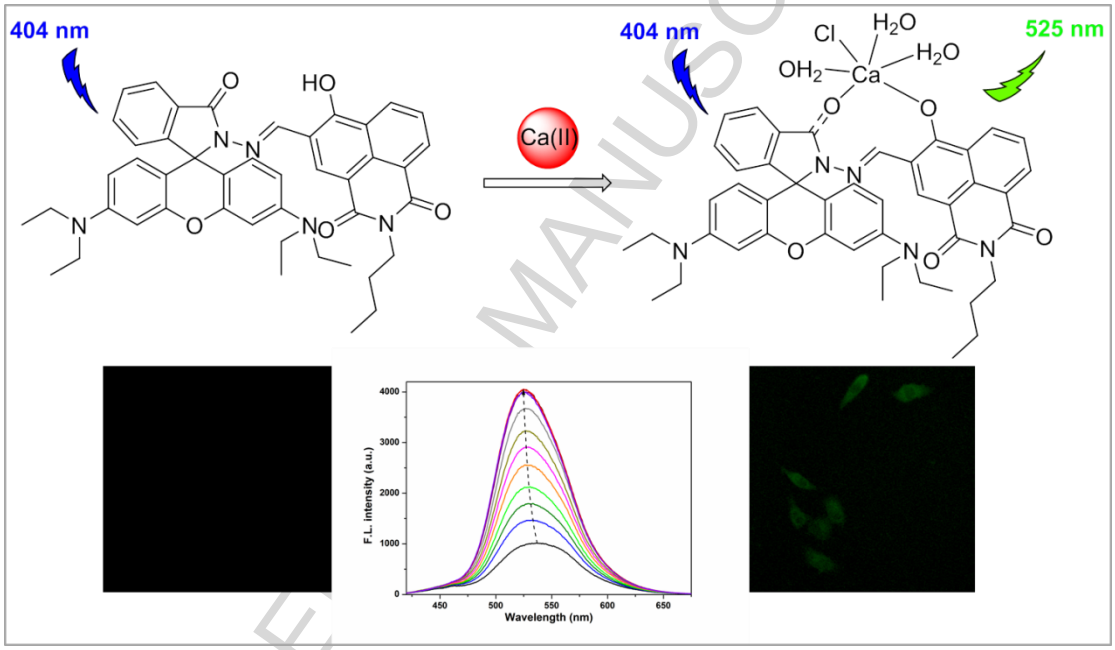
The Ttitle:

"Turn-on" fluorescent probe detection of Ca^{2+} ions and applications to bioimaging

List of Authors:

Huifang zhang[a,1], Caixia Yin[a,*], Tao Liu[a,1], Yongbin Zhang[b], Fangjun Huo[b,**]

The statement:



Based on the coordination reaction between probe and Ca^{2+} under mild conditions, we herein report a turn-on fluorescent probe which features a rapid signal response time (within 25 s), a good linearity range and a low detection limit (2.70×10^{-7} M). The application of this fluorescent probe was demonstrated by fluorescent imaging in HeLa cells.

Highlight

a) The compound is a novel fluorescent probe for the detection of Ca^{2+}.

b) The probe exhibited rapid detection time (within 25 s) and low detection limit: 2.70×10^{-7} M.

c) The ability of the probe to detect Ca^{2+} in living cells (HeLa cells) was also illustrated.