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ARTICLE TYPE

Rhodamine-based Fluorescent off/on Sensor for Fe³⁺ in Aqueous Solution and in Living Cells: 8-Aminoquinoline receptor and 2:1 binding

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8-aminoquinoline (8-AQ) and 2-aminopyridine (AmPyr) both as ionphoric agents were robustly used on development of cation sensor. To evaluate the effect of complexation with cations, flexible 2-aminopyridine and rigid 8-AQ moiety were directly introduced into rhodamine chromophore and two candidate fluorescent sensors were constructed successfully. **Rh-AQ** with a rigid 8-AQ fragment exhibited turn-on fluorescence and color responses to Fe³⁺ ion over other metal ions related to biology in aqueous solution. The sensor **Rh-AmPyr** with a flexible 2-aminopyridine did not give positive responses to cations although with a similar binding cave as on **Rh-AQ**. Furthermore, the 2:1 recognition mode of **Rh-AQ** with Fe³⁺ was proved according to the 1D and 2D COSY H-H NMR experiments. The live cells imaging experiments demonstrated that **Rh-AQ** could be successfully applied as a bioimaging agent for monitoring Fe³⁺ in living cells.

Introduction

Iron with chemical versatility is essential for the proper function of numerous biological systems in all organisms including bacteria, plants and mammals and in the whole of the biological space and time [1,2]. Iron overload in a living cell can lead to generation of reactive oxygen species (ROS) via the Fenton reaction, which can cause damage to lipids, nucleic acids, and proteins. The cellular toxicity of iron ions has been connected with serious diseases, including Alzheimer's, Huntington's, and Parkinson's disease [1b,3]. Therefore mapping of iron in normal cell or distorted cell will help us to discover the therapy strategies and to promote drug development [4-5].

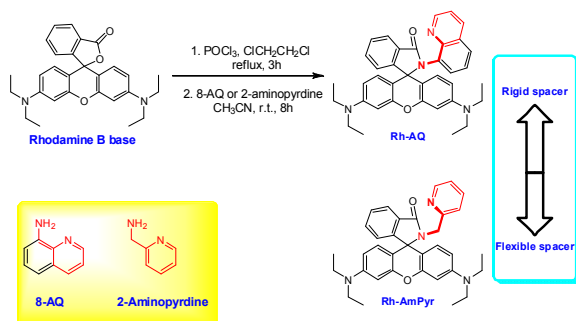
Optical fluorescent imaging is a major and powerful tool for studying localization, trafficking and expression levels of biomolecules and metal ions inside living cells by using confocal or common fluorescent microscopy [6]. Highly selective and sensitive fluorescent chemosensors for metal ions, such as calcium [7], zinc [8], copper [9] and mercury [10], have been developed for assessing their biological functions and locating in living system. Surprisingly, Fe³⁺-specific fluorescent sensors are still rare now. Even then, lots of reported Fe³⁺ sensors are fluorescent quenching due to the paramagnetic nature of ferric ion [11]. Recently, the fluorescence "turn on" probes for Fe³⁺ are attracting increasing attention since the "off-on" type signal is superior compared to "on-off" type due to their better overall signal processing in biosystems and specificity of mode of action. Up to now, although many fluorescent-amplified Fe³⁺ sensors have been reported [12], most of them do not meet the requirements for

applications in living cells due to the low selectivity, sensitivity and non-biocompatibility. To the best of our knowledge, only few of turned-on fluorescent Fe³⁺ sensors was involved in cell bioimaging [12d,l,m]. Therefore, the development of new fluorescent probes with high selective Fe³⁺-amplified emission and good bio-compatibility for imaging *in vivo* is still a challenge.

Rhodamine chromophore has been used extensively for fluorescent labelling owing to its unique properties such as long-wavelength of excitation (more than 550nm) and emission (590 nm), good bio-availability, large absorption coefficient, and high fluorescent quantum yields [13]. Recently, rhodamine-based fluorescent chemosensors have received increasing interest and are designed to sense metal ions, such as Pb²⁺ [13f], Cu²⁺ [13g-j], Hg²⁺ [13a-e], Fe³⁺ [12a-d]. The recognition mechanism is based on the switch on/off of the spirocyclic moiety mediated by guests. When guest bounds to the rhodamine-based fluorescent sensor, the colorless and non-fluorescent spirocyclic form of rhodamine amide converts to the open form, which is pink and strongly fluorescent [13, 14].

8-aminoquinoline (8-AQ) [8] and 2-aminopyridine [12e] and their derivatives have been used traditionally as ionphoric agents or binding receptors/ligands for the quantitative chemical assay of Zn²⁺, Cu²⁺ and other metal ions [8d]. For example, Lipard's group [8a] (combined Aldehyde-fluorescein and 8-AQ), Qian's group [8c] (combined an alkoxyethylamino chain to 8-AQ) and Liu's group (constructed a 8-aminoquinolino-β-cyclodextrin/1-adamantaneacetic acid (1/ADA) system) have developed the Zn²⁺ ions sensor used 8-AQ as the fluoroionphore. Also, 2-aminopyridine was introduced to the rhodamine skeleton and developed a HTM cations sensor [8e]. With these considerations in

mind, we integrated flexible 2-aminopyridine and rigid 8-AQ moiety to Rhodamine to obtain target molecule **Rh-AmPyr**, **Rh-AQ**^[15] (Scheme 1) and to evaluate the effect of flexible and rigid spacer on the complexation with cations.



Scheme 1. Synthesis of **Rh-AQ** and **Rh-AmPyr**.

Experimental

General

All the reagents and solvents were commercially available and used without further purification. ¹H-NMR spectra were collected in CDCl₃ or CD₃CN at 25°C on a Bruker AV-400 spectrometer. Electrospray ionization (ESI) analyses were performed in Mass Instrumentation Facility of Analysis&Research Centre of ECUST. UV-vis spectra were obtained using a Varian Cary 500 spectrophotometer (1 cm quartz cell) at 25°C. Melting points were determined by using a Büchi Melting point B-540 apparatus (uncorrected when using). pH was measured with a Sartorius basic pH-Meter PB-10.

The metal salts employed were LiClO₄, NaClO₄, KClO₄, Mg(ClO₄)₂, Cd(ClO₄)₂·6H₂O, Hg(ClO₄)₂·3H₂O, Mg(ClO₄)₂, Fe(NO₃)₃·3H₂O, FeSO₄·7H₂O, Cr(ClO₄)₃·6H₂O, Zn(ClO₄)₂·6H₂O, AgClO₄·H₂O, Co(ClO₄)₂·6H₂O, Mn(ClO₄)₂·6H₂O, Cu(ClO₄)₂·6H₂O, Ni(ClO₄)₂·6H₂O, and Pb(ClO₄)₂·3H₂O, respectively. Metal ions and sensors were dissolved in water or MeCN to obtain 1 mM stock solutions.

Syntheses and characterization

Rh-AmPyr and **Rh-AQ** were prepared according to the established literature procedure^[13,15].

To a solution of rhodamine B base (2.0 g, 4.6 mmol) in dry 1,2-dichloromethane (12 mL) was stirred, and phosphorus oxychloride (2.0 mL) was added dropwise at room temperature. The solution was refluxed for 4 h. The reaction mixture was cooled and evaporated in vacuo to give rhodamine B acid chloride, which was not purified and used directly for next step. The crude acid chloride was dissolved in dry MeCN (200 mL and divided into two 100 mL parts);

Synthesis of Rh-AmPyr: A part of 100 mL crude acid chloride in dry MeCN was added dropwise over 1 h to a solution of 2-aminopyridine (3 mL) in CH₃CN at room temperature. The reaction mixture was stirred overnight. After the solvent was evaporated under reduced pressure, the crude product was purified by column chromatography (DCM/MeOH,100/5, v/v) to give 477 mg of **Rh-AmPyr** (yield; 40%) as a slight pink solid: mp 101-102 °C; ¹H-NMR (CDCl₃, 400 MHz): δ 1.121 (12H, t, *J* =

7.6Hz), 3.28 (8H, q, *J* = 7.6Hz), 4.49(2H, s), 6.06(2H, dd, *J* = 8.8 Hz, *J*₂ = 2.8Hz), 6.26-6.28(4H, m), 6.82-6.85(1H, m), 7.06(1H, d, *J* = 8.0Hz), 7.10-7.13(1H, m), 7.26-7.29(1H, m), 7.44-7.48(2H, m), 7.97-7.99(1H, m), 8.17-8.19(1H, m). ¹³C-NMR(CDCl₃, 400 MHz): δ 12.62, 44.34, 45.60, 97.65, 105.36, 107.83, 120.97, 122.42, 122.80, 123.00, 123.94, 126.46, 128.08, 128.96, 131.41, 132.50, 135.56, 137.29, 148.28, 148.50, 153.10, 153.42, 157.68, 167.85. HRMS (ESI): Calcd for MH⁺, 533.2916; Found, 533.2900.

Synthesis of Rh-AQ: Another part of 100 mL crude acid chloride in MeCN was added dropwise over 1 h to a solution of 8-aminequinoline (1.0 g, 7.0 mmol) and TEA (3 mL) in MeCN at room temperature. The reaction mixture was stirred overnight. After the solvent was evaporated under reduced pressure, the crude product was purified by column chromatography (DCM/MeOH,100/3, v/v) to give 588 mg of **Rh-AQ** (yield: 45%) as a slight pink solid: mp 89-91 °C. ¹H-NMR (CD₃CN, 400 MHz): δ 1.10 (12H, t, *J* = 7.0Hz), 3.33 (8H, q, *J* = 7.0Hz), 6.162(2H, s), 6.38(2H, d, *J* = 8.0 Hz), 6.76(1H, dd, *J*₁ = 7.2Hz, *J*₂ = 1.2Hz), 6.93(1H, d, *J* = 8.8Hz), 7.17-7.19(1H, m), 7.33(1H, t, *J* = 8.8Hz), 7.40(1H, dd, *J*₁ = 8.0 Hz, *J*₂ = 4.0 Hz) 7.65-7.68(2H, m), 7.81(1H, dd, *J*₁ = 8.0 Hz, *J*₂ = 1.0Hz), 7.97-7.99(1H, m), 8.18(1H, dd, *J*₁ = 10.0Hz, *J*₂ = 1.6Hz), 8.75(1H, dd, *J*₁ = 4.0Hz, *J*₂ = 1.8Hz). ¹³C-NMR(CDCl₃, 400 MHz): δ 11.71, 43.92, 67.83, 96.78, 107.75, 121.42, 122.76, 13.91, 125.67, 128.12, 128.23, 128.45, 128.78, 130.40, 131.65, 132.83, 135.13, 135.80, 144.39, 148.79, 149.70, 153.15, 153.83, 166.98. HRMS (ESI): Calcd for MH⁺, 569.2916; Found, 569.2912.

Results and discussion

Compounds **Rh-AmPyr** and **Rh-AQ** were easily synthesized according the published procedure^[13,15] as showed in scheme 1. The structures of two compounds were confirmed by ¹H-NMR, ¹³C-NMR and HR-ESI (see S1). As expected, **Rh-AmPyr** and **Rh-AQ** showed colorless and a very low fluorescence in CH₃CN or in MeCN/HEPES buffer solution. Preliminary experiment showed that only **Rh-AQ** with rigid 8-AQ moiety could react easily with several cations, while **Rh-AmPyr** with flexible methylene spacer 2-aminopyridine fragment could not react with cations even at high concentration^[16]. Therefore the followed experiments would be focused on **Rh-AQ**.

The pH response of **Rh-AQ** in MeCN/water solution (5/5, v/v) was first evaluated as shown in Figure S2 (Supporting Information). The acid-base titration experiments revealed that **Rh-AQ** did not emit any obvious and characteristic (excitation at 530 nm) fluorescence in the pH range from 6.0 to 12.0, suggesting that it was insensitive to pH near 7.0 and could work in approximate physiological conditions with a very low background fluorescence and its Pka is 4.7. Therefore, further UV/vis and fluorescent studies in water contained system were carried out in MeCN/HEPES mixed buffer solution (HEPES 20 mM, pH 6.95, containing 50% CH₃CN).

Upon addition the various metallic cations to **Rh-AQ** in CH₃CN solution, Fe³⁺, Cr³⁺, Hg²⁺, Pb²⁺, and Cu²⁺ could induce color change from colorless to purple and an enhancement of fluorescence intensity. When the titration performed in HEPES buffer solution (20 mM, pH6.95), contained 50% CH₃CN as the cosolvent, Fe³⁺ ion could specifically bind to **Rh-AQ** and caused

the notable color and fluorescence dual responses. Unlikely in CH_3CN media, no significant responses were observed with Pb^{2+} and Hg^{2+} , but Cu^{2+} and Cr^{3+} caused a mild response. The remarkable responses and selectivity exhibited that the sensor **Rh-AQ** could use as a naked-eyes-detectable Fe^{3+} -responsive chemosensor for application in bioimaging. These results also revealed that (1) the reaction media could strongly affect coordination preference; (2) the trivalence cations (Fe^{3+} and Cr^{3+}) with strong positive charge could coordinate to **Rh-AQ** with high affinity. The similar results were observed by Czarnik^[13g] and Tong^[12b].

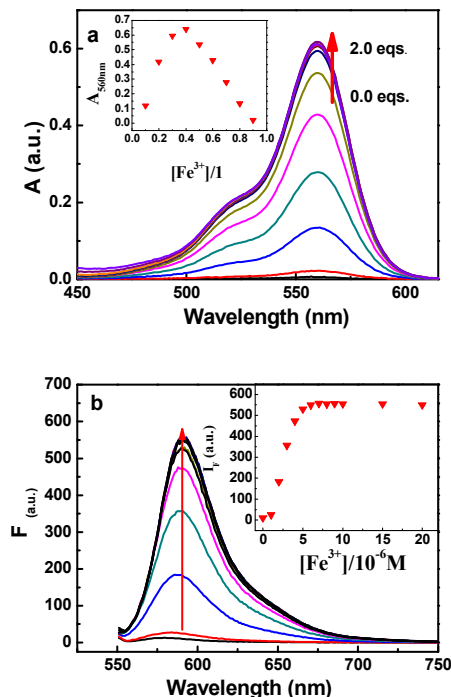


Fig. 1. (a) Absorption spectra of **Rh-AQ** (10 μM) in CH_3CN in the presence of different amounts of Fe^{3+} ion. Inset: Job's plots. Total concentration of $[\text{Rh-AQ} + \text{Fe}^{3+}]$ was kept constant at 20 μM . (b) Fluorescence spectra of **Rh-AQ** (10 μM) under the same conditions. Excitation is performed at 550 nm. Inset: fluorescence enhancement at 590 nm as a function of Fe^{3+} concentrations.

UV spectra (Fig.1a) of free **Rh-AQ** in CH_3CN showed no apparent absorption above 450 nm, which ascribed to its spiro lactam form in solution^[13] (confirmed by $^{13}\text{C-NMR}$, see S6). With the addition of Fe^{3+} ion, a new strong absorption band was formed at 560 nm which corresponded to the color changes from colorless to purple, it showed that the open-ring form was the main species^[13] (confirmed by $^{13}\text{C-NMR}$ see S6). The emission spectra of **Rh-AQ** responses to Fe^{3+} in acetonitrile were also recorded. The fluorescent emission of free **Rh-AQ** displayed a very low fluorescence centered at 578 nm owed to the existence of trace open-ring **Rh-AQ** or its aggregation. When Fe^{3+} was added to the solution of **Rh-AQ**, a significant fluorescent increase at 590 nm was observed instantly with 50-folds emission enhancements upon addition of 0.5 eqqv. Fe^{3+} ion. The inset in Figure 1b exhibited the dependence of the intensity of emission at 590 nm, which indicated the formation of an **Rh-AQ/Fe**³⁺ adduct of 2:1 stoichiometry. The binding stoichiometry was further

confirmed by the Job's plot as shown in inset of Fig.1a. Meanwhile, the detection limit was found to be 3.2×10^{-7} (see S 8) in MeCN.

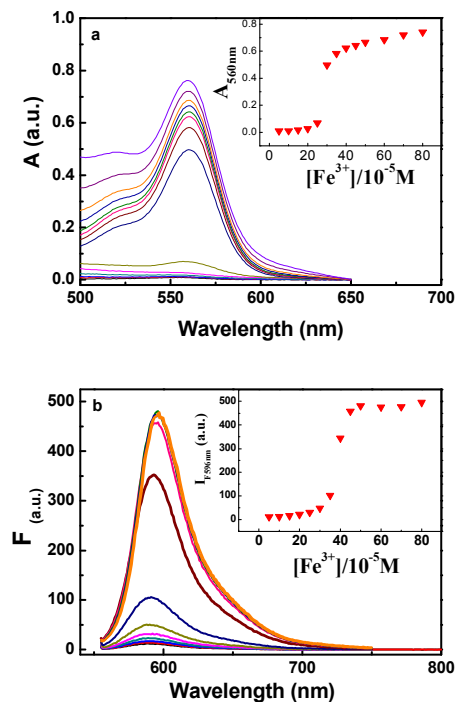


Fig.2. (a) Absorption spectra of **Rh-AQ** (10 μM) in HEPES (20 mM, pH 6.95) buffer containing 50% CH_3CN in the presence of different amounts of Fe^{3+} ion. Inset: Absorption enhancement at 560 nm as a function of Fe^{3+} concentration, it should be noted that the UV spectra suffered a slight interference by the color of Fe^{3+} itself and resulted in the systematic increase in UV intensity. (b) Fluorescence spectra of **Rh-AQ** (10 μM) under the same conditions. Excitation was performed at 550 nm. Inset: fluorescence enhancement at 590 nm as a function of Fe^{3+} concentration.

To improve the selectivity, an optimized $\text{CH}_3\text{CN/HEPES}$ buffer solution (HEPES 20 mM, pH 6.95, containing 50% CH_3CN) was used for the further spectroscopic investigations. As expected, the similar UV/vis and fluorescent emission profiles were obtained as in MeCN. In $\text{CH}_3\text{CN/HEPES}$ buffer solutions (Fig. 2), the FEF (Fluorescent Enhancement Factors) of 50-folds at 590nm was gained upon the addition of 60 equiv. of Fe^{3+} . As shown in Fig. 3b, only Fe^{3+} was able to induce the remarkable fluorescent enhancement. Cr^{3+} , Cu^{2+} and Hg^{2+} induced minor (less than 18-folds) fluorescent enhancements (Fig. 3c), which much lowered that of Fe^{3+} . The competition experiments, which were conducted in the presence of 60 equiv. Fe^{3+} mixed with Cr^{3+} (100 equiv.), Hg^{2+} (100 equiv.) and other cations (200 equiv.), respectively, indicated that no significant variation in its fluorescence intensity was found by comparison with that without the other metal ions besides Fe^{3+} (Fig. 3d). Also, the detection limit was found to be 3.5×10^{-6} M (see S 9) in $\text{CH}_3\text{CN/HEPES}$ buffer solutions.

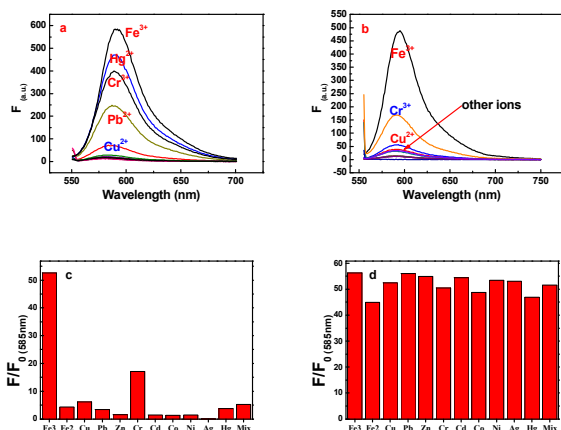


Fig. 3. Fluorescent spectra of **Rh-AQ** (10 μ M) in the absence and presence of different metal ions, (a) in CH_3CN , Fe^{3+} and the other ions are 0.5 equiv. (b) In HEPES (20 mM, pH 6.95) buffer containing 50% MeCN, 60 equiv. Fe^{3+} ion, 100 equiv. Cr^{3+} , 100 equiv. Hg^{2+} and 200 equiv. other cations were added. For all of the tests, excitation wavelength was performed at 550nm. The bar graphs (c) and (d) showed that **Rh-AQ** have the high selectivity, Mix is the mixture of Ca^{2+} , K^+ , Na^+ , Mg^{2+} .

The coordination structure could be analysed by 1D- $^1\text{H-NMR}$ and 2D $^1\text{H-}^1\text{H-COSY}$ experiments (Fig. 4 and S4-5). As showed in Fig. 4, a set of new peaks appeared with the increases of Fe^{3+} ion. When more than 0.5 equiv. Fe^{3+} was added, NMR signals kept unchanged, which further confirmed the 2:1 stoichiometry. H_8 (Fig. 5) on the quinolyl fragment displayed a small upperfield shift ($\Delta\delta = 0.078$ ppm, upon adding 0.5 equiv of Fe^{3+} ion), while the other protons (H_9 to H_{13}) on the quinoline displayed downfield shift, which were similar to the complexation of pyridine^[12e] and quinoline^[17] based ligands with metal cations. The downfield shift was due to the decrease in electron density of the quinolyl moiety upon coordination with metal ions. The upfield shift of H_8 was similar to the Artaud's^[17] and shiraishi's works^[12e] and revealed H_8 was shielded by complexation with Fe^{3+} , while the other quinolyl resonances were not^[12e]. This shielded effect should ascribe to the anisotropic effect caused by ring currents from adjacent π electrons on another **Rh-AQ** moiety within the **Rh-AQ/Fe³⁺** complex. Upon the addition of Cr^{3+} ion, the similar changes were observed (S7)^[18]. Taken the above results together, a plausible interaction model of **Rh-AQ/Fe³⁺** was proposed as shown in Fig. 5, in which Fe^{3+} ion was coordinated with quinolyl N and carbonyl O and the assignments of proton signals were made by the correlations of 2D COSY experiments. The similar coordination mode was reported^[12e, 17].

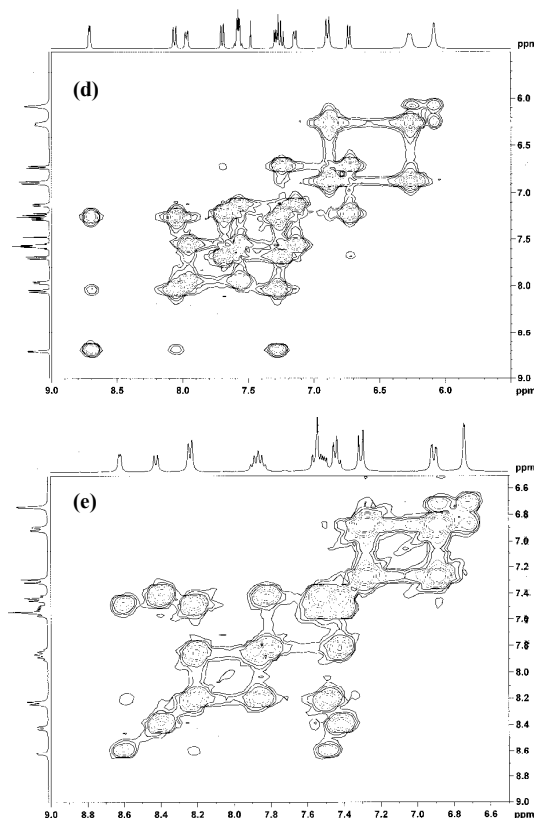
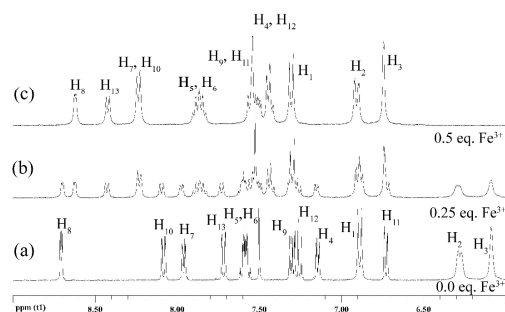


Fig. 4a. Fe^{3+} - $^1\text{H-NMR}$ titration of **Rh-AQ** (20 mM) in CD_3CN and CDCl_3 (1:1). (a) **Rh-AQ** only; (b) **Rh-AQ** + 0.25 equiv. of Fe^{3+} ion; (c) **Rh-AQ** + 0.5 equiv of Fe^{3+} ion; (d) 2D-COSY spectrum of **Rh-AQ** (20 mM) in CD_3CN and CDCl_3 (1:1) in absence of Fe^{3+} . (e) 2D-COSY spectrum of **Rh-AQ** (20 mM) in CD_3CN and CDCl_3 (1:1) in the presence of 0.5 equiv. Fe^{3+} ion.

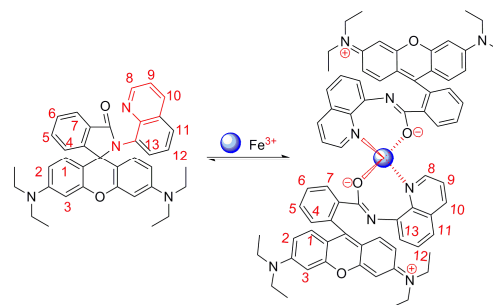


Fig 5. The proposed coordination mode between **Rh-AQ** and Fe^{3+} ion.

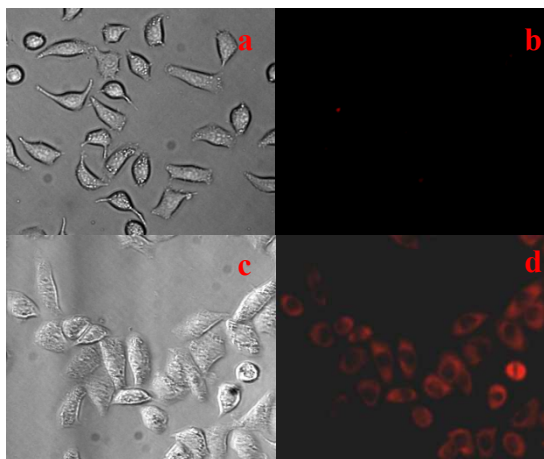


Fig.6. Fluorescence images of live HeLa cells. (a) Brightfield image: Fe(NO₃)₃ supplemented cells pretreated with 500 μM of the competing metal chelator, TPEN, for 5 min at 37°C before staining with 10 μM **Rh-AQ** for 5 min at 37 °C. (b) The fluorescent image of (a), the results showed that no fluorescent increased inside cells. (c) Brightfield image: Cells supplemented with 200 μM Fe(NO₃)₃ in the growth media for 2 h at 37 °C and stained with 10 μM **Rh-AQ** for 30 min at 37°C. (d) fluorescent image of “c”: the results showed that **Rh-AQ** was a specific and cell-permeable intracellular Fe³⁺ probe in living cells system.

Inspired by the above experimental results and its excellent chemical and spectroscopic properties (such as $\lambda_{\text{ex}} > 550 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$) of **Rh-AQ**, we next were interested in studying on the Fe³⁺ bioimaging of **Rh-AQ** in living cells system. Here HeLa cells were used as the living system. After incubation with 10 μM **Rh-AQ** at 37 °C for 30 min, there showed weak intracellular fluorescence. The cells were then supplemented with 200 μM Fe(NO₃)₃ in the growth medium for 2h at 37°C and loaded with **Rh-AQ** under the same conditions, the significant intracellular fluorescence was observed (Fig.6c, 6d). Iron-supplemented cells treated with 500 μM metal ion chelator TPEN as the control experiments were examined and also showed weak intracellular fluorescence (Fig. 6a, 6b). Brightfield measurements after loaded with iron and **Rh-AQ** confirm that the cells were viable in the bioimaging process (Fig.6c). Taken together, these experiments showed that **Rh-AQ** was cell-compatible and would be served as the Fe³⁺-responsive bioimaging probe.

Conclusion

In conclusion, we demonstrated a Fe³⁺-responsive fluorescent sensor **Rh-AQ**, which based on the rhodamine fluorophore moiety and integrated a rigid 8-aminequinine (8-AQ) unit as ion receptor. The 2:1 coordination mode of **Rh-AQ/Fe³⁺** was proposed based on Job's plot, and especially on 1D and 2D COSY H-H experiments. Moreover, fluorescent microscopic imaging experiments implied that **Rh-AQ** could be used as a bioimaging probe for monitoring Fe³⁺ in living cells. Alternatively, **Rh-AQ** with the ability for sensing Fe³⁺ over Fe²⁺ allowed us to monitor the redox potential in living system, and would facilitate new research in a variety of contexts associating iron^[19].

Acknowledgement

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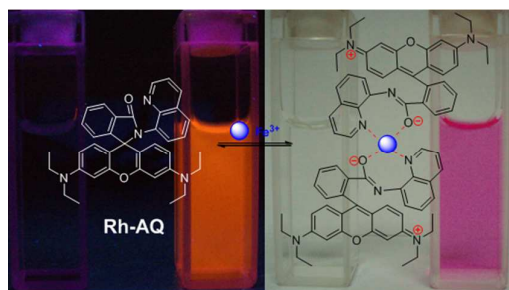
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Notes and references

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- † Electronic Supplementary Information (ESI) available: The spectra (¹H-NMR, ¹³C-NMR, ESI-MS) of **Rh-AmPyr** and **Rh-AQ**, pH-titration curves, full spectra of ¹H-¹H-COSY, ¹³C-NMR of **Rh-AQ** in CD₃CN and CDCl₃ (1:1) in the absence (a) or (b) presence of 0.5 eq Fe³⁺, Cr³⁺-titration [¹H-NMR experiment]. See DOI: 10.1039/b000000x
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- 85



A rhodamine-based Fe^{3+} sensor of a rigid 8- Aminoquinoline receptor shows a 2:1 binding according to 1D and 2D- ^1H NMR experiment.