Supporting Information of

Reasonably constructed fluorescent chemosensor based on dicyanoisophorone skeleton for discriminative sensing Fe^{3+} and Hg^{2+} as well as imaging in Hela cells and zebrafish

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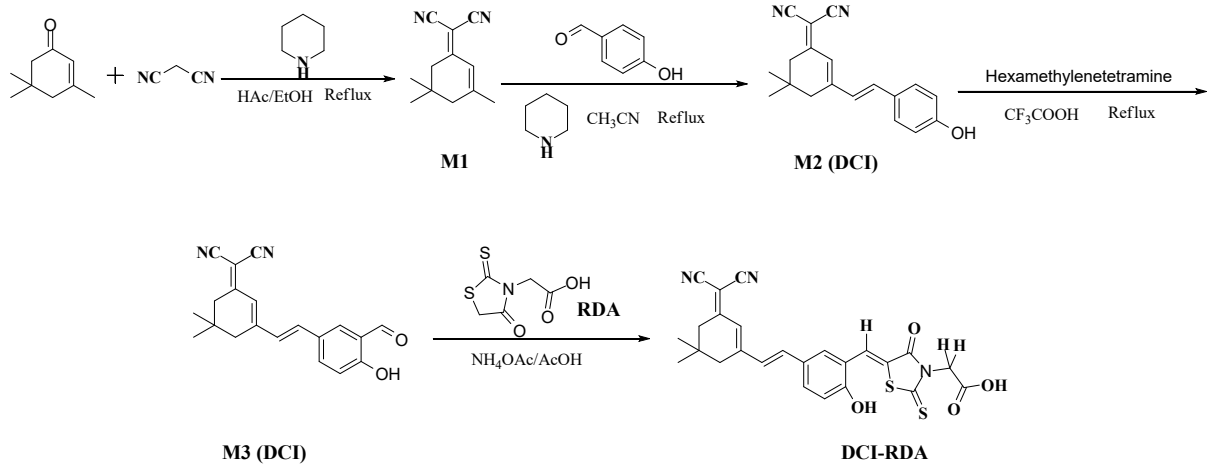
S1. General information

Unless stated otherwise, all analytical grad chemicals and solvents used in this paper were purchased from commercial vendors. The salts used in stock solutions of metal ions were Na_2SO_4, KNO_3, $AgNO_3$, $Pb(NO_3)_2$, $CoSO_4·7H_2O$, $ZnSO_4·7H_2O$, $MgSO_4$, $NiSO_4·6H_2O$, $CuSO_4·5H_2O$, $FeSO_4·7H_2O$, $MnSO_4·2H_2O$, $Cr(NO_3)_3·9H_2O$, $Al(NO_3)_3·9H_2O$, $Fe(NO_3)_3·9H_2O$ and $Hg(ClO_4)_2·3H_2O$. The ^1H and ^{13}C NMR spectra were collected on a Bruker AV-400(400 MHz) in a DMSO-d_6 solution with TMS as the internal standard. Fluorescence spectra was determined by a Hitachi F-4600 fluorescence spectrophotometer.

S2. Synthesis of compounds



Scheme S1. The synthetic pathways of probe DCI-RDA

S 2.1. Synthesis of compound 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)malononitrile (M1)

Isophorone (4.14 g, 30 mmol), malononitrile (2.64 g, 40.0 mmol), piperidine (0.4 mL, 4.0 mmol) and glacial acetic acid (0.24 g, 4 mmol) were dissolved in 80 mL EtOH. Then, the mixture was refluxed for 8 h under argon atmosphere. After the solvent was removed, the residue was dissolved with CH_2Cl_2, washed with water, and dried over Na_2SO_4. Finally, the solvent was evaporated under reduced pressure, and the crude product was purified by silica column chromatography (PE/EA = 1:8, v/v) to give a white solid (4.3 g, 77.1%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.56 (d, J = 1.3 Hz, 1H), 2.53 (s, 2H), 2.23 (s, 2H), 2.05 (s, 3H), 0.95 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): 171.3, 162.4, 119.4, 113.4, 112.6, 76.1, 44.7, 41.7, 31.9, 27.1, 25.0.

S 2.2 Synthesis of compound 2-(3-(4-hydroxystyryl)-5,5-dimethylcyclohex-2-en-1-ylidene)malononitrile (M2)

M1 (1.86 g, 10 mmol), p-hydroxybenzaldehyde (1.46 g, 12 mmol), five drops of piperidine were dissolved in 30 mL anhydrous acetonitrile. The mixture was refluxed for 5 h under argon atmosphere. Subsequently, the solvent was removed under reduced pressure. The resulting residue was dissolved in 20 mL dichloromethane, washed with water for three times, and dried over anhydrous Na_2SO_4. After the removal of the solvent, the crude product was purified by silica column chromatography (PE) to afford the **M2** as a red solid (2.42 g, 83.4%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.00 (s, 1H), 7.56 (d, J = 8.7 Hz, 2H), 7.35~7.12 (m, 2H), 6.80~6.79 (m, 3H), 2.60 (s, 2H), 2.52 (s, 2H), 1.01 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.2, 159.3, 156.7, 138.3, 129.9, 127.2, 126.1, 121.3, 115.8, 114.2, 113.4, 74.9, 42.3, 38.19, 3.62, 27.3.

S 2.3 Synthesis of compound 2-(3-(3-formyl-4-hydroxystyryl)-5,5-dimethylcyclohex-2-en-1-ylidene)malononitrile (M3)

Hexamethylenetetramine (0.50 g, 3.6 mmol) was added to solution of **M2** (0.87 g, 3 mmol) in trifluoroacetic acid (20 mL). The mixture was refluxed for 5 h. After complete reaction, the solvent was evaporated under a rotary evaporator, diluted with water and then neutralized with NaOH until the pH reached 7.0. Subsequently, the solution was extracted with dichloromethane. The organic layer was washed with water for three times, dried over anhydrous sodium sulfate. The crude product was purified by silica gel column chromatography with dichloromethane as the eluent to afford **M3** as a yellow solid

(0.45g, 47.2%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.32 (s, 1H), 10.31 (s, 1H), 7.98 (d, J = 2.2 Hz, 1H), 7.89 (dd, J = 8.9, 2.4 Hz, 1H), 7.31 ~ 7.25 (m, 2H), 7.10 (d, J = 8.9 Hz, 1H), 6.89 (s, 1H), 2.60 (s, 2H), 2.52 (s,2H), 1.01 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 190.7, 170.4, 161.9, 156.2, 136.9, 134.8, 128.8, 127.8, 127.7, 122.7, 122.2, 118.2, 113.9, 113.3, 75.6, 42.4, 38.2, 31.7, 27.4.

S2.3 Synthesis of probe DCI-RAD

Compound **M3** (343.4 mg, 1.08 mmol) and rhodanine-3-acetic acid (203.3mg, 1.07 mmol) were added into the acetic acid (25 mL) in the presence of ammonium acetate (83mg) under nitrogen atmosphere. The mixture was stirred 24h at 100 ℃. After the mixture cooled to room temperature, the reaction was quenched using ice water. The precipitate was filtered and washed thoroughly with water. The solvent was then evaporated, and the product was purified by column chromatography on silica with petroleumether/ethyl acetate (1/1 in v/v) as the eluent. The solvent was evaporated, and probe DCI-RAD was obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 13.33 (s, 1H), 11.46 (s, 1H), 7.98 (s, 1H), 7.80 (d, J=8.5 Hz, 1H), 7.60 (s, 1H), 7.28 (dd, J=41.9, 16.1 Hz, 2H), 7.02 (d, J=8.6 Hz, 1H), 6.83 (s, 1H), 4.74 (s, 2H), 2.59 (s, 2H), 2.55 (s, 2H), 1.03 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ=194.2, 170.6, 167.7, 167.0, 159.4, 156.6, 137.4, 132.4, 130.8, 129.6, 128.7, 128.3, 122.6, 121.7, 120.7, 117.5, 114.4, 113.6, 76.1, 45.4, 42.8, 38.7, 32.1, 27.9; HRMS(M+Na-H): m/z = 513.0792.

S3. Calculations

S3.1 Determination of Detection Limit

The detection limit was calculated based on fluorescence titration as a function the solubility of Hg^{2+}/Fe^{3+} at $\boldsymbol{\lambda_{em}}$ 622 nm. The fluorescence emission spectrum of free probe DCI-RAD was measured over 3 times to determine standard deviation for blank measurement. A linear plot was constructed with average values of the intensities against the concentration of Hg^{2+}/Fe^{3+} ions for determining the slope. Using the slope the detection limit was calculated from the following equation. [Dalton Trans. 2014, 43, 1881-1887; Analyst, 2017, 142, 2067-2089. Anal. Chem. 1996, 68, 1414-1418.]

$$LOD = \frac{3\sigma}{K}$$

Where, σ is the standard deviation of the blank solution and K is the slope between intensity versus sample concentration.

S3.2 Determination of Binding Constant

The binding constant of the DCI-RAD + Hg^{2+}/Fe^{3+} complex formed in solution has been determined by using the standard Benesi-Hildebrand (B-H) equation. [Anal. Chem. 1996, 68, 1414-1418.]

$$\frac{1}{F - F_o} = \frac{1}{K_a(F_{max} - F_o)\,[M]} + \frac{1}{F_{max} - F_o}$$

Where, F_o is the fluorescence intensity of free probe DCI-RAD, F is the observed fluorescence intensity at any given concentration of Hg^{2+}/Fe^{3+}, F_{max} is the intensity at saturation point with the Hg^{2+}/Fe^{3+}, K_a is the association constant and [Hg^{2+}/Fe^{3+}] is the concentration of the Hg^{2+}/Fe^{3+} ions in micromolar.

S3.3 Determination of Stoichiometry by Continuous Variation Plot (Job's plot) Measurement

The stoichiometric binding ratio of probe DCI-RAD and Fe^{3+}/Hg^{2+} ion was confirmed by continuous variation emission analysis at λ_{em} 622 nm, the resulting data was plotted as change in fluorescence intensity, emission intensity in the vertical axis against the mole fraction, X_M of Hg^{2+}/Fe^{3+} ions in the horizontal axis. [Anal. Chem. 1996, 68, 1414-1418.]

S4. **Cell Cultures and Imaging**

Hela cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (0.5U·mL^{-1} of penicillin and 0.5 g·mL^{-1} strepomycin) on a cell culture flask at 37℃ in an atmosphere of air with 5% CO_2 and constant humidity. Each cell line was seeded in a 6-well plate for 24 h. The cells were initially incubated with DCI-RAD (10 μM) in culture medium for 30 min at 37℃. After washing three times with PBS to remove the remaining DCI-RAD, the Hela cells were incubated in the absence and presence of Hg^{2+}/Fe^{3+} (100 μM) in culture medium for another 30 min at 37 ℃. The imaging was carried out using inverted fluorescence microscopy (Olympus IX71, Japan).

S5. **Imaging of Zebrafish**

The 3-7 days old zebrafishes post-fertilization were purchased from Eze-Rinka Company (Nanjing, China). The zebrafishes were cultured in 5 mL of embryo medium supplemented with 1-phenyl-2-thiourea

(PTU) in 6-well plates for 24 h at 30 ℃. Zebrafishes were divided into three groups and both incubated with **DCI-RAD** (10 μM) for 1 h. After washing three times to remove the remaining **DCI-RAD**, one group as control group and the others group further treated with Hg^{2+} and Fe^{3+} (100 μM) for another 1 h, respectively. The fluorescence images were acquired with stereo microscopy (Olympus SZX16, Japan).

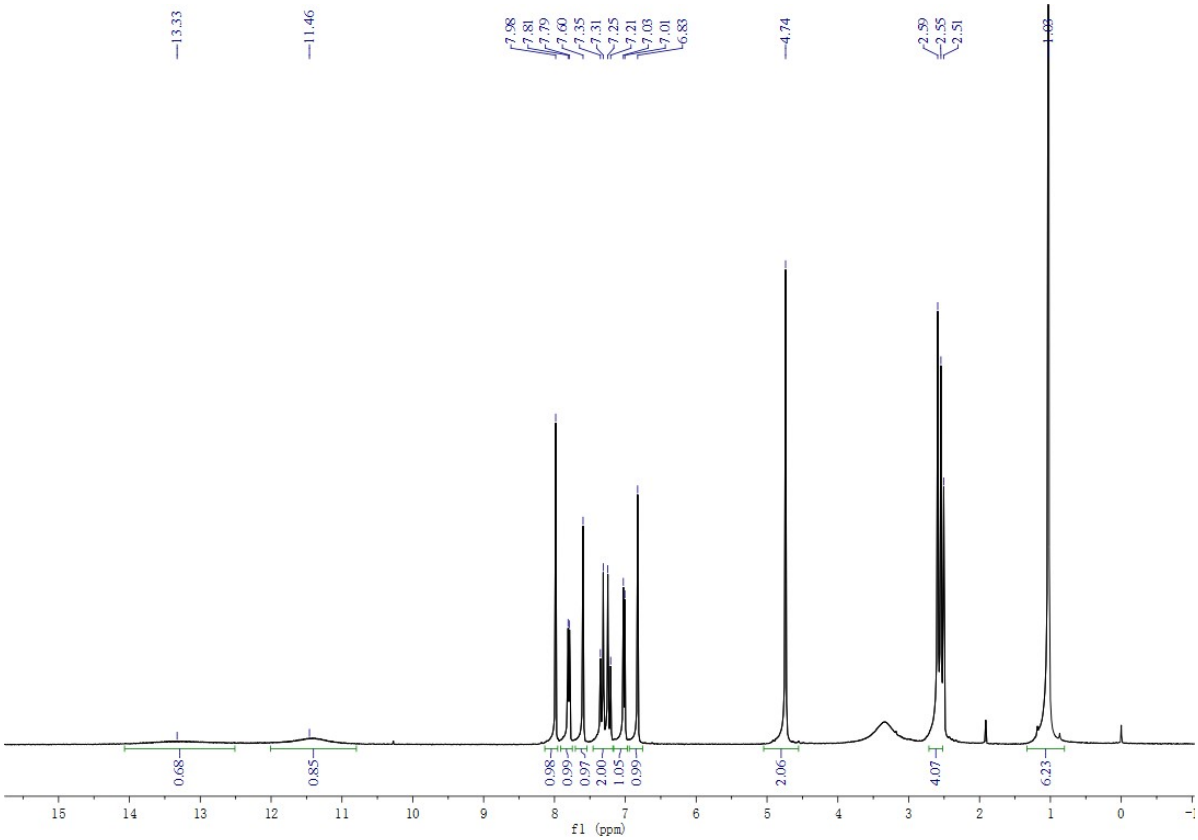


Figure S1. ^{1}H NMR Spectrum of probe DCI-RAD in DMSO-d_6

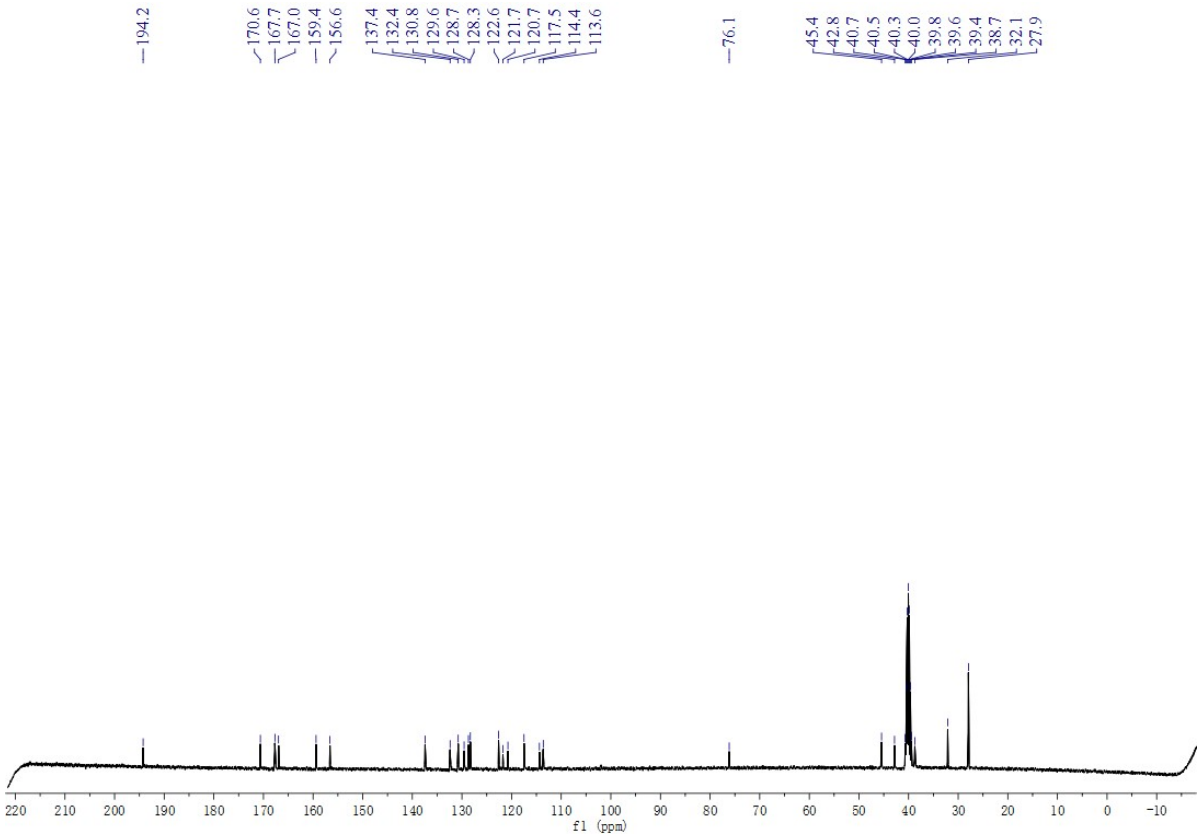


Figure S2. ^{13}C NMR Spectrum of probe DCI-RAD in DMSO-d_6

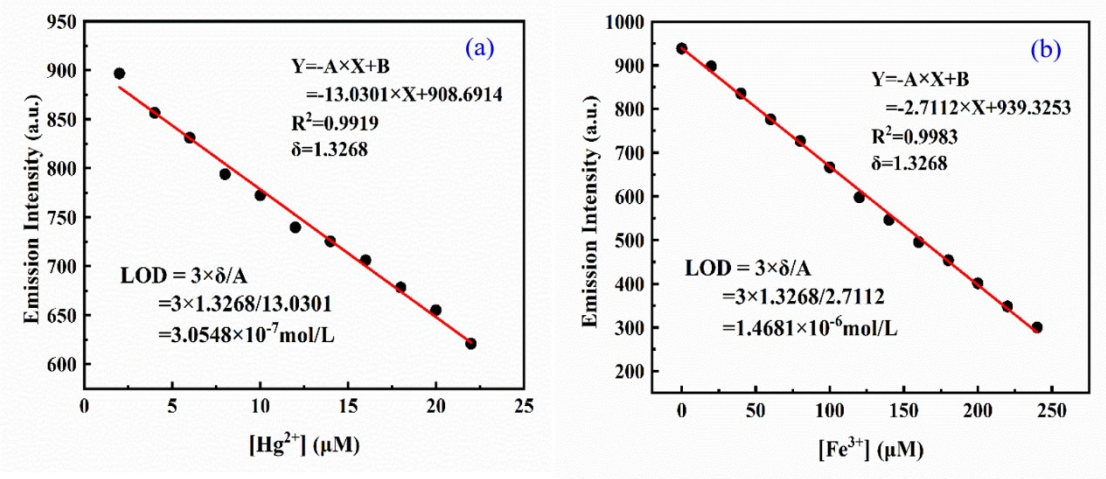


Figure S3. The LOD of DCI-RDA against Hg^{2+}(A) and Fe^{3+} (B).

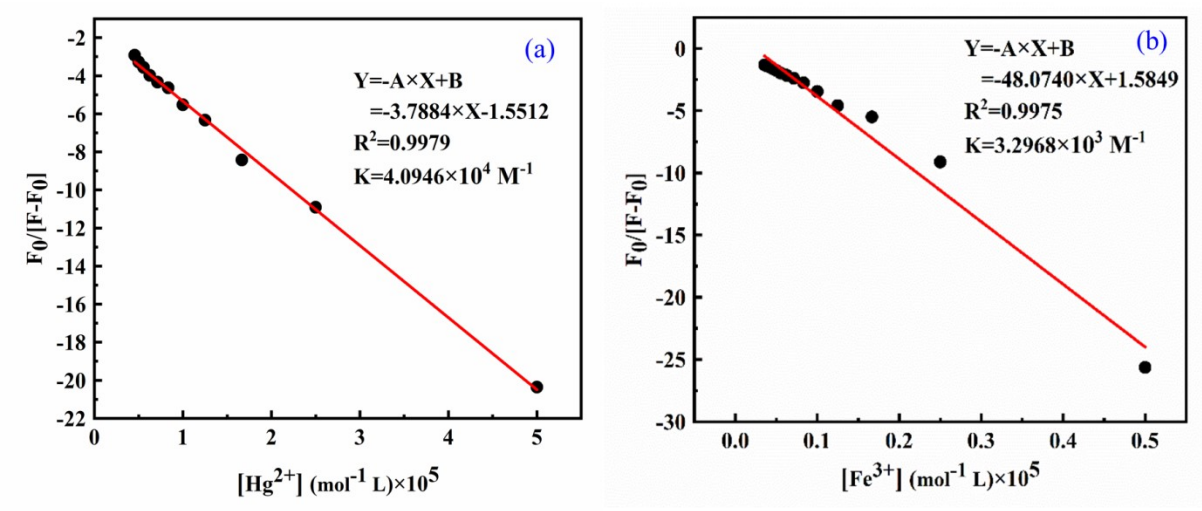


Figure S4. The association constant (Ka) of DCI-RDA combined with Hg^{2+}(A) and Fe^{3+} (B).

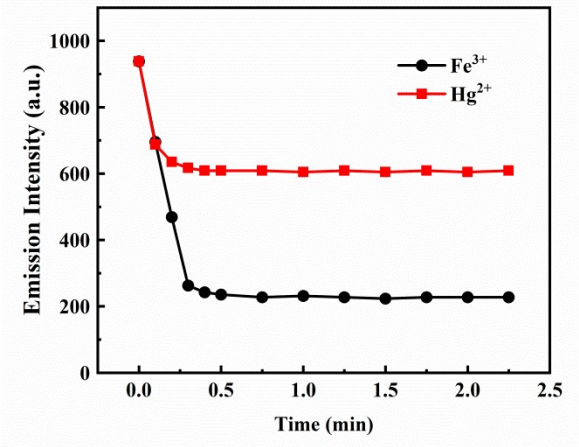
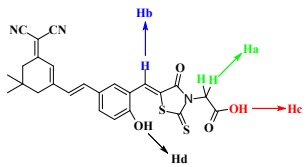


Figure S5. Effect of response time on the fluorescence intensity of DCI-RDA (20 μM)

Table S1 The chemical shift changes on values of change before and after



Proton chemicalshift(ppm)	Ha	Hb	Hc	Hd	CH_3	CH_2	=CH	HC=CH	Ph
Free DCI-RDA	4.74	7.98	13.33	11.46	1.03	2.55	7.60	7.28	7.80,7.02,6.83

DCI-RDA-Hg^{2+}	4.38	8.10	disappearing	11.45	1.03	2.55	7.60	7.28	7.80,7.02,6.83

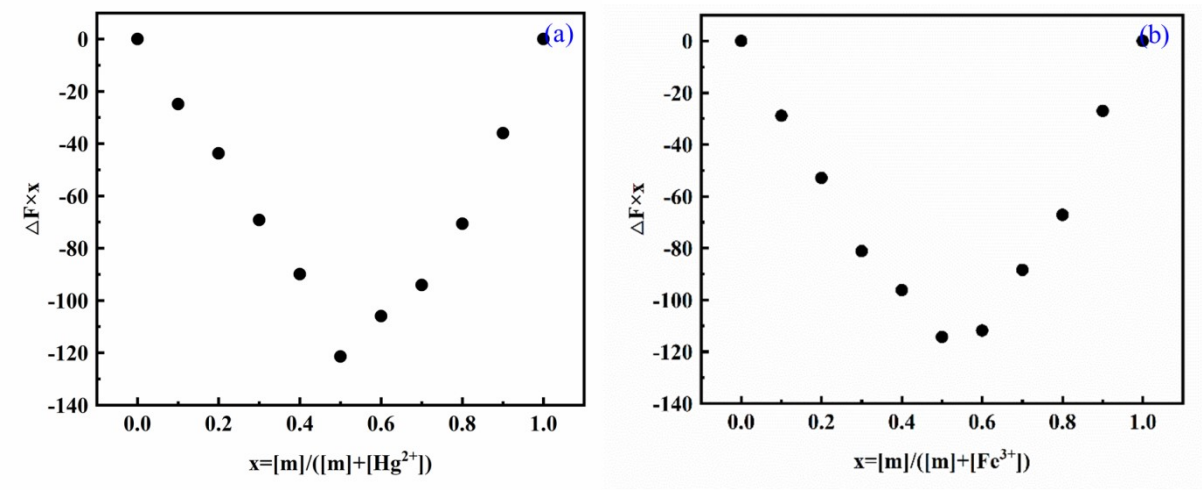


Figure S6 Job's plot for the complex of DCI-RDA with Hg^{2+}(A) or Fe^{3+} (B)

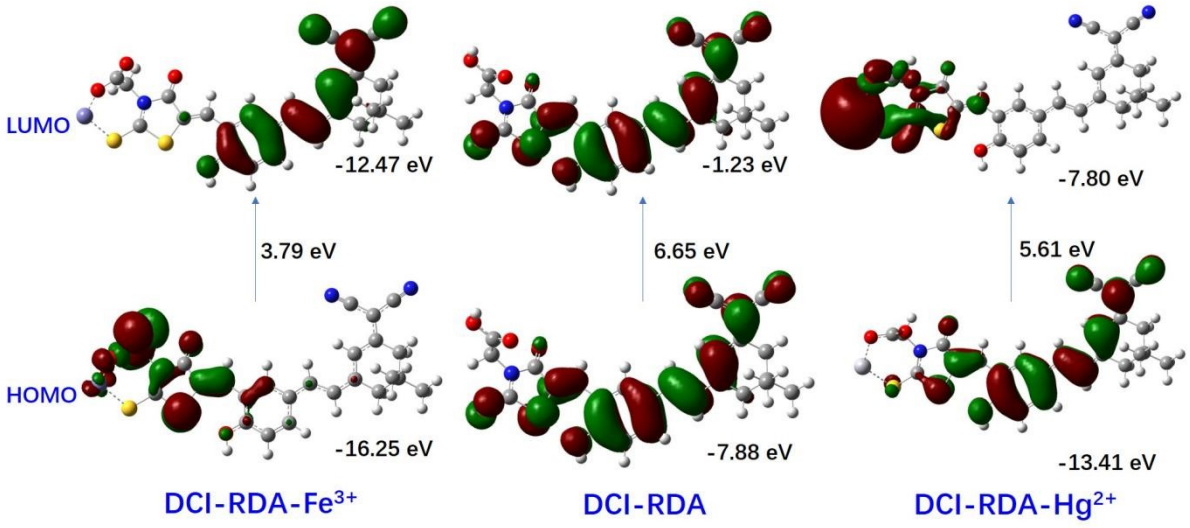
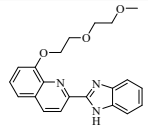
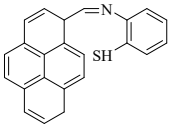
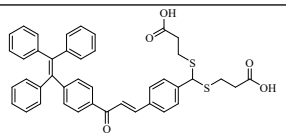
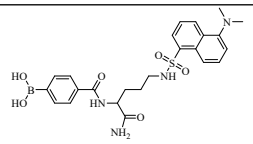
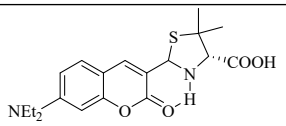
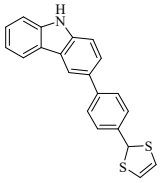
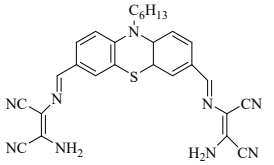
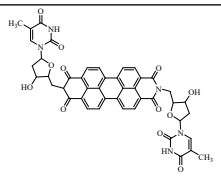
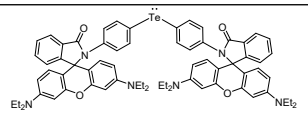
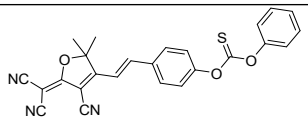
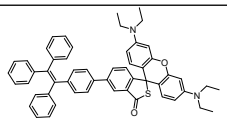
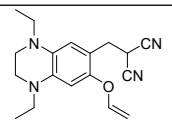
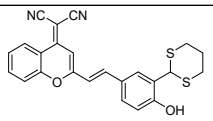
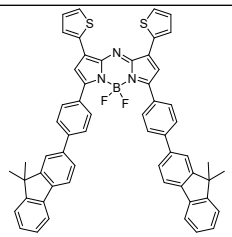
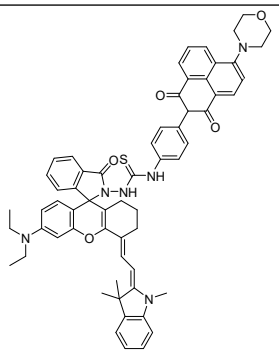
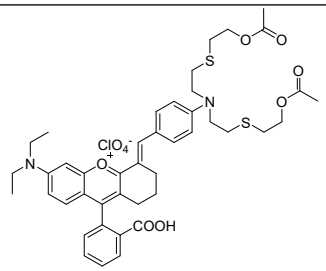
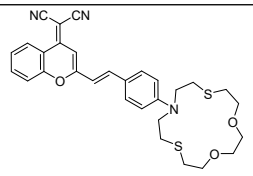
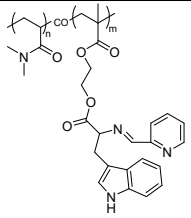
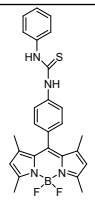
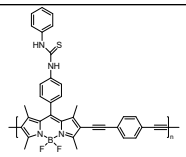
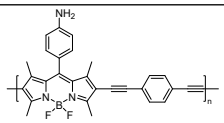
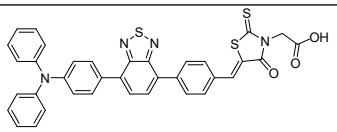
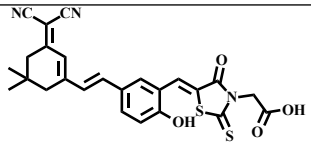


Figure S7. HOMO–LUMO energy diagrams of DCI-RDA with Fe^{3+}/Hg^{2+}.

Table S2 Comparison on detection limits of reported fluorescent sensor for Hg^{2+} ion.

Probes	λ_{em} (nm)	λ_{ex} (nm)	Stocks shift(nm)	LOD	Cell imaging	Zebrafish experiment	References in main manuscript
	475	348	127	2.54×10^{-7} M	No	No	RSC.. Advances., 4(2014)16612.
	427	347	80	2.82×10^{-6} M	Yes	No	New. J. Chem., 39(2015) 2523
	538	395	143	4.16×10^{-6} M	No	No	RSC. Adv., 9 (2019) 23316
	500/ 570	365		7nM	No	No	Dyes. Pigments. 191 (2021) 109374
	502	477	25	10nM	Yes	No	Chinese Chem. Lett., 05 (2021) 047.
	480	346	134	2.05×10^{-7} M	No	No	J. Photochem. Photobio. A., 416 (2021) 113322.
	575	450	125	3.5×10^{-8} M	Yes	No	RSC. Adv. 6 (2016) 7668.
	538	360	178	5nM	No	No	Chem. Commun. 2008, 1413-1415

	582	515	67	1.71μM	No	No	Dyes and Pigments. 159 (2018)121
	604	560	44	17 nM	No	No	Luminescence. 33 (2018)1122
	592	355	237	43pM	No	No	Analyst. 144 (2019) 1353
	625	475	150	7.1 nM	Yes	Yes	Org. Biomol. Chem., 16 (2018) 5036
	659	514	145	68nM	Yes	No	Dyes Pigm. 163 (2019) 118
	750	650	100	2.51μM	No	No	Dyes Pigm. 103 (2014) 145
	746	670	76	1.91×10^{-7} M	No	No	New. J. Chem. 41 (2017) 13495
	661	500	161	9.95nM	Yes	No	Dyes Pigm.160 (2019) 86

	645	517	128	0.14μM	Yes	Yes	Dyes. Pigm. 172 (2020) 107658.
	364 , 464	285	79	4.71nM	No	No	Polym Chem. 11 (2020) 2015
	529	319	210	2.4μM	Yes	Yes	Bioanal. Chem. 412 (2020) 881
	615	470	145	0.22 μM	Yes	Yes	
	618	470	148	2.86 μM	Yes	No	
	657	480	177	13.1nM	Yes	Yes	Org. Biomol. Chem., 32 (2020) 6357
	460	622	162	0.305 μM	Yes	Yes	This Work