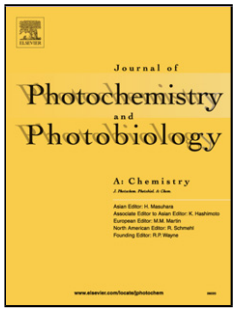

Title: Cascade OFF–ON–OFF fluorescent probe: Dual detection of Fe^{3+} ions and thiols

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Title page

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Cascade OFF–ON–OFF fluorescent probe: dual detection of Fe^{3+} ions and thiols

Authors:

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Highlights

> One dual-functional fluorescence probe based on Rhodamine B was designed

> **LDF** shows a highly selective fluorescent response to trace amounts of Fe^{3+} ions

> [**LDF+Fe**$^{3+}$] ensemble is also further successfully utilized for detection of the thiols

> This system can also be applied in Hela Cells

Cascade OFF–ON–OFF fluorescent probe: dual detection of Fe^{3+} ions and thiols

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Abstract

A new novel Rhodamine B-based fluorescent probe was synthesized for the selective cascade signaling of Fe^{3+} and thiols (glutathione, homo-cysteine, cysteine). Non-fluorescent rhodamine derivatives can selectively detect Fe^{3+} over some other metal ions in ethanol-water (4/1, v/v, pH 7.0) media, leading to prominent fluorescence OFF–ON switching. The obtained **LDF-Fe^{3+}** ensemble can subsequently serve as a sensitive and selective chemosensor for thiols, exhibiting complete signal quenching (fluorescence ON–OFF switching). The practical use of its Fe^{3+} ions ensemble was demonstrated by its application to the detection of thiols in the living cells.

Keywords: rhodamine; Fe^{3+} ions ; thiols; Hela Cells

Introduction:

Development of chemosensors is used for sensing and recognizing of environmentally and biologically important heavy and transition metal ions. On account of its simplicity, high sensitivity and instantaneous response, fluorescence is increasingly important for sensing various metal ions and biological thiols [1-10].

Iron has its own biological significance and it directly involved in the cell function [11-12]. For example, Fe^{3+} acts as oxygen carrier in hemoglobin, and plays important roles in enzyme catalysis and cellular metabolism, a deviation of its concentration from normal levels can lead to several disorders, including Alzheimer's, Huntington's, and Parkin-son's disease. Furthermore, Fe^{3+} can cause various diseases depending on its concentration, such as fibrosis with irreversible damage to various organs

and tissue damage. In this regard, the development of chemosensor that can detect Fe^{3+} is very important to prevent and solve environmental and health problems induced by Fe^{3+}.

Biological thiols, including glutathione (GSH), cysteine (Cys) and homocystein (Hcy), play crucial roles in maintaining the appropriate redox status of biological systems [13-14]. The most abundant cellular thiol is GSH. It is an essential endogenous antioxidant that plays a vital role in cellular defense against toxins and free radicals. Abnormal levels of GSH can lead to cancer, aging, heart problems, and other diseases. Anabnormal level of cysteine may cause skin lesions and liver damage. In addition, homocysteine is a risk factor for Alzheimer'sand cardiovascular diseases.

Rhodamine derivatives, which are ideal chemosensors due to their particular spirolactam structures and excellent photophysical properties, offer a 'naked-eye' straightforward detection method used extensively in the detection of metal ions. The spirolactam structure of rhodamine derivatives provides very weak fluorescence strength and UV absorption, while ring-opening of the spirolactam brings about strong UV absorption and fluorescence enhancement along with an apparent color change. Based on this mechanism, chemosensors based on rhodamine have been developed extensively.

Nowadays, although a number of probes have been used for the fluorescence sensing of Fe^{3+} and thiols by various workers [15-20], the majority of probes currently available cannot be operated in water let alone in buffered solution or organic solvents, which limited their practical applications. Hence, there is a great demand for the development of novel fluorescent chemosensors for Fe^{3+} which has good water solubility and be suitable for cell imaging studies. In addtion, relatively few studies have been devoted to the development of fluorescence sensors which are sensitive to Fe^{3+} ions and thiols. It is necessary to create a probe which is capable of simultaneous discrimination of Fe^{3+} ions and thiols from others.

Herein, we report a simple cascade "OFF–ON–OFF" fluorescent chemosensor for Fe^{3+} ions and thiols based on the equilibrium between spirolactam (non-fluorescent) to the ring open amide (fluorescent) form of rhodamine chromophore. In general, the chemosensor showed a specific selectivity toward Fe^{3+} ions over other common metal ions, leading to prominent fluorescence OFF–ON switching. The resultant **LDF-Fe^{3+}** ensemble could selectively recognize thiols, exhibiting complete ON–OFF signal quenching.

2. Experimental

2.1 Materials and General methods

Rhodamine B was purchased from Aladdin Reagent Co.,Ltd and used without purification. Organic solvents were purchased from Shanghai Lingfeng chemical reagent Co., Ltd. were all analytical reagent grades. The salts used in stock solutions of metal ions were $NaNO_3$, $CdCl_2{\cdot}H_2O$, $Co(NO_3)_2{\cdot}6H_2O$, $Mg(NO_3)_2$, $CuSO_4$, $Zn(NO_3)_2{\cdot}2H_2O$, $Pb(NO_3)_2$, $Ni(NO_3)_2{\cdot}6H_2O$, $AgNO_3$,

$Mn(NO_3)_2{\cdot}4H_2O$, $AlCl_3{\cdot}6H_2O$ and $FeCl_3.6H_2O$. All of them were of analytical reagent grade and used without purification. Water used in experiment was double distilled water. The UV-visible spectra measurements were performed with a UV-visible spectrophotometer (Unico UV-2800H) equipped with 1.0 cm quartz cells. Fluorescence was recorded on a spectrofluorimeter (Shimadzu RF-5301PC) equipped with 1.0 cm quartz cell.

2.2 Synthesis of probe

Synthesis of probe **LDF** is outlined in **Scheme1**. 2-amino-3', 6'-bis (diethylamino) spiro [isoindoline-1, 9'-xanthen]-3-one (LDF-1) was first synthesized following literature procedures (Fig.S1) [21-23].

To a solution of LDF-1(456mg,1mmol) in glacial acetic acid(20mL) were added maleic anhydride (98mg,1mmol) and were heated under reflux for 4 h. The solvent was removed in vacuum to give a pink solid[24]. The crude product was purified by flash chromatography (petroleum/ ethyl acetate=3:1, v/v) to afford a yellow solid LDF (322.6mg, yield: 60%). **^{1}HNMR**(Fig.S2) (400 MHz, C_3D_6O) δ 7.97 (m, 1H), 7.69 (dtd, *J*=28.6, 7.5, 1.1 Hz, 2H), 7.22 (d, *J* = 7.6 Hz, 1H), 6.86 (s, 2H), 6.65 (d, *J* = 8.9 Hz, 2H), 6.40 (dd, *J* = 8.9, 2.6 Hz, 2H), 6.29 (d, *J* = 2.6 Hz, 2H), 3.39 (q, *J* = 7.0 Hz, 8H), 1.15 (t, *J* = 7.0 Hz, 12H).**^{13}CNMR**(Fig.S3)(100MHz,C_3D_6O) δ(ppm) 167.27,164.63, 154.78,149.96, 141.00, 134.65, 134.60, 131.89, 129.93, 127.85, 125.86, 123.84, 108.78, 105.40, 97.71, 76.66, 57.73, 44.70, 12.86. **ESI-MS** (Fig.S4): calcd for $C_{32}H_{32}N_4O_4$ $[M+H^+]^+$:537.2424; found: 537.2499.

3. Results and discussion

3.1 Fluorescence OFF–ON sensing for Fe^{3+}

3.1.1 Proposed mechanism

As some literatures reported [25-27], the equilibrium between the non-fluorescent spirolactam form and the fluorescent ring-open amide form of rhodamine derivatives has been proven to be an efficient platform for the construction of fluorescent sensors for numerous heavy metal cations due to its large absorption coefficient and high fluorescence quantum yield. In our present work, this typical ion-recognition mechanism was utilized for the successive detection of Fe^{3+} ions, and then Michael addition of thiols damage the conjugate structure of the probe which led to the absence of the fluorescence. This mechanism rise fluorescence OFF–ON–OFF switching. The mechanism is shown in Scheme 2.

3.1.2 Fluorescence titration investigaton

Fluorescence titration of probe **LDF** was conducted in the solution of ethanol-water (4/1, v/v, pH 7.0) media. The free **LDF** itself was almost non-fluorescent due to its ring-closed spirolactam structure. After the addition of Fe^{3+}, probe **LDF** exhibited a color change from almost colorless to pink as well

as a bright jacinth fluorescence. A strong emission band centered at 581 nm was observed with an extreme fluorescence enhancement compared to the metal-free **LDF**.

The relative affinities of Fe^{3+} toward sensor **LDF** were evaluated from fluorescence spectroscopic titration experiments in ethanol-water (4/1, v/v, pH 7.0) media, as shown in Fig.1(A). The concentration of **LDF** was maintained at 10μM, while the concentration of Fe^{3+} was varied between 0 and 60μM. The fluorescence spectra were recorded at an excitation wavelength of 455 nm and emission wavelength of 525–700 nm. For free **LDF**, no obvious characteristic emission of rhodamine derivatives was observed. With increasing concentrations of Fe^{3+}, the fluorometric titration reaction curve showed a steady and smooth enhancement, which was used as the basis of Fe^{3+} sensing. The recognition interaction was completed immediately after the addition of Fe^{3+}.When the concentration of Fe^{3+} was greater than 30μM, the fluorescence intensity did not increase any further and a plateau was reached. The chart in Fig.1 shows that the dependence of fluorescence intensity at 581 nm on Fe^{3+} concentration. Plotting fluorescence intensity versus Fe^{3+} concentration (0.1-1 equiv.) afford a good liner relationship (R^2=0.98704) Fig. 1(B).

3.1.3 Selectivity investigation

To get an insight into the selectivity, fluorescence spectrum measurements of the probe **LDF** were carried out with the addition of various metal ions. Among the metal ions investigated 2 equiv. Fe^{3+} selectively enhance the fluorescence intensity of **LDF** (Fig. 2). While other metal ions, such as, Na^{+}, K^{+}, Ag^{+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} , Ni^{2+} and Al^{3+} do not enhance fluorescence intensity or change fluorescence color under the same spectroscopic condition used for the Fe^{3+}.

In order to further explore the selectivity and anti-interference of the sensor, some competitive ions were first added to the detection solution, and then Fe^{3+} ions were added after half an hour. As shown in Fig. 3, most of the detection systems exhibited minimum interference in the detection of Fe^{3+} (20μM each) also did not induce any obvious interference in the fluorescence sensing of Fe^{3+}. The association constants was 5.827×10^5 for **LDF-Fe^{3+}**.

To determine the interaction stoichiometry between sensor **LDF** and Fe^{3+}, Job's method was employed using an absorbance intensity at 558 nm as a function of molar fraction of **LDF** because the total concentration of **LDF** and Fe^{3+} ion was located at 20μM[28]. The maximum absorbance was observed when the molar fraction of **LDF** reached 0.50 (Fig. 4.), which was indicative of a 1: 1stoichiometry complexation between **LDF** and Fe^{3+}.

*3.1.4. UV-vis absorption spectra of **LDF** with Fe^{3+} ion*

The sensing property of **LDF** was investigated in detail utilizing the Fe^{3+}. As shown in Fig.5. The absorption spectrum of **LDF** changes upon Fe^{3+} addition. Sensor **LDF** exhibited almost no absorption peak in the visible wavelength due to the spirolactam form of **LDF**. However, a new band centered at

about 558nm emerged upon the gradual addition of Fe^{3+} indicating the sensor **LDF**-Fe^{3+} complex formation and Fe^{3+} induced spirolactam ring-opening processes. Moreover, the titration solution displayed a characteristic color change of rhodamine derivatives from colorless to pink, indicating that probe **LDF** could serve as a "naked-eye" indicator for Fe^{3+}in ethanol-water (4/1, v/v, pH 7.0) media., with increasing proportionally and levelled off when the concentration of Fe^{3+} reached 50uM.

3.1.5 Fluorescence response to pH

Appropriate pH condition for the successful operation of the fluorescence sensing was evaluated [29-30]. Without the addition of the Fe^{3+}, the ring opening of the Rhodamine B-based sensor **LDF** occurred under acidic conditions(PH<5.0) due to protonation, while no fluorescence change was observed with pH values over 5 (Fig. 6). However, the gradual addition of Fe^{3+} led to an obvious fluorescence enhancement over a wide pH range from5.0 to 8.0, which was attributed to the similar opening of the spirolactam structure. Because the most remarkable Fe^{3+} induced OFF–ON fluorescence changes occurred under the physiological pH window, all the fluorescence measurements were conducted at pH 7.0.

3.2 Fluorescence ON-OFF sensing for thiols

*3.2.1 Fluorescence spectra of **LDF-Fe^{3+}** with thiols*

Interestingly, when the **LDF–Fe^{3+}** complex was treated with thiol-containing small molecules such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) , the color of the solution changed from pink to colorless, and the emission was almost completely quenched within 1 min (Fig. 7A), while other natural amino acids including L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan and L-valine did not show any interference to the detection. This observation suggested that the probe **LDF** could not only act as a sensor for Fe^{3+} but also for thiols in a successive manner. Once the Fe^{3+} ion interacted with the sensor **LDF**, the spirolactum ring was opened, yielding a high fluorescence emission. However, when treated with the thiols, the conjugate structure of maleic anhydride was broken, leading to the absence of fluorescence. The thiols sensing capability of the obtained complex was further investigated in detail with fluorescence titration analysis (Fig.8). The titration curve showed an excellent linear decrease (Fig.7B.) and about 2 equiv. of thiols (compared with **LDF**) was required to obtain almost completely fluorescence quenching.

3.2.2 Application in living cells

To test the capability of LDF to image in living cells, Hela Cells were treated with various amounts of Fe^{3+} and GSH [31-34]. Initially, Hela Cells were incubated with probe **LDF** (10μM; Fig. 9B) in

PBS buffer containing 1/100 DMSO for 30 minutes at room temperature and then it was treated with $FeCl_3.6H_2O$ (10μM, Fig. 9C) for 10 minutes. Their fluorescence images became bright (Fig. 9C and 9D), implying that the intracellular uptake of Fe^{3+}ions complexed with probe **LDF** yielded pink fluorescence. Upon further incubation of Hela Cells with various amounts of GSH (20uM) for 10 min, fluorescence images became dim (Fig. 9E and F), indicating that the uptake of GSH resulted in the decomplexation of intracellular [**LDF** + Fe^{3+}] ensemble to fluorescent **LDF**. Therefore, the off–on–off fluorescence imaging of probe **LDF** was accomplished in Hela Cells by the intracellular complexation / decomplexation interaction modulated by $Fe^{3+}$and GSH. Furthermore, these results indicate that probe **LDF** is cell membrane permeable and able to response to Fe^{3+}/GSH in the living cells.

Conclusions

In summary, we report the synthesis and characterization of a new probe for the cascade fluorogenic detection of Fe^{3+} ion and thiols. The probe **LDF** showed excellent "OFF–ON" fluorescence signals with high sensitivity and selectivity in the presence of Fe^{3+} ion, whereas it remained silent in the presence of other cations such as Na^+, K^+, Ag^+, Mg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} , Ni^{2+}. A successive "ON–OFF" fluorescence switching was then observed in the presence of thiols in ethanol-water (4/1, v/v, pH 7.0) media.

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List of Scheme and Figures

Scheme 1. Synthetic route for probe **LDF**

Scheme 2. Proposed mechanism for the recognition of Fe^{3+} ion and **LDF- Fe^{3+}**

Figure.1 Fluorescence spectra of **LDF** (10μM) in the presence of various metal ions (10μM each) in ethanol-water (4/1, v/v, pH 7.0).

Figure.2 Fluorescence spectral data of 3 mL (10μM) probe **LDF** at pH=7.0 with 4 equiv metal ions Fe^{3+}, Al^{3+}, Cr^{3+}, Na^{+}, K^{+}, Ag^{+},Mg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} in ethanol-water (4/1, v/v, pH 7.0).

Figure.3 (A)Fluorescence titrations of **LDF** (10μM) with Fe^{3+} ions in ethanol-water (4/1, v/v, pH 7.0). Inset:fluorescence emission intensity changes with increasing Fe^{3+} ions. (B)Fluorescence intensity as a function of Fe^{3+} concentration.

Figure.4 Job's plot obtained for the determination of binding stoichiometry between LDF and Fe^{3+} in ethanol-water (4/1, v/v, pH 7.0). The total concentration of **LDF** and Fe^{3+} was maintained at 30μM. Absorbance was measured at 565nm.

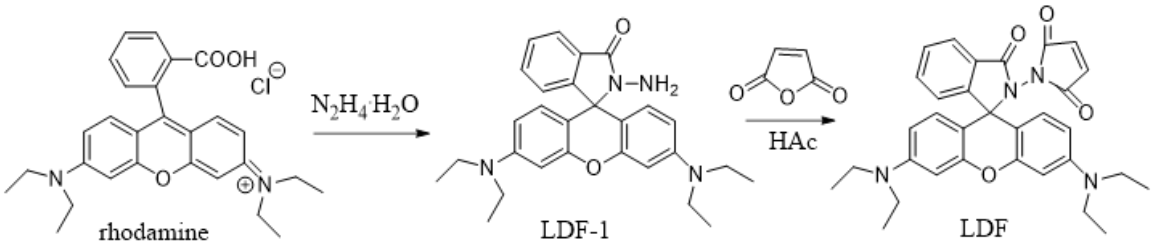
Figure.5 UV-vis spectra and absorbance changes (565nm) recorded for **LDF** (10μM) in ethanol-water (4/1, v/v, pH 7.0).

Figure.6 Fluorescence intensities recorded for **LDF** (10μM, ethanol-water 4:1, v/v) at various pH values in the absence and presence of 4 equiv.Fe^{3+}.

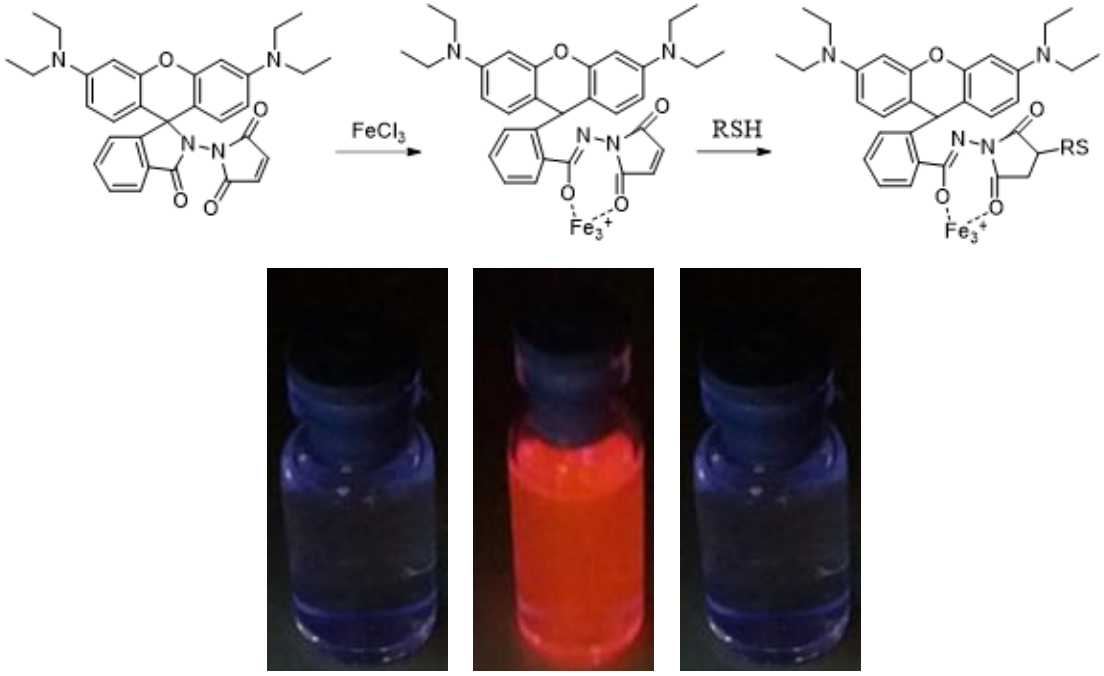
Figure.7 (A)Fluorescence intensity recorded for **LDF** (10μM) with 4 equiv Fe^{3+} ions upon gradual addition of GSH (0-50equiv. for Fe^{3+}) in ethanol-water (4/1, v/v, pH 7.0). The spectra were obtained 1 min after Fe^{3+} addition.(B)Linear response of fluorescence intensity at 586 nm of the LDF-Fe^{3+} complex to the GSH concentration changes in ethanol-water (4/1, v/v, pH 7.0). [L] = 10 μM,[Fe^{3+}] = 40 μM. The unit of x is 10^{5}mmol/L.

Figure.8 Fluorescence spectra obtained for **LDF** (10μM) in the presence of Fe^{3+} (40μM) in ethanol-water (4/1, v/v, pH 7.0) 10min after the addition of various amino acids (40μM each).

Figure.9 (A) Fluorescence microscope images of Hela cells only, (B) images of cells + **LDF** (10μM), (C) images of cells + **LDF** + Fe^{3+}(1μM), (D)images of cells + **LDF** + Fe^{3+}(4μM), (E) images of cells + [LDF + Fe^{3}] + GSH(5μM), (F) images of cells + [LDF + Fe^{3}] + GSH(20μM).



Scheme 1. Synthetic route for probe **LDF**



Scheme 2. Proposed mechanism for the recognition of Fe^{3+} ion and **LDF- Fe^{3+}**

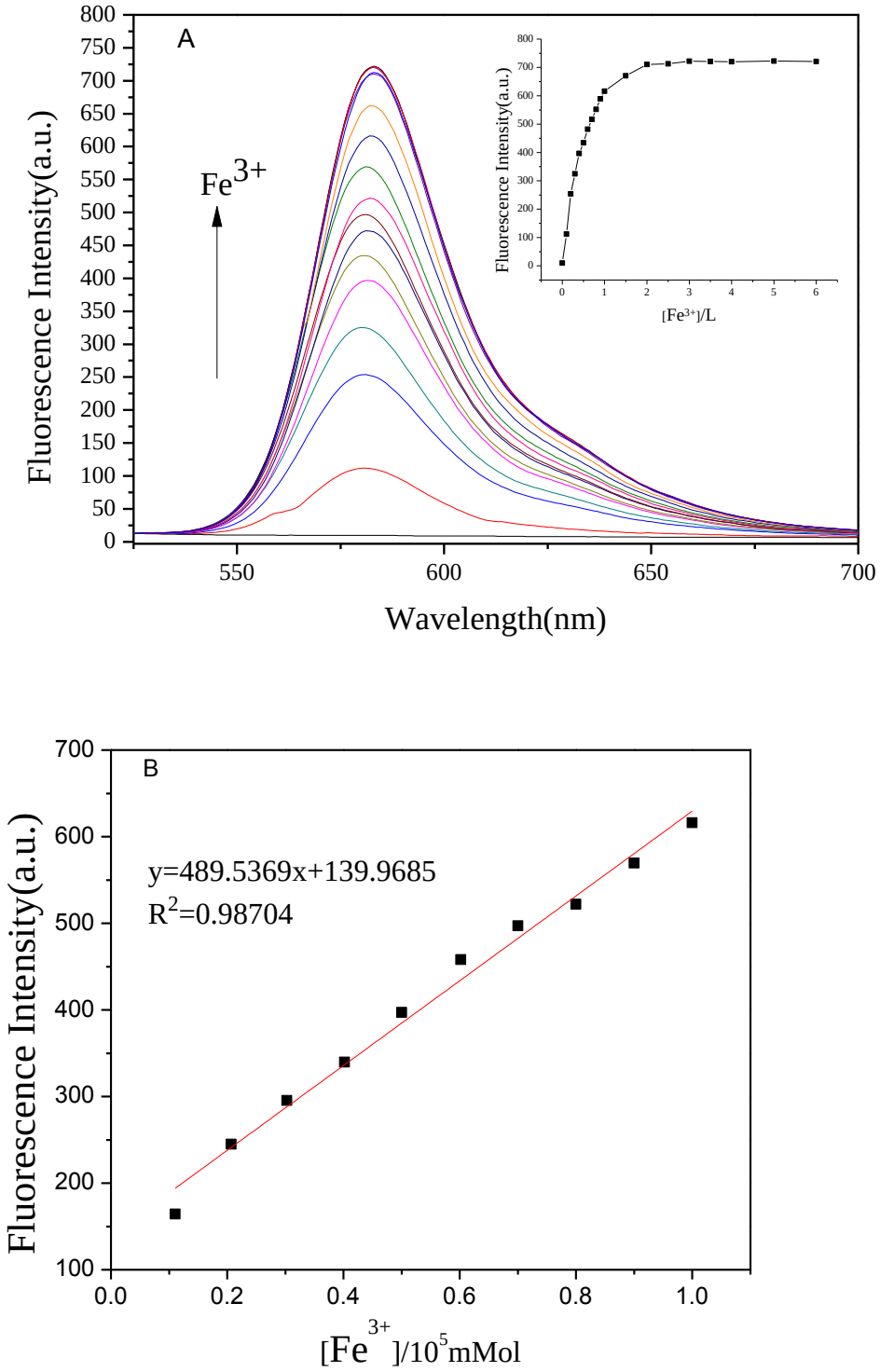


Fig.1. (A) Fluorescence titrations of 3mL **LDF** (10μM) to Fe^{3+} ions (0,0.1,0.2,0.3,0.4,0.5, 0.6,0.7,0.8,0.9,1,2,3,4,5equiv.) in ethanol-water (4/1, v/v, pH 7.0). (B)Fluorescence intensity as a function of Fe^{3+} concentration.

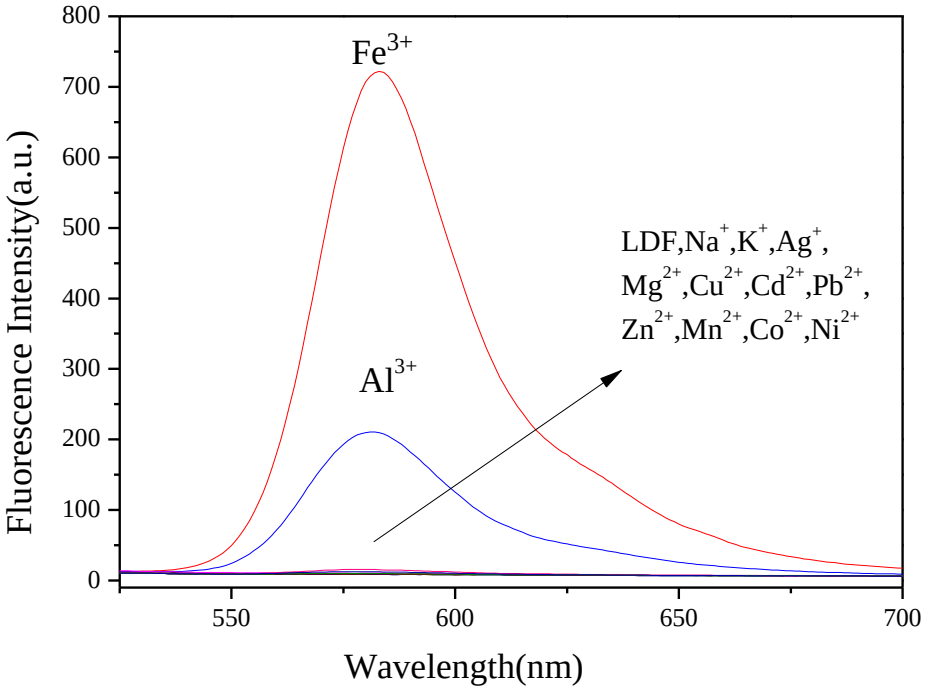


Fig.2. Fluorescence spectra of **LDF** (10μM) in the presence of various metal ions (20μM each) in ethanol-water (4/1, v/v, pH 7.0).

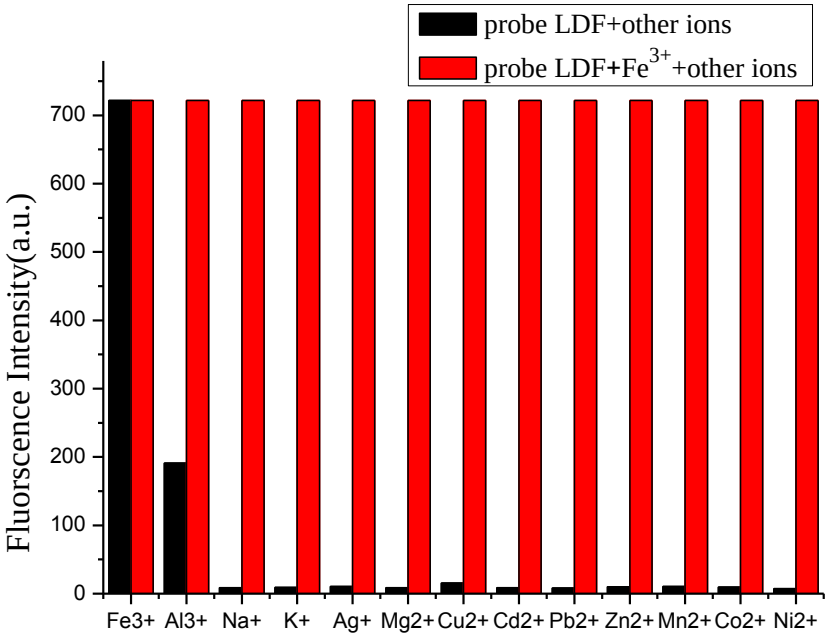


Fig.3 Fluorescence spectral data of 3 mL (10μM) probe LDF at pH=7.0 with 2 equiv metal ions Fe^{3+}, Al^{3+}, Na^{+}, K^{+}, Ag^{+},Mg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} in ethanol-water (4/1, v/v, pH 7.0).

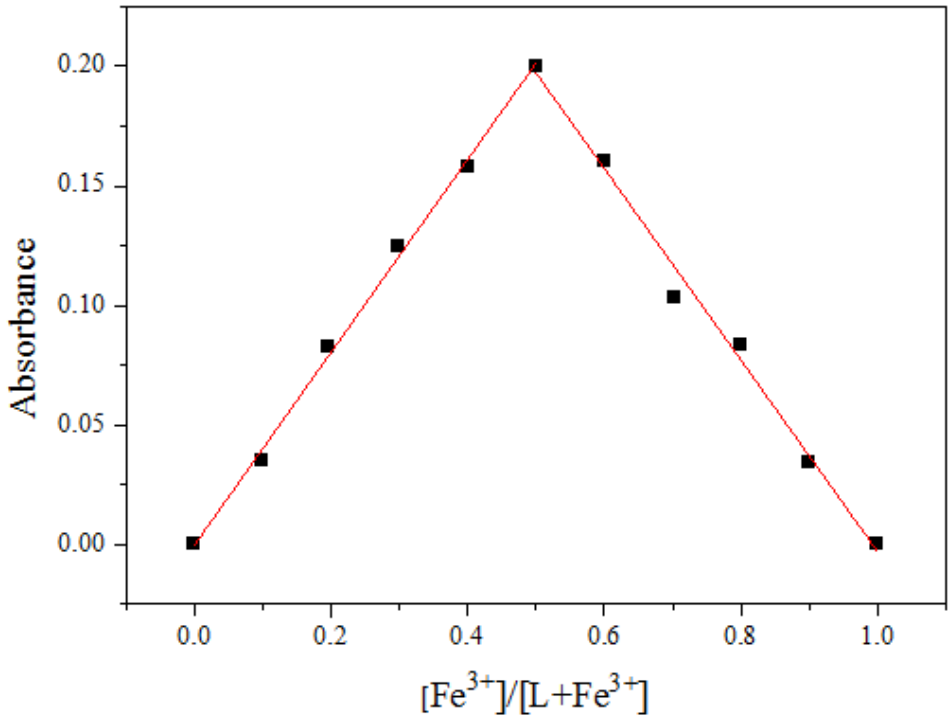


Fig.4 Job's plot obtained for the determination of binding stoichiometry between **LDF** and Fe^{3+} in ethanol-water (4/1, v/v, pH 7.0). The total concentration of **LDF** and Fe^{3+} was maintained at 20μM. Absorbance was measured at 565nm.

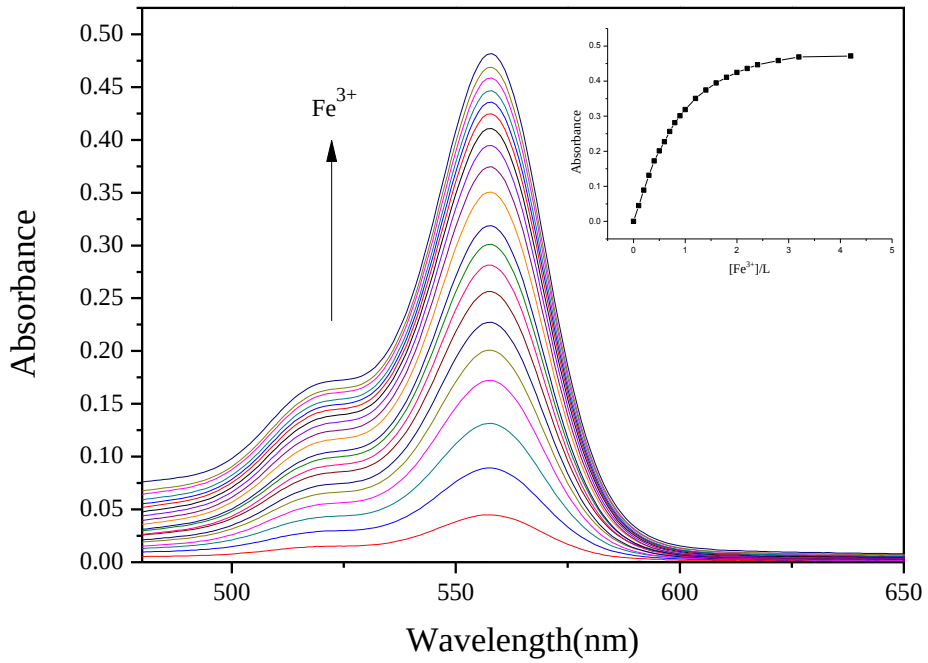


Fig.5. UV-vis spectra and absorbance changes (565nm) recorded for **LDF** (10μM) in ethanol-water (4/1, v/v, pH 7.0).

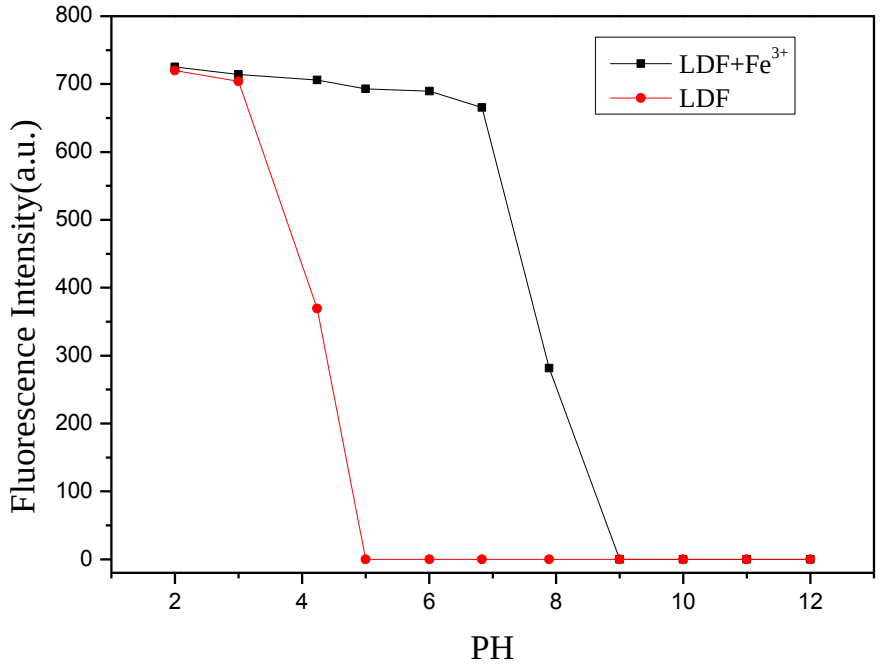
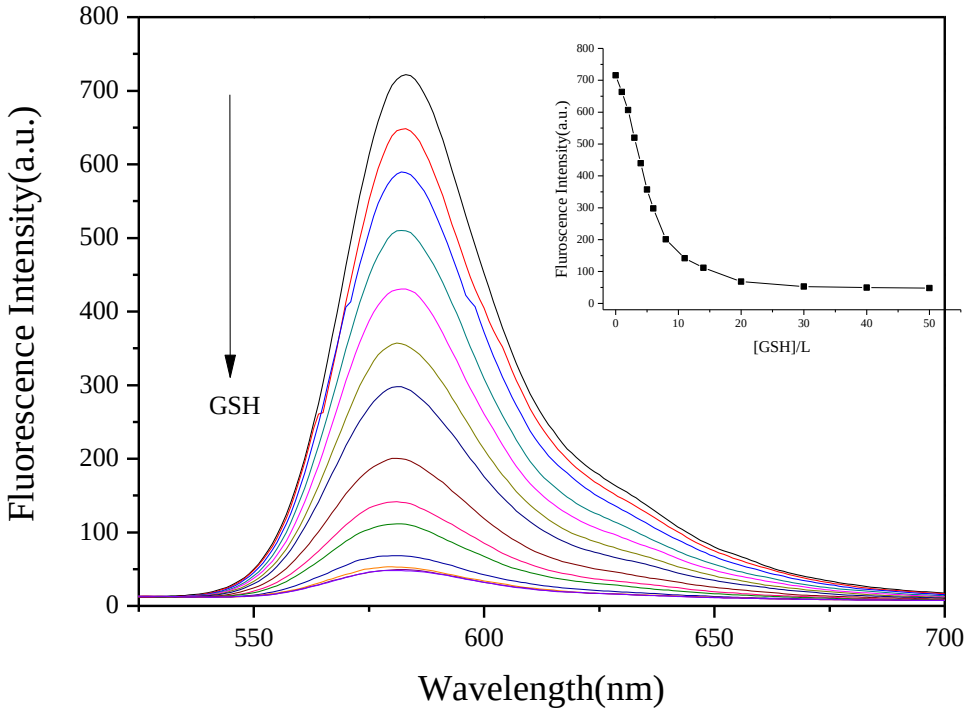


Fig.6. Fluorescence intensities recorded for **LDF** (10μM, ethanol-water 4:1, v/v) at various pH values in the absence and presence of 2 equiv.Fe^{3+}.



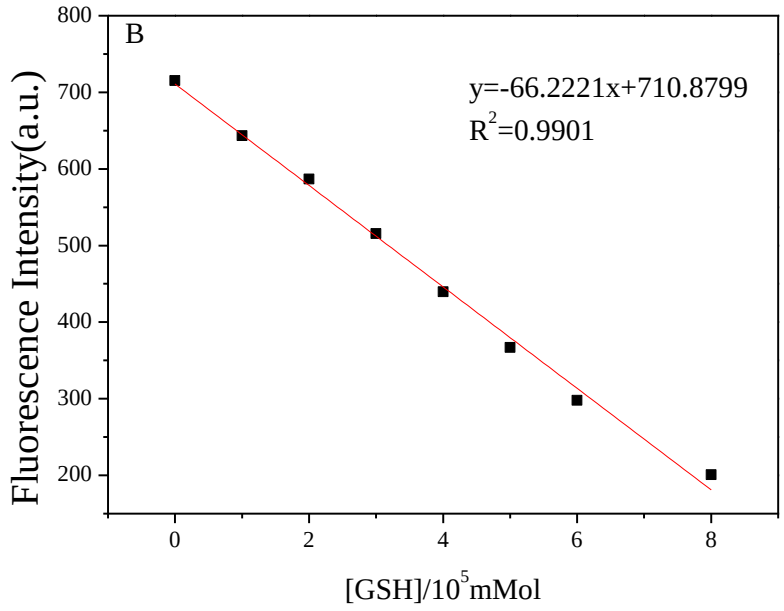


Fig.7.(A)Fluorescence intensity recorded for **LDF** (10μM) with 2 equiv Fe^{3+} ions upon gradual addition of GSH (0-5 equiv. for Fe^{3+}) in ethanol-water (4/1, v/v, pH 7.0). The spectra were obtained 1 min after Fe^{3+} addition.(B)Linear response of fluorescence intensity at 586 nm of the **LDF-Fe^{3+}** complex to the GSH concentration changes in ethanol-water (4/1, v/v, pH 7.0). [L] = 10 μM,[Fe^{3+}] = 20 μM. The unit of x is 10^5mmol/L.

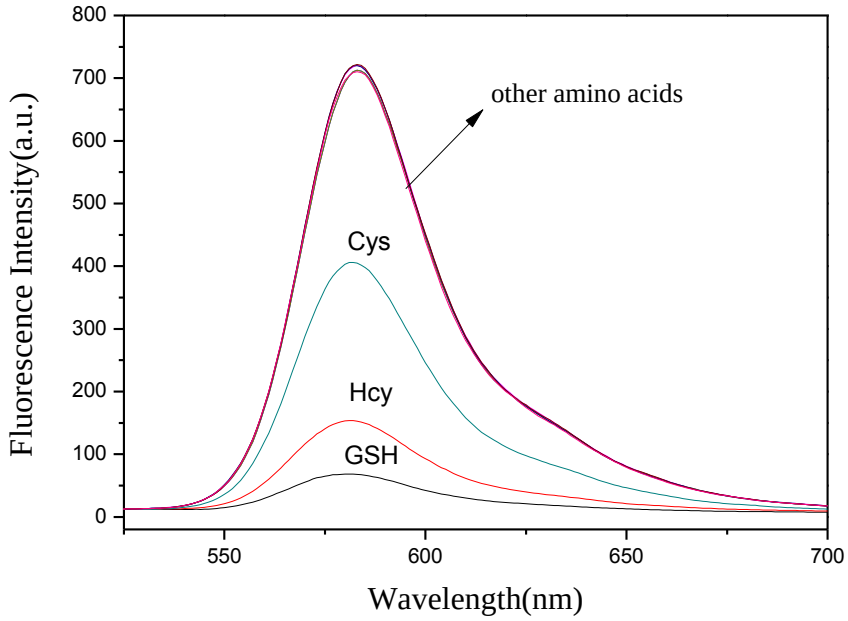


Fig.8.Fluorescence spectra obtained for **LDF** (10μM) in the presence of Fe^{3+} (20μM) in ethanol-water (4/1, v/v, pH 7.0) 10min after the addition of various amino acids (40μM each).

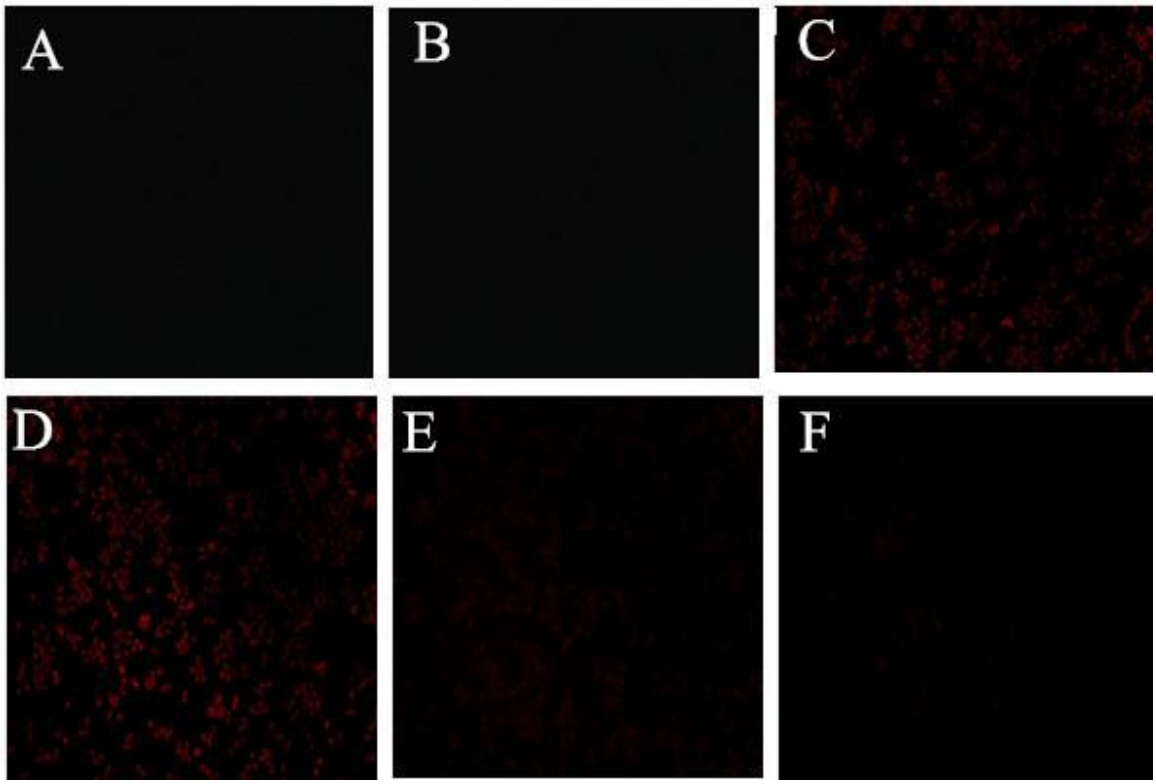


Fig.9. (A) Fluorescence microscope images of Hela cells only, (B) images of cells + LDF (10μM), (C) images of cells + LDF + Fe^{3+}(10μM), (D)images of cells + LDF + Fe^{3+}(20μM), (E) images of cells + [LDF + Fe^{3}] + GSH(20μM), (F) images of cells + [LDF + Fe^{3}] + GSH(40μM).