Cation Sensors

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Selective Detection of Iron(III) by Rhodamine-Modified Fe_3O_4 Nanoparticles**

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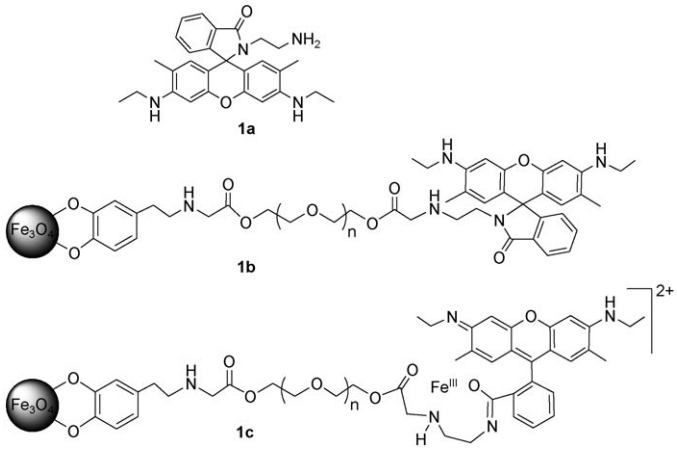
The highly selective and sensitive detection of metal ions in aqueous solution is of great importance in tracking and studying the physiological functions of these ions in living organisms.[1] Fe^{III} is a biologically important metal ion and plays an essential role in oxygen uptake, oxygen metabolism, and electron transfer.[2] However, the presence of Fe^{III} in biological systems has to be efficiently moderated as both its deficiency and overloading can induce various biological disorders.[3] Currently, the detection of Fe^{III} relies on small organic molecules,[4] but these organic molecules are poorly soluble in water and cannot provide the necessary selectivity and sensitivity for Fe^{III} detection in biological environments. Recently, we reported that magnetic Fe_3O_4 nanoparticles (NPs) that were modified with polyethylene glycol (PEG) derivatives were highly soluble and stable in physiological solutions.[5] These properties indicate that the problems related to the low solubility of small organic molecules in biological solutions can be resolved by coupling these molecules to PEG-Fe_3O_4 NPs. The increased solubility should thus greatly improve the detection sensitivity. The presence of magnetic Fe_3O_4 NPs in the sensor-PEG-Fe_3O_4 NP conjugate should also facilitate the magnetic separation of the Fe^{III}-sensor-PEG-Fe_3O_4 from biological solutions, leading to the selective detection of Fe^{III} at ultralow concentrations.[6]

Herein, we report that a rhodamine 6G derivative, *N*-(rhodamine-6G)lactam-ethylenediamine (Rh6G-LEDA) (**1a**), can be coupled to the PEG-Fe_3O_4 NPs (**1b**) and act as an Fe^{III}-selective fluorescent sensor (**1c**; Scheme 1). Rhodamine-based sensors have received ever-increasing interest in sensing Pb^{2+}, Cu^{2+}, Hg^{2+}, Fe^{3+}, Cr^{3+}, NO, and OCl^- ions.[7] Their detection is based on the on/off switch of the spirocyclic moiety, mediated by the metal ion.[7] For example, the spirocyclic form of Rh6G-LEDA (**1a**) is nearly nonfluorescent and colorless. But once bound to a metal ion, it is converted into the open-cyclic form with much better intramolecular conjugation. As a result, the complex is strongly fluorescent.[7] Unfortunately, most of the reported rhodamine-based sensors that were intended for the detection of metal ions suffer from poor water solubility, thus preventing them from having practical applications in biological systems. We demonstrate that in the presence of various metal ions in water, **1b** can selectively bind to Fe^{III} (**1c**), thereby leading to the sensitive detection of Fe^{III} with the detection limit reaching below 2 ppb. Such a sensitive Fe^{III} probe could also be applied to intracellular Fe^{III} detection using the enhanced fluorescence from **1c**.



Scheme 1. Structural illustration of Rh6G-LEDA (**1a**), its coupling with PEG-Fe_3O_4 NP (**1b**), and its complex with Fe^{III} (**1c**) along with the structural change that enhances its fluorescence property.

Fe_3O_4 NPs were synthesized from the thermal decomposition of [Fe(acac)$_3$] (acac = acetylacetonate) in benzyl ether in the presence of oleylamine.[5] The oleylamine-coated 12 nm Fe_3O_4 NPs, as shown by transmission electron microscopy (TEM; see the Supporting Information, Figure S1 A), were functionalized by replacing oleylamine with dopamine-PEG, which had been synthesized from the acylation of PEG (MW = 2000) with bromoacetyl chloride, followed by a nucleophilic substitution reaction in which a bromo substituent was displaced by the dopamine NH_2. The bromo-PEG-Fe_3O_4 reacted with **1a** by a nucleophilic substitution reaction between the bromide and amine groups to give **1b** (see the Supporting Information, Scheme S1). **1b** was readily dispersed in water, with its solubility reaching 1.23 mg mL^{-1}, and it stayed in the dispersion state for at least half a month. TEM

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showed that there was no change in the morphology of the NPs during the surface replacement reaction (see the Supporting Information, Figure S2B).

The successful conjugation of **1a** onto the surface of the Fe_3O_4 NPs was further confirmed by infrared (IR) and fluorescence spectroscopy. **1b** exhibited a ν_{lactam}(C=O) vibration at 1673 cm^{-1}, red-shifted from 1684 cm^{-1} in **1a** (see the Supporting Information, Figure S2). Two aromatic C=C peaks at 1628 and 1463 cm^{-1} in the IR spectrum of **1b** were blue-shifted compared to those of the free **1a** (1619 and 1423 cm^{-1}, respectively). These shifts indicate that **1a** couples to PEG-Fe_3O_4 NP through the amino group. A fluorescence titration of iron(III) chloride was conducted using a 4 μM solution of **1b** in water at pH 7.0. In the absence of Fe^{III} ions, **1b** showed a rather weak fluorescence signal in the range from 520 to 600 nm. However, after addition of 1 equivalent of iron(III) chloride, the fluorescence intensity of the solution increased approximately 56-fold and the emission maxima shifted from 544 to 549 nm (Figure 1a). Clearly, the addition of Fe^{III} ions

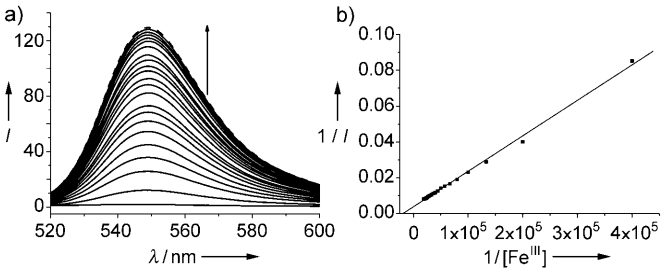

Figure 1. a) Fluorescence response of **1b** (4 μM) upon addition of Fe^{III} in water at pH 7 (excitation at 500 nm). Slit: excitation/emission = 1.5:1.5. b) Fitted line in the calculation of the binding constant by monitoring the fluorescence intensity changes at 545 nm.

leads to the formation of **1c** with enhanced fluorescence properties. As a control, we also added 1 equivalent of iron(III) chloride to **1a**, but the fluorescence intensity of the solution increased by only roughly 16-fold (see the Supporting Information, Figure S3). By fitting the data to the Benesi–Hildebrand expression,[8] the stability constant of **1c** was determined to be 5.0×10^6 M, with a good linear relationship (R = 0.997; Figure 1b). This observation also indicates that **1b** and Fe^{III} have a 1:1 binding ratio. The fluorescence enhancement in **1c** is the result of the spiro-ring-opening that was induced by coordination of Fe^{III} ions with a N atom and two carbonyl O atoms.[7,4b] It should be noted that the Fe_3O_4 NPs in **1b** did not release any detectable amount of Fe^{III} under the current conditions.

Changes in the fluorescence properties of **1b** as a result of the addition of other metal ions, including Fe^{II}, Cu^{II}, Zn^{II}, Ni^{II}, Ca^{II}, Cd^{II}, Mg^{II}, Co^{II}, Mn^{II}, Cr^{III}, Pb^{II}, Hg^{II}, K^{I}, and Na^{I} were also measured. Fluorescence spectra of a 4 μM solution of **1b**, recorded within 5 minutes of the addition of 1 equivalent of each of these metal ions, are shown in Figure 2a. Of all the ions tested, only the addition of Cr^{III} ions gave a small increase in fluorescence intensity. The addition of other metal ions to **1b**, including La^{III}, Ce^{III}, Eu^{III}, Pr^{III}, Ho^{III}, Er^{III}, and

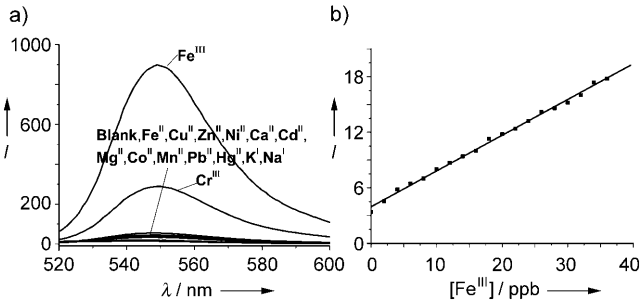

Figure 2. a) Fluorescence spectra (excitation at 500 nm) of **1b** (4 μM) in water at pH 7 in the presence of 1 equivalent of Fe^{II}, Cu^{II}, Zn^{II}, Ni^{II}, Ca^{II}, Cd^{II}, Mg^{II}, Co^{II}, Mn^{II}, Cr^{III}, Pb^{II}, Hg^{II}, K^{I}, or Na^{I}. Slit: excitation/emission = 5.0:5.0. b) Fe^{III} concentration (at the parts per billion level) dependent fluorescence intensity change at 545 nm.

Yb^{III} ions (see the Supporting Information, Figure S4), did not lead to measurable fluorescence emissions. In addition, the enhancement in fluorescence intensity resulting from the addition of Fe^{III} ions is not influenced by subsequent addition of other metal ions. The selectivity observed for Fe^{III} over other ions is remarkably high (see the Supporting Information, Figure S5). The detection limit based on the formation of **1c** was also evaluated. The fluorescence titration profile of **1c** (10^{-7} M) with Fe^{III} (see the Supporting Information, Figure S6) demonstrates that detection of Fe^{III} ions is at the parts per billion level. Under these conditions, the fluorescence intensity of the solution of **1c** is still proportional to the amount of Fe^{III} added (Figure 2b). As a comparison, in the presence of other metal ions, **1a** was not as selective as **1b** for iron detection (see the Supporting Information, Figures S7 and S8); its detection limit was only at the 0.4 ppm level (see the Supporting Information, Figures S9 and S10).

The sensitive binding of Fe^{III} ions to **1b** was then explored for the possible detection of Fe^{III} ions in living cells. HeLa cells were incubated with and without 10–160 μM Fe^{III} solutions for 5 hours in Dulbecco's modification of Eagle's medium (DMEM) at 37 °C then washed with a phosphate-buffered saline (PBS) buffer (pH 7.4) to remove any excess Fe^{III} ions. The treated cells were incubated with **1b** (10 μM) in DMEM for 30 minutes. After being washed with PBS buffer, the cells were imaged by a confocal fluorescence microscope. As a control, the cells were treated with an Fe^{III} solution and those incubated with pure **1b** in the absence of Fe^{III} ions were also imaged. Figure 3 shows the fluorescence images of these cells. The cells from the controlled experiments show no fluorescence (Figure 3a), but those treated with Fe^{III}/**1b** display strong red fluorescence (Figure 3b). The fluorescence image grew brighter as the concentration of Fe^{III} increased (see the Supporting Information, Figure S11). These results suggest that **1b** can penetrate the HeLa cell membrane and bind to intracellular Fe^{III} ions, leading to a strong intracellular fluorescence. Such highly sensitive Fe^{III} detection, based on the **1b** fluorescence enhancement upon its binding to Fe^{III} ions, should be important for monitoring intracellular iron concentration and for studying iron toxicity or bioactivity in living cells.

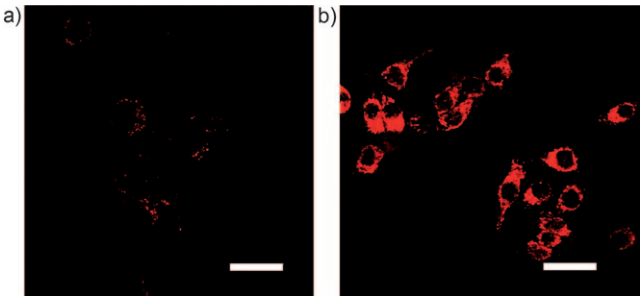

Figure 3. Fluorescence image of HeLa cells after incubation with a) **1b** for 5 h, and b) **1b**/Fe^{III} for 5 h. In (b), the cells were first incubated with Fe^{III} for 5 h, washed three times with PBS and then further incubated with **1b** for 30 min (scale bar = 40 μm). The image was acquired under 490 nm excitation and 560 nm emission.

In summary, we have synthesized a NP conjugate of Fe_3O_4-Rh6G-LEDA and demonstrated that such a NP-rhodamine conjugate is sensitive and selective in detecting Fe^{III} ion concentrations amongst various metal ions in water with the detection limits reaching as low as 2 ppb. As a comparison, the free Rh6G-LEDA can only detect iron at a concentration above 0.4 ppm, owing to its low solubility, and the detection selectivity is poor in the presence of other metal ions. The sensitive detection of Fe^{III} by the NP conjugate is further demonstrated in HeLa cells, thus indicating the potential applications of this detection method in the biological monitoring and tracking of iron. This detection technique can also be coupled with the magnetic separation capability of Fe_3O_4 NPs (see the Supporting Information, Figure S12) to offer a simple route to highly sensitive Fe^{III} enrichment and detection in various biological environments.

Experimental Section

Chemicals: Iron(III) acetylacetonate was purchased from Strem Chemicals, Inc. Triethylamine, potassium iodide, bromoacetyl chloride, potassium carbonate, benzyl ether, oleic acid, oleylamine, polyethylene glycol (MW = 2000), rhodamine 6G, ethylenediamine, and all cationic compounds were purchased from Sigma–Aldrich. DMEM was purchased from Hyclone in China. All chemicals were used without further purification. *N*-(rhodamine-6G)lactam-ethylenediamine was synthesized according to the published method.[9]

Instrumentation: ^{1}H NMR spectra were acquired on a Varian 300 MHz NMR spectrometer. TEM images were taken on a Philips EM 420 (120 kV). UV/Vis spectra were recorded on a Varian Cary 100 Conc spectrophotometer. The fluorescence spectra were recorded on a Hitachi RF-4500 spectrofluorophotometer. Infrared spectra (4000–400 cm^{-1}) were determined using KBr disks on a Therrno Mattson FTIR spectrometer. Fluorescence measurements were taken on Zeiss Leica inverted epifluorescence/reflectance laser scanning confocal microscope.

Synthesis of Fe_3O_4 nanoparticles: [Fe(acac)$_3$] (0.706 g, 2 mmol) was dissolved in a mixture of benzyl ether (10 mL) and oleylamine (10 mL). The mixture was dehydrated at 110 °C for 1 h under a flow of nitrogen; under a blanket of nitrogen, the mixture was heated at 300 °C for 2 h. The black-brown mixture was cooled to room temperature by removing the heating source. Ethanol (40 mL) was added and the precipitate was collected by centrifugation at 8000 rpm and was washed three times with ethanol. Finally, the product was redispersed in *n*-hexane.

Synthesis of PEG-DBrAc: PEG2000 (10 g) was dissolved in anhydrous dichloromethane (20 mL). Triethylamine (2.09 mL) was added drop-wise, followed by the drop-wise addition of 1.25 mL of bromoacethyl chloride under nitrogen. The reaction was stirred overnight in the dark. The product was purified by precipitation in diethyl ether. Next, the product was dissolved in water, and the solution was adjusted to a pH value of 6 using saturated Na_2CO_3 solution. The product was then extracted three times with 20 mL of dichloromethane, precipitated by addition of diethyl ether, and stored at −20 °C. ^{1}H NMR (300 MHz, $CDCl_3$: $\delta = 3.3$–3.7 (232 H, OCH_2CH_2O), 4.15 (s, 4 H, $2\times CH_2Br$), 4.20 ppm (t, 4 H, $2\times CH_2COO$).

Synthesis of DPA-PEG-DBrAc: PEG-DBrAc (448 mg) was dissolved in anhydrous dichloromethane (20 mL), and then dopamine (DPA) hydrochloride (18.9 mg), KI (16.6 mg), K_2CO_3 (70 mg), and *N*,*N*-dimethylformamide (0.5 mL) were added to the solution. The mixture was stirred for 10 h at 25 °C under a nitrogen atmosphere. The insoluble product was filtered, and the filtrate was added to diethyl ether. The precipitate was collected and dissolved in water. DPA-PEG-DBrAc was extracted with dichloromethane (3×10 mL), and precipitated with diethyl ether on dry ice. The product was then stored at −20 °C. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.634$ (t, 2 H, CH_2N), 2.86 (t, 2 H, $PhCHCH_2$), 3.4–3.6 (234 H, OCH_2CH_2O), 4.07 (s, 2 H, CH_2Br), 4.24 (t, 4 H, $2\times CH_2COO$), 6.54 (d, 1 H, Ph), 6.75 ppm (m, 2 H, Ph).

Preparation of Fe_3O_4-DAP-PEG-DBrAc: Fe_3O_4 (10 mg) was added to a solution of DPA-PEG-DBrAc (50 mg) in dichloromethane (5 mL). The mixture was stirred overnight at room temperature. The modified Fe_3O_4 nanoparticles were precipitated by adding *n*-hexane, collected by centrifugation at 6000 rpm, and washed with dichloromethane *n*-hexane (1:5, v/v) three times. Finally, the product was redispersed in dichloromethane.

Preparation of Fe_3O_4-DAP-PEG-DAcNH-Rh6G-LEDA: N-(rhodamine-6G) lactam-ethylenediamine (Rh6G-LEDA; 10 mg), KI (4.4 mg), and K_2CO_3 (15 mg) were added to a solution of Fe_3O_4-DAP-PEG-DBrAc (10 mg) in dichloromethane (5 mL), and the mixture was stirred for 12 h at room temperature. The product was precipitated by adding *n*-hexane, collected by centrifugation at 6000 rpm, and washed three times with dichloromethane/*n*-hexane (1:5, v/v). Finally, the product was redispersed in deionized water.

Calculation of binding constant between Fe^{III} and **1b**: The stability constant of the complex was calculated by the linear Benesi–Hildebrand expression

$$\frac{I_0}{I-I_0}=\frac{I_0}{[\mathrm{L}]}+\frac{I_0}{[\mathrm{L}]\mathrm{Ks}}\frac{1}{[\mathrm{M}]} \qquad (1)$$

where I is the change in the fluorescence intensity at 545 nm, Ks is the stability constant, and [L] and [M] are the concentrations of **1b** and Fe^{III}, respectively. I_0 is the fluorescence intensity of **1b** in the absence of Fe^{III}. $I-I_0$ can be approximated to I because at 545 nm I_0 is very weak. On the basis of the plot of $1/I$ versus 1/[Fe], the stability constant can be obtained.

Determination of the concentration of **1a**: The concentration of **1a** was determined based on the Beer–Lambert law. The values of ε were obtained through measurement of the emission of **1a** at different concentrations (1.0×10^{-5}, 2.0×10^{-5}, 3.0×10^{-5}, 4.0×10^{-5} M) in a mixed water/methanol solution (9:1, v/v). The ε value was calculated to be $4.9\times 10^{6}\,M^{-1}cm^{-1}$.

Excess **1a** was suspended in water (50 mL) and stirred at 70 °C for 5 h. The insoluble **1a** was filtered off. The concentration of **1a** was obtained by measuring the fluorescence intensity of the solution at 545 nm. The saturated concentration of **1a** in H_2O is 2.1×10^{-5} M.

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