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Design and synthesis of fluorescence-based siderophore sensor molecules for Fe^{III} ion determination*

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Abstract: The design principles of fluorescence-based siderophore sensor molecules for detection of heavy transition-metal (HTM) ions are first reviewed. As an example, fluorescein-desferrioxamine (FlDFO), a highly efficient fluorophore molecule combining a specific Fe ion receptor and a fluorescence-sensitive signalling site has been designed, synthesized, and used for dosing with Fe ions. Its response test shows its high selectivity and sensitivity to Fe^{III} ions and its potential for nanobiosensor design. This work clearly identified that among two FlDFO positional isomers differing by the attachment of DFO at the 5- or 6-position of the bottom benzene ring of Fl, the fluorescence of 6-FlDFO is insensitive to the complexation with Fe ions. This is independent of the linkage used between Fl and DFO. Only 5-FlDFO could be a highly potential sensor molecule since it has been revealed that in a free state without complexation with Fe ions, this fluoroionophore sensor molecule gave a maximum fluorescent signal. With successive Fe ion complexing, the fluorescence of 5-FlDFO decreased very sensitively and proportionally with ion concentration. The response speed has been evaluated as a function of Fe ion concentration. Responses to other metal ions present in the solution, such as Cu^{2+}, Ca^{2+}, Ni^{2+}, and Al^{3+}, and the effect of pH value on the efficiency of the sensor molecules have also been investigated.

Keywords: ferric ion dosing; fluorescein-desferrioxamine (FlDFO); fluoroionophore sensor; heavy transition metals; molecular probe; photoinduced electron transfer (PET).

INTRODUCTION

Metal ion detection is of great interest due to the fact that various metal ions are involved in metabolical processes such as oxygen transportation, transmission of nerve impulses, muscular contraction, signal transmission, regulation of cell activity, photosynthesis, nitrogen fixation, etc. and can be a kind of metalloenzyme. Some metal ions can also play the role of enzyme co-factors. A proper concentration—no more, no less—of these metal ions is thus crucial to guarantee in a correct manner these biological

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processes. Furthermore, some metal ions such as mercury, lead, and cadmium are known to be toxic for the organisms. Early detection in the environment is becoming a priority for society.

The quantification of metal ions can be achieved by a series of chemical and analytic methods such as colorimetry, flame photometry, UV–vis spectrophotometry, atomic absorption spectrometry, ion-sensitive electrodes (ISEs), electron microprobe analysis, neutron activation analysis, inductively coupled plasma-mass spectrometry (ICP-MS), etc. However, these methods have certain shortcomings because they are generally expensive and voluminous and often require samples of large size and sometimes the destruction of the samples. Furthermore, these methods do not allow continuous monitoring.

The development of sensitive and selective fluorescent nanosensors for dosing with heavy transition-metal (HTM) ions has recently attracted great attention due to their importance in the fields of environmental monitoring, clinical toxicology, wastewater treatment, and industrial process monitoring [1–4]. The principle advantages of this technology rely on its high sensitivity, which allows easy measurement of low analyst concentrations and its selectivity due to the excitation and emission wavelengths of each fluorescent species [1–4]. Fluorescent organic chelators, proteins, and peptides have emerged as powerful receptors for dosing with HTM ions, and remarkable progress has been made in developing fluorophore molecules for metal ions such as Ca^{++}, Zn^{++}, Pb^{++}, Hg^{++}, Cu^{++}, and Ag^{+} ions [5–18]. These systems are generally composed of a substrate binding unit and one or more chemophore molecules which can enhance (or generate) or quench the fluorescence signal when complexing HTM ions. These modular systems are of particular interest since such an approach allows tuning sensitivity and selectivity through the choice of the functional subunits. When employing a fluorophore as the transduction unit, all of the advantages of highly sensitive and versatile methods of fluorimetry can be utilized for the application of interest. Such fluorescent sensors consisting of a fluorophore linked to an ionophore are called fluoroionophores. Unfortunately, serious problems such as low sensitivity, low selectivity, and low stability after complexation with HTM ions and the difficult regeneration of these molecules render the concept of a simple, stable, cost-effective, and long-lived nanobiosensor unattainable. The design and synthesis of sensitive and selective fluorosensor molecules with increased selectivity for various environmentally or biochemically relevant metal ions thus remain a significant challenge.

In the design of fluoroionophore sensor molecules, both ion recognition units and signalling moieties should be taken into account. An excellent review has been entirely devoted to the design principles of fluoroionophore molecules for cation recognition [19]. There are two kinds of fluoroionophore sensor molecules: the signalling moiety (fluorophore) can be linked via a spacer to the recognition unit (ionophore), called "spaced fluoroionophores", or they can be linked directly to the recognition unit, called "integrated fluoroionophores". The signalling moiety, acting as a signal transducer, is responsible for converting the information into an easily detectable optical signal. For the design of a better fluorophore moiety, different considerations relating to the field of photophysics of photoinduced processes such as electron transfer, charge transfer, energy transfer, etc. should be taken into account. On the other hand, the recognition unit is responsible for the selectivity and efficiency of binding. Aspects relating to supramolecular chemistry such as the ligand topology, the characteristics of cations (ion size, valence, coordination number, hardness, etc.), the nature of the solvent, etc. should be considered.

Figure 1, published in an excellent review paper authored by Valeur and Leray, summarizes in an excellent way different aspects to be considered for the design of a highly selective and sensitive fluoroionophore sensor molecule [19]. The underlying concept of fluoroionophore sensor molecules is that upon metal complexation, the fluorescence signal F_o is usually deactivated, yielding $-\Delta F = F_o - F$. If the absolute $-\Delta F$ results from high affinity complexation, it can be directly equated with quencher concentration, irrespective of the probe levels [20].

On the basis of the above information, the present paper illustrates, as an example of a monitor, a highly selective and efficient fluoroionophore sensor molecule for dosing with Fe^{III} ions from its design, synthesis, and structural and spectroscopic characterization to its response test to Fe ions.

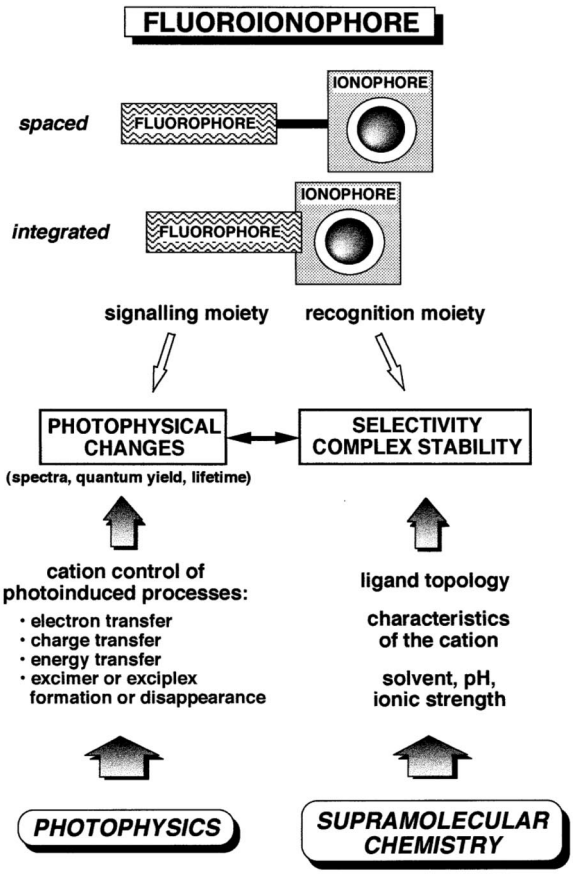


Fig. 1 Different aspects to be considered for the design of a highly selective and sensitive fluoroionophore sensor molecule [19].

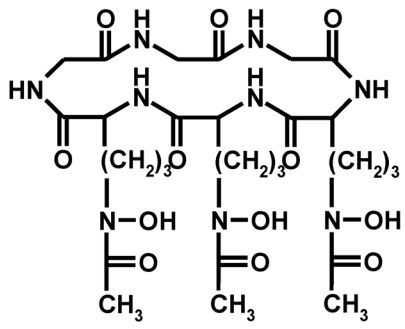
DESIGN OF FLUOROIONOPHORE SENSOR MOLECULE FOR DOSING WITH Fe^{III} IONS

Fe ions are indispensable to human and animal life as they play a critical role in oxygen transportation. A low level of Fe ions in the blood provokes important anemia, with symptoms such as tiredness and hair loss, while too high a level of Fe ions will also be toxic and leads to other health problems such as hemochromatosis, which can induce liver cancer. The concentration of Fe ions in the blood is therefore crucial.

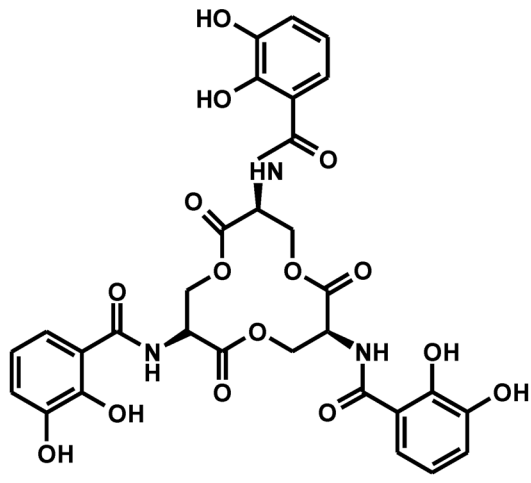
As regards the recognition moiety, some highly specific natural ion chelators exist, usually transferrin or microbial siderophores. Transferrin is a blood plasma protein for Fe ion delivery and binds Fe ions very tightly and reversibly. This glycoprotein has a molecular weight of around 80 kDa and contains two specific high-affinity Fe^{III} binding sites. The affinity of transferrin for Fe^{III} ions is extremely high (10^{23} M^{-1} at pH 7.4) but decreases progressively when pH value decreases below neutrality.

Siderophores are small, high-affinity Fe-chelating compounds secreted by microorganisms such as bacteria, fungi, and grasses. They are amongst the strongest soluble Fe^{III} ion binding agents known.

There are, in general, three kinds of siderophores—ferrichromes, enterobectines, and ferrioxamines (Fig. 2). Ferrichrome (Fig. 2A) is a cyclic hexapeptide compound of three glycine and three modified ornithine residues that can bind Fe^{III} with hydroxamate groups [21]. Enterobactin (Fig. 2B) is the strongest siderophore, binding to the ferric ions with the affinity ($K = 10^{52}$ M^{-1}) [22,23]. Due to its



(A)



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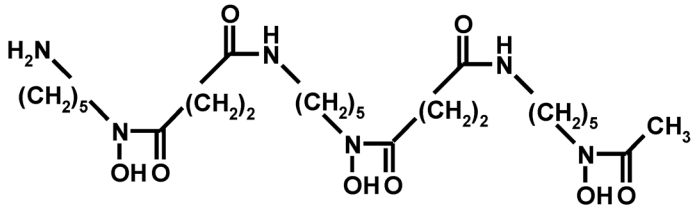

(C)

Fig. 2 Chemical structure of three siderophores: (A) DFO; (B) enterobactin; and (C) DFO B.

high affinity, enterobactin is capable of chelating even in environments where the concentration of Fe^{III} ions is very low. However, the complexation is only reversible with difficulty.

Desferrioxamine (DFO) (Fig. 2C) is a drug currently used for the treatment of hemochromatosis patients. It is a natural product extracted from *Streptomyces pilosus* and is quite efficient for the complexation of Fe ions and their elimination from the human body. In general, DFO displays an extended conformation while a quite contracted configuration is adopted around a metal ion since the six oxygen atoms of –(N–OH)–(C=O)– are placed at six summits of an octahedral complex with metal ions located at the octahedral center. DFO has a very high complexation constant (log *K*) of 30.7 for Fe^{III} ions compared to Fe^{II} (7.2), Ni^{II} (10.9), Cu^{II} (14.1), Zn^{II} (10.1), and Al^{III} (23.1). It can be a highly efficient and selective receptor for Fe^{III} ions [24].

Concerning the signalling unit, commonly used fluorophores are derivatives of rhodamine (Fig. 3A), coumarine (Fig. 3B), cyanine (Fig. 3C), and fluorescein (Fl) (Fig. 3D). Rhodamines are a

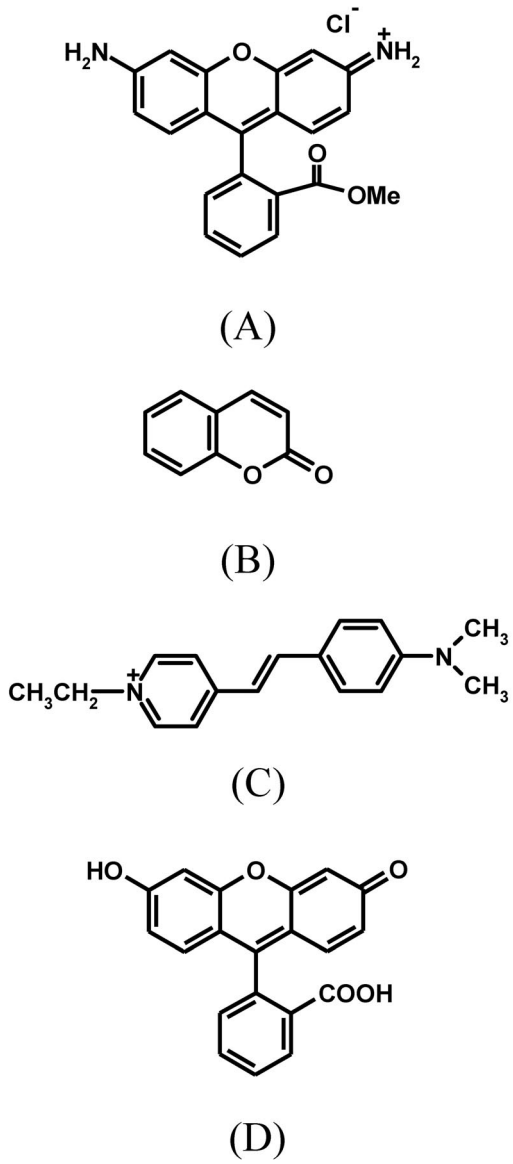

Fig. 3 Chemical structure of four fluorophores: (A) rhodamine 123 (hydrochloride); (B) coumarin (2*H*-chromen-2-one); (C) cyanine; and (D) Fl.

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family of fluorone dyes and are generally toxic, which limits their utilization. Coumarines can be found in many plants. Their moderate toxicity can also limit their applications [25]. Cyanines, a synthetic dye family, have many uses as fluorescent dyes, particularly in biomedical imaging. Fl is largely used in analytical chemistry as adsorption indicator for the titration of chloride ions with silver nitrate. This fluorescent molecule was also employed as a sensor for bromide vapor. In fact, free bromide can convert the yellow dye Fl to the red tetrabromofluorescein or eosin. Filter paper impregnated with Fl solution is therefore prepared for signalling the presence of bromide vapor since the paper acquires a red color [26]. Due to their high fluorescent emission, the Fl molecules are also used in hydrology and speleology. Fl with a very high molar absorption coefficient of 63 900 l/(mol cm), is water-soluble, often employed as a laser dye and can be excited at rather long wavelengths (~500 nm). The reason for selecting these probes includes high quantum yields and resistance to photobleaching. Most importantly, Fl retains a high quantum yield after conjugation to the chelating moiety and exhibits 70–90 % quenching upon stoichiometric metal titration [20]. In addition, the long wavelength of absorption and emission minimizes the problems of background fluorescence and eliminates the need for quartz optics.

Among the four different types of fluorophores discussed above, Fl shows its high potential as a signalling unit in the fluoroionophore molecular sensor design for fluorescent detection of Fe^{III} ions.

Combining a fluorosensitive reporter unit with a high selective Fe ion receptor such as DFO molecules allows for efficient fluorescence quenching and Fe ion concentration determination. It is of great interest from a physicochemical point of view to combine receptor-based (chemical) selectivity with analyte-mediated signalling (spectroscopic) sensitivity in bifunctional probe molecule conception. DFO as an Fe ion chelator has been used recently to combine with a series of fluorescent molecules such as Phen Green SK (PG SK), *N*-methylanthracene (MA), and 7-nitrobenzo-2-oxa-1,3-diazole (NBD). NBD-DFO and MA-DFO were used for selective scavenging of Fe^{III} ions from parasized erythrocytes [27,28]. A pyoverdin molecule was employed for the recyclable uptake of Fe ions from water [29]. Fl's have been proposed for assessing chelation therapy or for antimalarial actions [28,30]. However, the combination of Fl molecules with DFO component for Fe ion quantitative dosing and further, for the development of Fe ion sensors, is not yet well explored.

The present paper reports, on the basis of the above review and analysis, the synthesis and structural and spectroscopic characterization of the highly selective and sensitive fluoroionophore molecule FlDFO with a fluorophore signalling group (Fl) and a receptor unit (DFO), as well as the fluorimetric tests and the study of their potential for dosing with Fe ions. Moreover, at the bottom benzene ring of Fl, there are two different positions, 5 and 6. The DFO molecule or other reactive groups can be attached at the 5- and 6-positions. Thus, FlDFO has two different positional isomers. If DFO is attached at the 5-position, the isomer obtained is labeled as isomer 5 or 5-FlDFO, whereas if is attached at the 6-position, the isomer is labeled as isomer 6 or 6-FlDFO. Figure 4 depicts these two positional isomers. Figure 4A depicts that the DFO group is linked to Fl via an amide group at the 6-position (a of Fig. 4A) and at the 5-position (b of Fig. 4A). Figure 4B shows that DFO is linked to Fl via a thio-urea group at the 6-position (a of Fig. 4B) and at 5-position (b of Fig. 4B). The question can then arise as to whether both isomers can be used as fluoroionophore sensor molecules for dosing with blood Fe^{III} ions. The properties of these two isomers are indistinguishable in terms of excitation and emission spectra while they can have quite different behavior upon the complexation with Fe^{III} ions when Fl is coupled with DFO. The response to this question is of great importance in the conception of highly sensitive and selective sensors.

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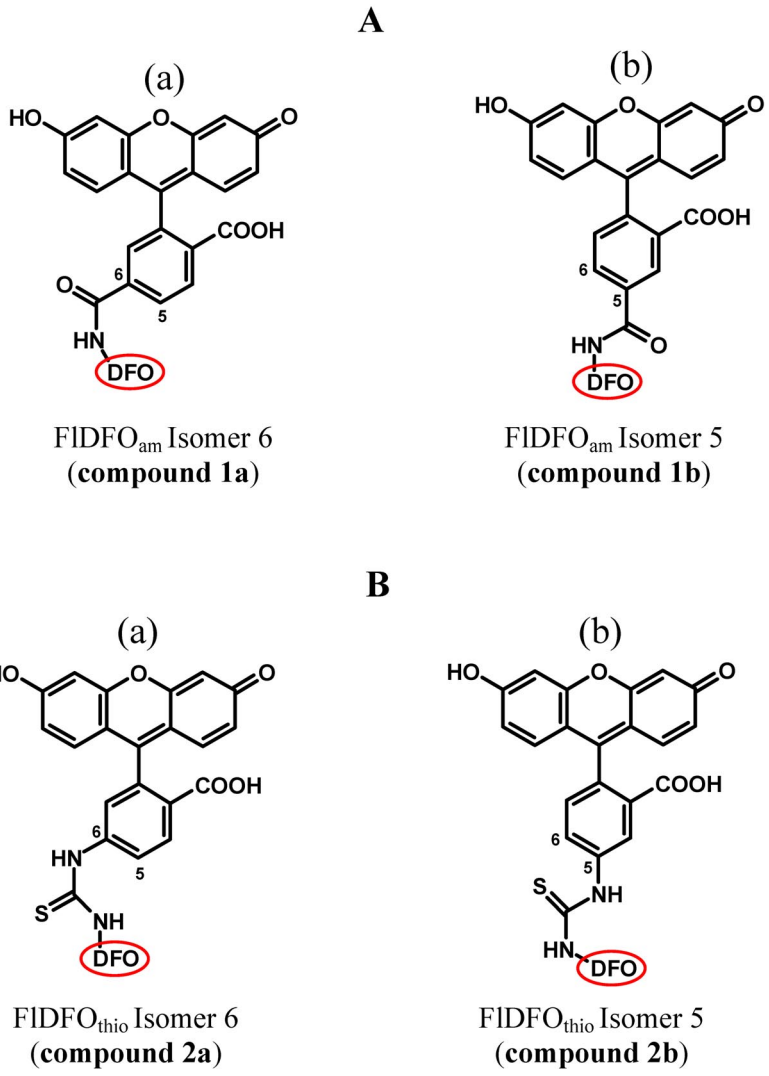

Fig. 4 Chemical structure of two FlDFO positional isomers with (A) amide linker (FlDFO$_{am}$) and (B) thio-urea linker (FlDFO$_{th}$) with clear indication of 5- and 6-position at the bottom of the benzene ring of Fl.

EXPERIMENTAL

Synthesis

FlDFO (isomers 5 and 6) synthesis

To test whether both isomers can be used as a fluoroionophore unit, mixtures of the two isomers with different ratios were synthesized. The combination of FlDFO was achieved from DFO mesylate salt with Fl by means of two different methods.

The first involves the use of a commercially available 5- and 6-carboxyfluorescein *N*-hydroxy-succinimide (NHS) ester isomer mixture to obtain an amide bond between Fl and DFO. A mixture of 5- and 6-FlDFO with an amide linkage and with an expected ratio of 5-/6-isomer of 60/40 is thus obtained (a mixture of compounds **1a** and **1b**, Fig. 4A).

The second one concerns the use of a commercially available Fl 5 and 6-isothiocyanate isomers mixture to obtain a thio-urea bond 5- and 6-FlDFO mixture (a mixture of compounds **2a** and **2b**, Fig. 4B) with an expected ratio of 5-/6-isomer of around 80/20.

In both cases, DFO mesylate (67.0 mg, 15.2 mmol) was dissolved in 5 ml of *N*,*N*-dimethylformamide (DMF) (99.9 %, Aldrich) with 1 equiv of triethylamine and the mixture was stirred for 1 h. 5(6)-Carboxyfluorescein NHS ester (83.6 mg, 15.6 mmol) or Fl 5(6)-isothiocyanate (60.3 mg, 15.5 mmol), both purchased from Sigma-Aldrich, were dissolved in 5 ml of DMF and added drop by drop to the DFO solution. The final mixture was stirred for 4 h at room temperature, with an Al-foil protection from the light, and concentrated under vacuum. The residue was washed with 3 × 100 ml acetonitrile and dried under vacuum at 40 °C overnight. The solid residue was chromatographed through a sephadex LH20 lipophilic column using CH_2Cl_2/MeOH (8:2) as eluent to provide pure FlDFO.

Synthesis of FlDFO isomer 5 with an amide linkage (b of Fig. 4A)

The synthesis of the pure 5-amido-FlDFO (compound **1b**) requires prior activation of 5-carboxyfluorescein by an NHS ester with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) to form a 5-carboxyfluorescein NHS ester [28]. To a solution of 5-carboxyfluorescein (50 mg, 0.13 mmol) in anhydrous *N*,*N*-DMF (5 ml) was added (EDC, 31.0 mg, 0.20 mmol), followed by NHS (17.4 mg, 0.15 mmol). The reaction was covered with foil and stirred under nitrogen for 20 h.

The reaction mixture was rinsed into a separatory funnel with DMF (1 ml) and diluted with acetone (20 ml). Buffer (0.05 M, pH 6 phosphate buffer, 25 ml) was added, and the mixture was extracted, with diethyl ether (Et_2O)/ethyl acetate (EtOAc) (2:1, 25 ml). The organic layer was separated, and the aqueous layer was extracted twice with Et_2O/EtOAc (25 ml). The combined organic extracts were washed with water (3 × 20 ml) and brine (1 × 20 ml), dried over Na_2SO_4, and filtered, and the solvents were removed in vacuo to create pure 5-carboxyfluorescein NHS ester. 5-Carboxyfluorescein NHS ester was then used to synthesize pure 5-amido-FlDFO (compound **1b**) with similar conditions of 5(6)-carboxyfluorescein NHS ester.

Synthesis of FlDFO isomer 5 with thio-urea linkage (b of Fig. 4B)

The same experimental conditions were used to synthesize the pure 5-thio-urea-FlDFO (compound **2b**). Instead of using the Fl 5(6)-isothiocyanate mixture as starting reagent, synthesis has been performed with the pure Fl 5-isothiocyanate (Aldrich).

Instruments for FlDFO characterization and Fe sensing test

^{1}H NMR spectra were obtained on a JEOL JNM EX-400 in d_6-DMSO. UV–vis spectra were recorded on a Perkin-Elmer Lambda 35 spectrometer, from 300 to 650 nm. Phosphate buffers were used for all pH values. The melting point was determined on an Electrothermal IA9000 digital melting point apparatus by heating a sample up to 120 °C at 10 °C/min, then up to 150 °C at 1 °C/min.

Spectrofluorimetry spectra were obtained on a Perkin-Elmer LS-45 light spectrometer with a variable slit width. The relative fluorescence intensity was recorded in the range of 480–600 nm (excitation at 460 nm). The response curve of FlDFO to the presence of Fe^{III} was obtained by successive additions of the methanolic Fe chloride solution (5.24 10^{-3} M L^{-1} of $FeCl_3$) or other metals. The calibration graph was obtained by plotting the variation of the fluorescence intensity at its maximum vs. Fe^{III} concentration. More details on measurement conditions are given in the figure captions.

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RESULTS AND DISCUSSION

Identification of synthesized FlDFO

^{1}H NMR studies

Four different products were synthesized: mixture 1 (Table 1), a mixture of 6-FlDFO (compound **1a**) and 5-FlDFO (compound **1b**) isomers with an amide linkage; mixture 2 (Table 1), a mixture of 6-FlDFO (compound **2a**) and 5-FlDFO (compound **2b**) isomers with a thio-urea bond and isolated pure FlDFO isomer 5 with an amide linkage (compound **1b**, b of Fig. 4A) and with a thio-urea linkage (compound **2b**, b of Fig. 4B).

Table 1 Chemical composition of FlDFO mixtures synthesized.

Mixture	$FlDFO_{am}$ (5)/$FlDFO_{am}$ (6)	$FlDFO_{thio}$ (5)/$FlDFO_{thio}$ (6)
1	60/40	
2		80/20

The analysis of NMR spectra of synthesized FlDFOs, whatever the linkage between Fl and DFO, revealed the displacement of the chemical shift value of methylene protons in the α-position of amino-terminal group of DFO after grafting of the Fl moiety, from 2.75 to 3.17 ppm (see Fig. 5 and Table 2).

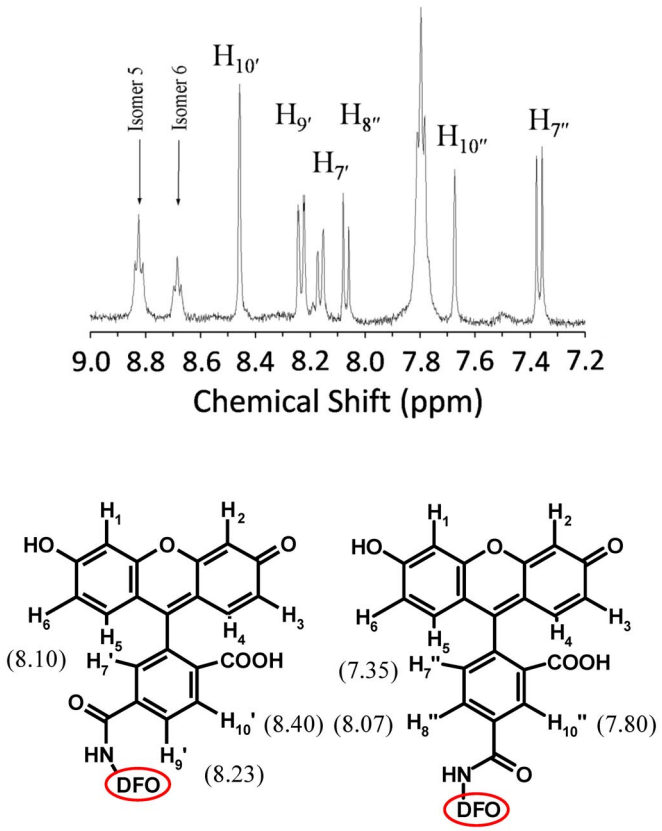

Fig. 5 ^{1}H NMR spectra of 5(6)-$FlDFO_{am}$, mixture 1 in d_6-DMSO. For a better understanding, H locants in 5- and 6-$FlDFO_{am}$ are clearly indicated in 5-(a)- and 6-(b)-FlDFO molecular structures. The number of H in FlDFO structure is given following ^{1}H NMR tradition and is used only for the facility of assignments of signal.

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A low-intensity signal was also observed at around 2.75 ppm after the reaction, which indicates that some DFO molecules remain unconsumed. Some changes were also observed in Fl phenyl residue after reaction. The three protons of the benzyl residue are affected by the substitution of DFO molecules; with different chemical shifts depending on isomers 5 or 6 (see Table 2). For $FlDFO_{am}$, the chemical shifts of benzyl of Fl moiety are detailed in Fig. 5. For a better understanding, the chemical structure with a precise indication of H atom location in 5-isomer and 6-isomer was given in this figure. It is noteworthy that there is no relationship between the numbering of H atoms for the purpose of NMR spectra interpretation and the nomenclature indicating 5- and 6-position (Fig. 4) at the bottom benzene ring of Fl. $FlDFO_{am}$ NMR revealed the presence of amide protons which were attributed to protons resulting from the grafting, for the two isomers, at 8.82 and 8.64 ppm. For $FlDFO_{th}$ mixed isomers, two peaks are observed for the thio-urea bond at 7.88 and 9.59 ppm, also indicating that the grafting had taken place.

Table 2 ^{1}H NMR characteristics of FlDFO mixture of isomers 5 and 6.

Compound	^{1}H NMR (DMSO-d_6) (ppm)
1	1.96 (CH_3C=O, 3H, s), 9.58-9.58 (N-O*H*, 3H, m), 3.4 (CH_2-NOH, with remaining water), 1.2-1.5 (CH_2, 18H, m), 3.0 (C=ONHCH_2, 4H, q), 7.8 (C=ON*H*, 2H, m), 2.3 (NHC=OCH_2, 4H, m), 2.6 (CH_2C=ONOH, with non-deuterated DMSO), 3.17 (CH_2NHC=O-fluorescein, 2H, m), 8.82 and 8.68 (N*H*C=O-fluorescein, isomers 6 and 5 respectively, 0.4 and 0.6 H, t), 6.5 (H_1 and H_2, 2H, m), 6.69 (H_3 to H_6, 4H, m), 8.4 (H_{10}', 0.6H, s), 8.23 (H_9', 0.6H, d), 8.1 (H_7', 0.7H, d), 7.67 (H_{10}", 0.4H, s), 8.07 (H_8", 0.5 H, d), 7.35 (H_7", 0.4H, d)
2	1.96 (CH_3C=O, 3H, s), 9.5-9.6 (N-O*H*, 3H, m), 3.44 (CH_2-NOH, 6H, m), 1.2-1.5 (CH_2, 18H, m), 7.81 (C=ON*H* in DFO, 2H, t), 2.26 (CH_2C=ONH, 4H, m), 2.52(CH_2C=ONOH, with non-deuterated DMSO, m), 3.21 (CH_2-NHC=S, 2H, t), 7.6 (N*H*C=SNH-fluorescein, 1H, m), 9.9 (C=SN*H*-fluorescein, 1H, t), 6.5-6.7 (H_1 to H_6 of FI, 6H, m), 8.2 (H_{10}', 1H, s), 8.1 (H_9', 1H, d), 7.1 (H_{10}', 0.8H, d), 7.80 (H_{10}", 0.2H, s), 7.87 (H_8', 1H, d), 7.85 (H_7', 1H, d)

^{1}H NMR analysis also gave the ratio of the two isomers of FlDFO. Indeed, after the reaction of Fl with DFO, the chemical shifts for H_7, H_8, and H_9, of the benzyl moiety of Fl for both isomers were different. The isomer 5/6 ratio was thus found to be equal to 60:40 for mixture 1, 80:20 for mixture 2, and 100:0 for both pure 5-$FlDFO_{am}$ (compound **1b**) and 5-$FlDFO_{thio}$ (compound **2b**), respectively. All the data are summarized in Table 1. Infrared studies allow us to establish the presence of chemical groups of FlDFO as described in Table 3. The fusion temperature was determined to be in the range of 130–140 °C for all compounds, confirming again the successful synthesis of FlDFO compounds.

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Table 3 Fourier transform-infrared (FT-IR) characteristics of FlDFOs.

Compound	FT-IR (KBr, cm^{-1})	Chemical groups
1	3442	Hydroxyl group O–H elongations
	3376 and 3130	Amide N–H elongations
	3086	Carboxylic O–H elongations
	2963–2924	Methylene C–H elongations
	2453–2369	Aromatic C–H elongations
	1710 and 1644	Carboxylic angular deformations
	1261	C–N elongations
	1133	C–C elongations
2	3440	Hydroxyl group O–H elongations
	3375 and 3127	Amide N–H elongations
	3076	Carboxylic O–H elongations
	2960–2925	Methylene C–H elongations
	2454–2359	Aromatic C–H elongations
	1720 and 1655	Carboxylic angular deformations
	1255	C–N elongations
	1129	C–C elongations

Optical properties of synthesized FlDFO

UV–vis spectroscopic study

Four protolytic forms of Fl have previously been identified, as the cation, neutral species, anion, and dianion [26]. Their equilibria have been studied by absorption spectrometry. The apparent equilibrium constants of protolytic forms have been determined as $pK_1 = 2.09$, $pK_2 = 4.30$, and $pK_3 = 6.41$. Figure 6 shows the absorption spectra of $FlDFO_{thio}$ recorded in a pH range of 5–11. FlDFO dianion has a main absorption peak at 495 nm, with a shoulder around 470 nm, and also has a weak absorption at 322 nm. The anion has a weaker absorption with peaks at 480 and 456 nm. At a low pH value, the monoanionic form predominates, while with the increase of pH value, the dianionic form becomes prevalent.

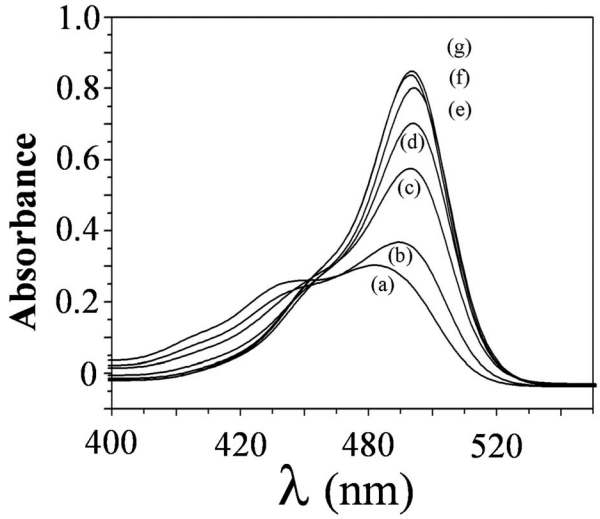

Fig. 6 UV–vis spectra of compound **2a** in the range of pH (a) 5.76, (b) 6.10, (c) 6.58, (d) 7.08, (e) 8.01, (f) 9.82, and (g) 10.50. The FlDFO standard solution was prepared by dissolving 5.6 mg FlDFO in 5 ml of phosphate buffer solution of different pH values.

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The present UV–vis analysis of $FlDFO_{thio}$ shows a spectrum identical to that of Fl absorption at the same pH value [26]. The absorption spectrum recorded for FlDFO clearly shows that grafting of DFO molecules onto Fl dyes does not modify their absorption properties.

Fluorescence study

Figure 7 shows the variation of the fluorescence emission spectra for pure compound **2a** in the pH range of 5–11. At pH 5.5, as the monoanionic form of Fl is mainly present in solution, its fluorescence emission is weak due to a low quantum yield. By increasing the pH value, the fluorescence emission is enhanced as the dianion appears in the solution to reach a maximum for a pH value of 8. For higher pH values, the fluorescence emission decreases slightly. The present analysis suggests that FlDFO gives a maximum emission at pH = 8, while a slight change of pH values does not substantially modify the fluorescence signal. The same observations were made for mixture **1** and both isolated isomers **1b** and **2b**. Thus, all the tests carried out in this study will use a pH value of 8.

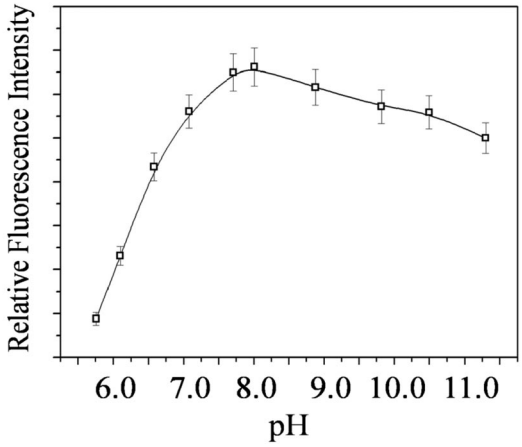

Fig. 7 Variation in fluorescence emission intensity of compound **2a** with pH. The FlDFO standard solution was prepared by dissolving 5.6 mg FlDFO in 5 ml of phosphate buffer solution of different pH values.

FlDFO response to Fe^{III} ions: Selection of sensor

Fluorometric titration of mixtures 1 and 2 and pure compound 1 with Fe^{III}

Figure 8 shows the variation of the fluorescence intensity of mixtures 1 and 2 compared with the intensity of single isomer 5-$FlDFO_{am}$, at 524 nm as a function of increasing iron concentration, with λ_{exc} = 460 nm. Both mixtures show a notable decrease in fluorescence intensity with higher μM concentrations in $FeCl_3$. For the mixture of isomers 5 and 6 with an amide linkage between Fl and DFO (mixture of compounds **1a** and **1b**, named mixture 1) at equimolar concentration, the fluorescence signal is maintained at around 44 % of its initial fluorescence intensity (Fig. 8a). Even higher Fe^{3+} ion quencher concentrations are unable to completely switch off the emission. The remaining fluorescence is attributed to an inefficient FlDFO isomer. The amido-DFO can be located at position 5 or 6 of the phenyl moiety of Fl (Fig. 4). By ^1H NMR spectra integration, the mixture of isomers has been determined to have a ratio of 60:40 in favor of the 5-position (Table 1). This coincides well with the fluorimetric titration with Fe^{III}, for which only around 60 % of the total light emission could be switched off at equimolar concentrations. This gives the first indication that only the isomer 5 responds to Fe^{3+} ion complexation by fluorescence quenching while the fluorescence of the isomer 6 is insensitive to the complexation with Fe^{3+} ions. In the same way, the mixture of isomer 5 and 6 of $FlDFO_{thio}$ (mixture 2) has been determined to have a ratio of 80:20 in favor of the 5-position by ^1H NMR (Tables 1 and 2), and a 20 % remaining

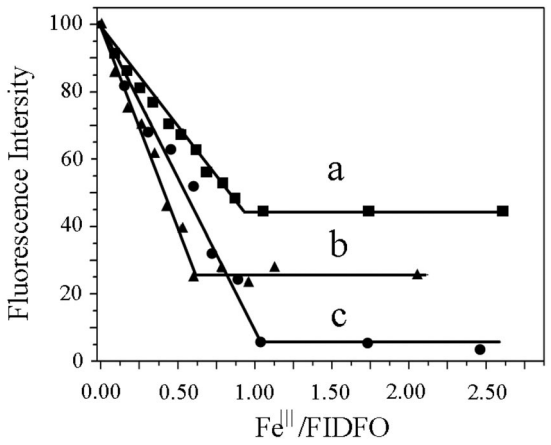

Fig. 8 Variation in fluorescence by titration with Fe^{III} methanolic solution of (a) mixture of 5- and 6-FlDFO isomers (compounds **1a** and **1b** mixed in a ratio: 60:40), (b) mixture of isomers 5- and 6-FlDFO isomers (compounds **2a** and **2b** mixed in a ratio of 80:20), and (c) pure compound **1b**. To the FlDFO standard solution, methanolic Fe chloride solution ($5.24\ 10^{-3}$ ML^{-1}) was successively added. (λ_{exc} = 460 nm).

fluorescent signal is observed at equimolar concentration in $FeCl_3$, as shown in Fig. 8b. Once again, the percentage of the initial intensity quenching coincides with the percentage of 5-isomer in the mixture.

Moreover, pure 5-FlDFO (either **1b** or **2b**) showed almost complete extinction of the fluorescence signal after addition of equimolar concentration of Fe^{III}. This indicates clearly that the disappearance of fluorescence is directly related to the complexation of 5-isomer with Fe^{3+} ions. This part of the study reveals a quite important piece of information that 6-FlDFO is unable to switch fluorescence off after complexation of an Fe ion and only isomer 5 responds sensitively to the complexation with Fe^{3+} ions by its fluorescence quenching, whatever the linkage used in the present study between Fl and DFO. This can be explained easily since when amino- or thio-urea-DFO is located at the 5-position of the bottom benzene ring of Fl (Fig. 4), the electron transfer from DFO upon complexation with Fe^{III} ions to very large conjugated π-system is possible and can switch off the fluorescence, while at the 6-position location, this is not the case. In the 6-position, the electron transfer from DFO complexing with Fe^{III} ions to the large conjugated π-system of Fl is impossible. That is why there is no response upon complexation with Fe^{III} ions.

Dynamic response to Fe^{III}

The sensitivity of a sensor, i.e., the response time to an ion, is essential for an efficient dosing and sensing. A study of the dynamic response with increasing Fe ion concentration was carried out on a pure 5-$FlDFO_{thio}$ (compound **2b**). The changes of fluorescence emission were followed at 524 nm, and characteristic responses were obtained for different concentrations as are shown in Fig. 9. An exposure of 10 min is sufficient to have maximum quenching at low concentrations such as 1.1×10^{-2} μM or less, but the response time increases up to 95 min to reach the level of stability of the fluorescence emission for higher concentrations. This could be due to the solubility of our sensor molecule in the methanolic aqueous solution. Parabactin extracted from *Paracoccus denitrificans* has been used as a fluorescence-based siderophore sensor molecule for the determination of bioavailable Fe ions in oceanic waters [31]. When this sensor molecule was encapsulated in a thin porous film prepared by the sol-gel method, for up to 600 min, the fluorescence intensity did not reach stability and continued to decrease no matter what the level of ferric ion concentration is in the solution. This shows that our fluoroionophore sensor molecule, FlDFO, is much more sensitive than parabactin. In a very comprehensive paper on Fe^{III} quantitative determination using three types of fluorescent signalling units, NBD, MA, and NCP (2-cyano-

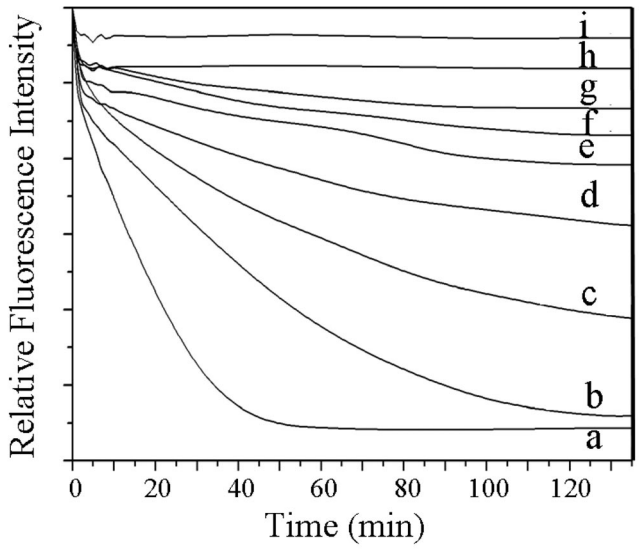

Fig. 9 Response time of compound **2b** monitored at 524 nm with increasing Fe ion concentration in solution. 5 ml Fe ion solution with different concentrations was successively added to the FlDFO standard solution. The measurement was realized as soon as the Fe ion solution was added to FlDFO standard solution. (a: 2.16×10^{-1}, b: 1.09×10^{-1}, c: 7.58×10^{-2}, d: 6.50×10^{-2}, e: 4.33×10^{-2}, f: 3.25×10^{-2}, g: 2.16×10^{-2}, h: 1.08×10^{-2}, and i: 0.54×10^{-2} μM).

naphtho[2,3-c]-2*H*-pyrroyl) coupled with DFO, the fluorescent intensity was measured in all the cases 10 s after the fluorescent siderophore molecules were in contact with a solution with or without containing Fe(III) ions. No detailed uptake data was presented [32]. The improvement of the solubility of our fluoroionophore molecules in a suitable solution can certainly enhance the contact of the sensor molecules with ions to be dosed. This study also shows clearly that our fluoroionophore sensor molecule responds to a very low concentration of Fe ion up to 2.16 10^{-2} μM although around 90 min is necessary to reach fluorescence stability. This means that our sensor is quite sensitive to a low concentration of Fe ion.

*Calibration of compounds **1b** and **2b***

The response curve of FlDFO to the presence of Fe^{III} was obtained by successive additions of the methanolic Fe chloride solution (5.24 10^{-3} M L^{-1} of $FeCl_3$). The calibration graph was obtained by plotting the variation of the fluorescence intensity at its maximum vs. Fe^{III} concentration. Figure 10 shows the fluorescence intensity variation (Fig. 10A) and the calibration graph of compound **1b** (Fig. 10B), after addition of $FeCl_3$ solution. No noticeable difference could be recorded between calibration of compounds **1b** and **2b**, which confirms that fluorescence quenching is not dependent on the chemical nature of the link between Fl and DFO. The fluorescence variation was shown to be linear for a wide range of Fe concentration from 1.10^{-8} to 3.10^{-6} M L^{-1}. This is in agreement with metabolic Fe concentration in the labile Fe pool of the body.

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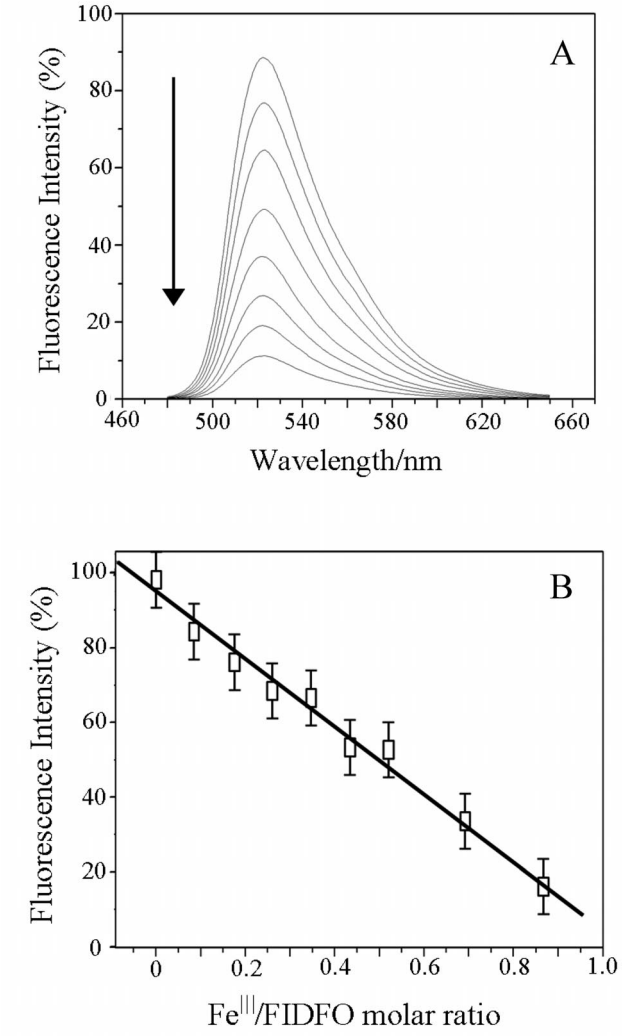

Fig. 10 Emission spectra of compound **1b** (excitation at 460 nm) and monitoring for increasing concentration of Fe ions in solution. To the FlDFO standard solution, methanolic Fe chloride solution ($5.24\ 10^{-3}$ ML^{-1}) was successively added.

Mechanism of fluorescence extinction

Recently, it has been shown that Fl is a directly conjugated electron donor and acceptor system. Furthermore, the fluorescence properties of Fl derivatives can be controlled by intramolecular photo-induced electron transfer (PET) [29]. PET is a well-known mechanism through which the fluorescence of a fluorophore is quenched by electron transfer from the donor to the acceptor. Studies suggested that the electron transfer is involved for molecules containing two groups, a fluorophore (electron acceptor) and its quencher (electron donor) located in close proximity and with no ground-state interaction with each other (Fig. 11) [19,22,23]. In the ground state (Fig. 11A), the highest occupied molecular orbital (HOMO) energy level of the electron donor (DFO) is lower than the HOMO energy level of Fl moiety, preventing electron transfer from the donor to acceptor site. Thus, when the fluorophore is photo-chemically excited no electron interaction between FI and DFO can occur, leading to a high fluores-cence emission.

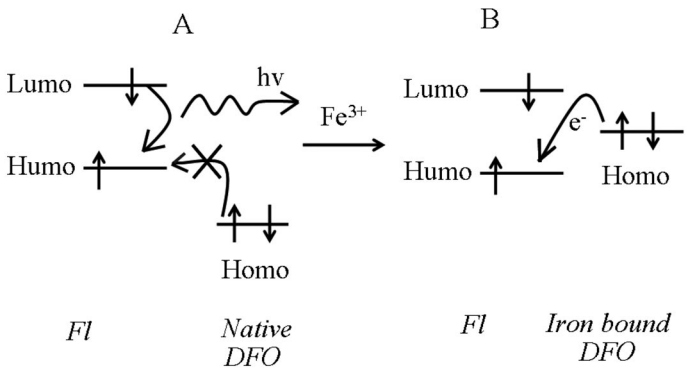

Fig. 11 Schematic representation of quenching mechanism of receptor unit (DFO) upon its complexation with Fe ions, (A) before and (B) after complexation.

When the DFO receptor unit binds an Fe^{III} atom (Fig. 11B), the electron donor HOMO energy level increases enough to overcome the electron acceptor HOMO energy level, which allows a single electron to be transferred from the donor to the excited fluorophore. Consequently, the fluorophore loses its energy thermally instead of emitting fluorescence. The use of the fluorescent Fe-responsive FIDFO molecule allows the direct determination of the Fe^{III} concentration in the solution.

Response to other metal ions

The following metal ions, Al^{III}, Cu^{II}, Ca^{II}, and Ni^{II}, were subsequently tested with FlDFO since all can be found at trace levels in the human organism and can bind with the DFO receptor unit, even though their affinity constants for DFO are lower than Fe^{III}. FlDFO solutions were prepared using the same procedure as for Fe^{III} titration, and increased amounts of methanolic solution of equal metal concentration were added and the fluorescence emission was recorded 150 min after addition (Fig. 12). Al^{III}, Ca^{II}, and Ni^{II} showed no substantial effect on fluorescence emission of FlDFO, although they all form complexes with DFO in solution. Cu^{II} ions are able to complex with DFO and to extinguish light emis-

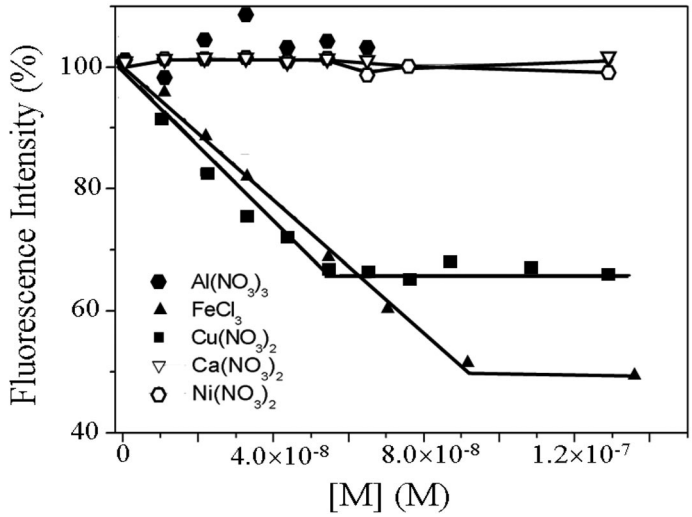

Fig. 12 Response of compound **2b** to different metallic ions at pH 8. To the FlDFO standard solution, methanolic metal ion chloride solution (5.24 10^{-3} ML^{-1}) was successively added. (λ_{exc} = 460 nm).

sion of FlDFO molecules, but only in a very narrow concentration range (less than 8.0×10^{-8} M L^{-1}). Furthermore, as Cu^{II} is the most unlikely to be found in the human body, we can suggest that it will not substantially affect the measurement of the concentration of Fe in the blood. Recently, a tryptophan-containing fluoroionophore sensor with high selectivity to and specific selectivity for Pb ions in water has been designed and tested. The detection limit is down to 0.15 μM [15]. While our fluoroionophore sensor molecule can detect to a very low concentration of Fe ion down to 2.16 10^{-2} μM, showing its high selectivity and sensitivity to Fe ion even at very low concentrations.

CONCLUSIONS

A novel fluoroionophore molecule, FlDFO, for dosing with Fe ions in solution has been designed, based on the combination of two subunits: fluorescein (Fl), a fluorescent probe, with a very high quantum yield, responsible for the transfer of information and desferrioxamine (DFO), an ionophore selected to provide the highest metal complexation ability. This molecule is highly fluorescent in a free state but light emission can be switched off by the complexation of Fe^{III} with DFO. FlDFO has two different isomers (5 and 6), and the fluorescence tests on both isomers have suggested that only isomer 5 was able to undergo a quenching in light emitted after Fe complexation. The dynamic studies of the probe pointed out that 10 min was enough for a low concentration in Fe, in order to reach a stabilized light emission, and more time is required if at increased concentration. On the basis of the above analysis, it is clear that the fluoroionofore molecules FlDFO designed in this study are highly sensitive and selective sensor molecules for dosing with Fe ions and sensing. The strategy developed by this study can be extended to the design of other efficient fluoroionophore molecules for other ion dosing and sensing and could open exciting new doors for the conception of new, portable, stable, regenerable, and simple cost-effective nanosensors.

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