New Pyridine-Bridged Ferrocene–Rhodamine Receptor for the Multifeature Detection of Hg$^{2+}$ in Water and Living Cells

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ABSTRACT: A challenge in the design of optical and redox-active receptors is how to combine a specific recognition center with an efficient responsive system to facilely achieve multifeature detection in biological and environmental analyses. Herein, a novel ferrocene–rhodamine receptor conjugated with a pyridine bridge was designed and synthesized. This receptor can sensitively sense Hg$^{2+}$ in aqueous media via chromogenic, fluorogenic, and electrochemical multisignal outputs with a low detection limit and fast response time. Moreover, it can be qualified as a fluorescent probe for effectively monitoring Hg$^{2+}$ in living cells. A plausible recognition mode was proposed and rationalized with theoretical calculations.

1. INTRODUCTION

Mercury is one of the most hazardous heavy metals because of its detrimental and accumulated features.$^{1,2}$ It is extensively distributed in water, soil, and atmosphere systems through natural phenomena and different human activities, resulting in serious public health and ecological environment problems.$^{3−5}$ Once inside the human body, Hg(II) tends to accumulate in vital organs and impairs human health even at low concentrations due to its strong affinity to thiols from enzymes and proteins.$^{6−9}$ For example, Hg(II) can cause the serious Minamata disease.$^{10}$ Therefore, it is significant to develop new strategies for efficiently monitoring Hg$^{2+}$ ions in aqueous media and biological systems.

Presently, many techniques have been developed for the detection of Hg$^{2+}$, involving liquid chromatography, atomic absorption spectrometry, solid-phase microextraction, high-performance plasma emission spectroscopy, etc. These techniques generally require the tedious sample preparation and expensive equipment.$^{11−15}$ By comparison, fluorescent or electrochemical probes are more popular methods used to survey Hg$^{2+}$ ions in view of their high sensitivity and specificity. However, most of them were built by a singly optical or electrochemical responsive model with some limitations for the practical usages.$^{16−22}$ As of now, there is a paucity of optical and redox-active receptors designed via a multiple signal model to expand the application scope. Thus, it is essential to develop multimodel-responsive receptors for the efficient analysis of Hg$^{2+}$ in water and living cells.

A key challenge in the design of multimodel-responsive receptors is how to combine a specific recognition center with a signal output system aimed to facilely achieve validly multifeature detection. In recent years, a number of fluorescent probes involving rhodamine dyes have been documented for sensing Hg$^{2+}$ by the optical response based on their spiral skeleton and distinctive photochemical features.$^{23−29}$ However, a few ferrocene–rhodamine receptors have been reported for the multifeature detection of Hg$^{2+}$ ions.$^{30−33}$ Herein, we present a multimodel-responsive ferrocene–rhodamine receptor FR-P (Scheme 1) that can efficiently monitor Hg$^{2+}$ ions in aqueous media and biological systems.

Scheme 1. Synthesis of Multiple Model Receptor FR-P

2,6-pyridinediamine

Rhodamine B chloride

Rhodamine B spirolactam I

FeCOCI

Et$_3$N, CH$_2$Cl$_2$

FR-P, Fe = ferroceny, yield 58%

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water with a low detection limit (4.14 \times 10^{-7} \text{M}) and fast response time (<5 min). Especially, it can be qualified for testing Hg^{2+} in living cells. Our strategy is to couple rhodamine fluorophore with an eminent electrochemical probe so as to construct a multimodel receptor that combines the ferrocenecarboxamide function and the rhodamine B spirolactam scaffold with 2,6-pyridinediyl moiety as a linkage and part of the recognition center.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization of FR-P. The pyridine-bridged ferrocene–rhodamine B receptor FR-P, as depicted in Scheme 1, was facilely synthesized via two steps. First, the treatment of rhodamine B chloride with excess 2,6-pyridinediamine and triethylamine in dichloromethane generated rhodamine B spirolactam I in 52% yield; then, it reacted with ferrocenylformyl chloride in dichloromethane with triethylamine as a base to produce FR-P in 58% yield. Its chemical structure was fully characterized by Fourier transform infrared (FT-IR), $^1$H nuclear magnetic resonance (NMR), and $^{13}$C NMR spectroscopy and high-resolution mass spectrometry (HR-MS) techniques (Figures S1–S4, Supporting Information).

The structure of FR-P was further identified by X-ray diffraction analysis (Tables S1 and S2, Supporting Information). In the solid state, FR-P crystallizes in the $P2_1_1_2_1$ space group with one-molecule solvent containing ca. 69% CH$_2$Cl$_2$ and 31% MeOH. As shown in Figure 1, the typical rhodamine B spirolactam is connected with a ferrocenecarboxamide function and the rhodamine B spirolactam scaffold with 2,6-pyridinediyl moiety as a linkage and part of the recognition center.

Notably, FR-P possesses a ferrocenecarboxamide function as the electrochemical probe and a rhodamine B scaffold as the optical probe that are smartly conjugated with a pyridine bridge to create a multimodel-responsive receptor. Its recognition properties were fully evaluated with UV–vis, fluorescence, and electrochemistry techniques as well as theoretical calculations.

2.2. UV–Vis and Fluorescence Evaluations of FR-P. The UV–vis and fluorescence assays were conducted in an optimized H$_2$O/dimethylformamide (DMF) (9:1, v/v) solution. FR-P (50 $\mu$M) shows one absorption peak at 311 nm in the UV–vis spectrum (Figure S5, Supporting Information), assigned to the ferrocenyl function. However, no absorption peaks in the visible region above 450 nm were found, confirming that FR-P exists as a spirolactam form in solution. This corresponds to the chemical shift at $\delta = 66.1$ ppm in its $^{13}$C NMR and belonged to the tertiary carbon. Next, its UV–vis absorption behaviors were studied in the presence of different metal ions including Na$^+$, K$^+$, Cs$^+$, Mg$^{2+}$, Ca$^{2+}$, Pb$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Cu$^{2+}$, Ag$^+$, Co$^{2+}$, Ni$^{2+}$, Nd$^{3+}$, La$^{3+}$, Ce$^{3+}$, and Eu$^{3+}$ (Figure 2). Gratifyingly, we found that by only adding Hg$^{2+}$, FR-P can generate a new maximum absorption peak at 567 nm, showing that its spirolactam ring opened and delocalized into the xanthene moiety. In this case, an apparent color change in the solution emerged from yellowish to pink (Figure 3, top), indicating that FR-P can be applied for the “naked-eye” detection of Hg$^{2+}$. However, upon the addition of other metal ions to the FR-P solution, no corresponding variations in both color and absorption spectra were observed.

![Figure 1. Crystal structure of FR-P with one-molecule solvent containing 31% MeOH and 69% CH$_2$Cl$_2$ showing the atom-numbering scheme. Displacement ellipsoids are drawn at the 30% probability level. Hydrogen atoms are omitted for clarity.](image1.png)

![Figure 2. UV–vis absorbance of FR-P (50 $\mu$M) in H$_2$O/DMF (9:1, v/v) at room temperature upon the addition of various metal ions (250 $\mu$M).](image2.png)

![Figure 3. Color changes of FR-P (50 $\mu$M) in H$_2$O/DMF (9:1, v/v) under visible (top) and UV (365 nm, bottom) light in the presence of various metal ions (250 $\mu$M), where 1–17 stand for Na$^+$, K$^+$, Cs$^+$, Mg$^{2+}$, Ca$^{2+}$, Pb$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Cu$^{2+}$, Ag$^+$, Co$^{2+}$, Ni$^{2+}$, Nd$^{3+}$, La$^{3+}$, Ce$^{3+}$, and Eu$^{3+}$, respectively.](image3.png)
Through the sequential titration of FR-P with Hg$^{2+}$ ions (Figure 4), the absorption peak at 567 nm gradually increased.

![Figure 4](https://dx.doi.org/10.1021/acsomega.0c02197)

**Figure 4.** UV−vis absorbance of FR-P (50 μM) in H$_2$O/DMF (9:1, v/v) at room temperature upon the titration of Hg$^{2+}$ (50−900 μM). Inset: absorbance at 567 nm as a function of Hg$^{2+}$ concentration.

This shows that FR-P possesses a strong binding affinity to Hg$^{2+}$ ions with high specificity. In addition, Job’s plot analyses (Figure S6, Supporting Information) confirm that a 1:1 complex formed between FR-P and Hg$^{2+}$ in solution with a coordination constant of $2.8 \times 10^9$ M$^{-1}$, calculated according to the nonlinear fitting of the titration profile. Significantly, the quick responsive time (<5 min) of FR-P toward Hg$^{2+}$ was obtained from the UV−vis absorption spectrum tests in various time intervals (Figure S7, Supporting Information).

Moreover, the fluorescence and sensing properties of FR-P were studied in a H$_2$O/DMF (9:1, v/v) solution. Free FR-P (25 μM) displays no distinct fluorescence in the range from 576 to 800 nm excited at 563 nm ($Φ_f = 0.014$, vs rhodamine B) (Figure S6, Supporting Information). $^{35−37}$ also proving that the spirolactam structure existed in solution. Upon the addition of Hg$^{2+}$ ions, FR-P yields a remarkable fluorescence-on response at 590 nm ($Φ_f = 0.029$, vs rhodamine B) with a color change to pink, verifying that the spirolactam ring of FR-P was opened upon sensing Hg$^{2+}$. In the identical conditions, the other metal ions failed to engender any vital fluorescent emission spectra of FR-P (Figure S9, Supporting Information), which is similar to the UV−vis results. This shows the high specificity of FR-P toward Hg$^{2+}$. To evaluate the sensitivity of FR-P to Hg$^{2+}$, fluorescence titration assays were further performed (Figure 5). Upon addition of 10 equiv of Hg$^{2+}$, the fluorescence intensity of FR-P increased up to ca. 130-fold at 590 nm, which confirms that FR-P also owns a high sensitivity for Hg$^{2+}$ ions.

![Figure 5](https://dx.doi.org/10.1021/acsomega.0c02197)

**Figure 5.** Fluorescence emission spectra of FR-P (25 μM) in H$_2$O/DMF (9:1, v/v) at room temperature upon addition of Hg$^{2+}$ (25−450 μM). Inset: fluorescence emission variations at 590 nm with the incremental addition of Hg$^{2+}$. $λ_{ex} = 563$ nm.

To examine the practical ability of FR-P as a fluorescent probe, competition experiments were also performed. As discussed above, FR-P only senses Hg$^{2+}$, while the other metal ions give a negligibly perceptible effect (Figure 6, purplish-red bars). Next, the fluorescence response of FR-P was measured separately when 250 μM of various interfering metal ions (Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, Cd$^{2+}$, Cu$^{2+}$, Eu$^{3+}$, Ag$^+$, Co$^{2+}$, Ni$^{2+}$, Nd$^{3+}$, La$^{3+}$, Ce$^{3+}$, and Eu$^{3+}$) and 125 μM Hg$^{2+}$ ions (Figure 6, green bars) were added. No remarkable variations in the fluorescence emission were found by the addition of most competitive ions, while only Cd$^{2+}$, Cu$^{2+}$, and Eu$^{3+}$ ions partially quenched the fluorescence owing to the spin−orbit coupling effect. $^{38,39}$

**2.3. Electrochemistry Evaluation of FR-P.** In view of FR-P having a redox-active ferrocenyl function, cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques were applied to study its electrochemistry and sensing properties. We found that FR-P displays a one-electron quasi-reversible redox peak at $E_{1/2} = 0.670$ V with $I_{pa}/I_{pc} = 1.04$, assigned to the ferrocene/ferrocenium (Fc/Fc+) redox couple. Upon the addition of 1.0 equiv of Hg$^{2+}$, the original CV wave of FR-P shifts negatively, forming a new CV wave at $E_{1/2} = 0.625$ V with $ΔE_{1/2} = −50$ mV (Figure S11, Supporting Information). Alternatively, the DPV assays show a similar shift with $ΔE = −70$ mV, confirming that FR-P can be used as an electrochemical sensor for the detection of Hg$^{2+}$ (Figure 7).

![Figure 7](https://dx.doi.org/10.1021/acsomega.0c02197)

**Figure 7.** DPV assays of FR-P (0.5 mM) in MeCN/CH$_3$Cl$_2$ (9:1, v/v) with 0.1 M n-Bu$_4$NClO$_4$ as a supporting electrolyte upon the addition of 1.0 equiv of Hg(ClO$_4$)$_2$ and 1.0 equiv of HClO$_4$. 

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equiv of H+°, demonstrating only a slight shift of Fc/Fc° with ΔE = ~20 mV. Thus, the negative shift can be mainly ascribed to FR-P binding Hg2+, which increases the electron density on the ferroceny group.40

2.4. Studies on the Sensing Mode of FR-P to Hg2+. As of now, two plausible modes have been documented for the rhodamine-based probes sensing Hg2+ in the literature: one is that the probe specifically coordinates Hg2+,41–44 and the other is that the probe decomposes with the selective catalysis of Hg2+.45–48 To verify how FR-P senses Hg2+, we have designed and carried out a few supplementary studies including HR-MS, FR-IR, and theoretical calculations.

First, a binding mode between FR-P and Hg2+ was initially confirmed by the HR-MS analysis based on a typical peak at m/z 982.2125 corresponding to a 1:1 complex of FR-P with Hg2+, [FR-P-Hg(II) + Cl]⁺ (Figure S12, Supporting Information). Next, FR-IR spectra of FR-P and its Hg(II) complex were measured to understand the coordination mode (Figure S13). In the FR-IR spectrum of FR-P, the stretching frequency of the spirolactam C=O bond appears at 1696 cm⁻¹, while it shifts to 1647 cm⁻¹ in that of its Hg(II) complex. Simultaneously, the pyridine C=N peak moves from 1605 to 1584 cm⁻¹. This implied that the spirolactam ring opens with Hg2+ binding to FR-P.49,50 Thus, we deduce that the pyridine N atom and two amide O atoms of FR-P possibly coordinate Hg2+ to yield a chelating complex assisted with the solvent molecule (Figure 8A).

Figure 8. Plausible binding mode of FR-P to Hg2+ (A) and the DFT-optimized structure of complex [FR-P-Hg(II)-DMF]2+ (B), calculated with the B3LYP/LANL2DZ basis set.

To rationalize the mode of FR-P binding Hg2+, as depicted in Figure 8A, structures of FR-P and its Hg(II) complex (Figure S14, Supporting Information) were optimized with the density functional theory (DFT) calculations via the Gaussian 09W program. Figure 8B demonstrates an optimized structure of complex [FR-P-Hg(II)-DMF]2+ generated with a multibonding mode between FR-P and Hg2+. In this complex, one Hg2+ ion bonds with two amide O atoms and one pyridine N atom of FR-P together with one amide O atom of DMF, creating close contacts of dHg-O1 = 2.278 Å, dHg-O2 = 2.227 Å, dHg-N2 = 2.383 Å, and dHg-O3 = 2.306 Å, as well as bond angles of N2-Hg-O3 = 154.1° and O1-Hg-O2 = 153.5°. The opening of the spirolactam ring caused a significant electronic delocalization that can be identified by the variation of the N3-C4 distance in FR-P and its Hg(II) complex, which is shortened from 1.401 to 1.310 Å upon coordinating Hg2+. The addition of Hg2+ opened the spirolactam ring and extended its conjugate system.

The molecular orbitals of FR-P and its Hg(II) complex were also optimized (Table S3, Supporting Information). As shown in Figure 9, the highest occupied molecular orbital (HOMO) of FR-P is largely located on the xanthene unit of rhodamine, while the lowest unoccupied molecular orbital (LUMO) is mainly distributed over the pyridine ring and the ferrocene-carboxamide function, with a LUMO–HOMO gap of 3.94 eV (Figure 9A). For [FR-P-Hg(II)-DMF]2+, its HOMO is also located on the xanthene ring; however, the LUMO is diffused over the pyridine and amide groups, with a lower energy gap of 2.79 eV (Figure 9B), confirming that [FR-P-Hg(II)-DMF]2+ is more stable than FR-P.

Figure 9. HOMO–LUMO distributions of FR-P (A) and [FR-P-Hg(II)-DMF]2+ (B), calculated by the 6-31G** basis set for C, H, N, and O atoms and the LANL2DZ basis set for Fe and Hg atoms.

2.5. Bioimaging Evaluations of FR-P. After extensive evaluations, we found that FR-P has high sensitivity, good selectivity, and fast response time for monitoring Hg2+ in aqueous media. Encouraged by these advantages, we continue to examine the potential applications of FR-P in fluorescence imaging of Hg2+ in living cells. The evaluation of FR-P to survey intracellular Hg2+ in HeLa cells was assessed by fluorescence imaging studies. As shown in Figure 10, the HeLa cell lines incubated with FR-P (50 μM) at 37 °C for 30 min show no fluorescence (Figure 10a), confirming that the spirolactam scaffold of FR-P is stable enough to tolerate the HeLa cell lines. However, when adding Hg2+ ions (100 μM) to the preincubated HeLa cells for less than 10 min, strong fluorescence appears (Figure 10d). These results imply that FR-P can be qualified as a fluorescence imaging probe for the survey of intracellular Hg2+ ions in living cells.

3. CONCLUSIONS

A novel optical and redox-active ferrocene–rhodamine receptor linked by a pyridine moiety has been successfully designed and prepared for the multifeature detection of Hg2+ in water and living cells. The pyridine ring plays a key role in both creating the recognition center and regulating the responsive system. This receptor features a high selectivity, a low detection limit (4.14 × 10⁻⁷ M), and a fast response time (<5 min) to Hg2+. Moreover, theoretical calculations are applied to understand and rationalize the sensing mode.
4. EXPERIMENTAL SECTION

4.1. Materials and Instruments. All starting materials and solvents were commercially available and utilized without further purification. Stock solutions of FR-P and all metal salts were prepared in DMF and distilled water, respectively. 1H NMR and 13C NMR spectra were obtained using BRUKER ADVANCE 300/400 spectrometers (CDCl3, TMS as an internal standard). FT-IR spectra (KBr pellets) were recorded in the range of 400–4000 cm⁻¹ with a PerkinElmer 1600 FT-IR spectrometer. Electrospray ionization mass spectra (ESI-MS) were obtained using a maXis UHR-TOF system. Melting points were measured using a Yanaco MP-500 micromelting point instrument and uncorrected. UV−vis spectra were recorded using a TU-1900 UV−vis spectrometer. Fluorescence spectra were measured using an RF-600 Shimadzu fluorescence spectrometer. Electrochemical analyses were carried out using a CHI660 electrochemical analyzer.

4.2. Synthesis of FR-P. To a solution of rhodamine B spirolactam I (prepared as the known procedure,51 0.320 g, 0.6 mmol) in anhydrous CH2Cl2 (10.0 mL) and solvents were commercially available and utilized without further purification. 1H NMR (400 MHz, CDCl3, TMS): δ = 8.27 (d, J = 7.8 Hz, 1H), 8.02–8.00 (m, 1H), 7.92 (s, 1H), 7.75 (d, J = 7.7 Hz, 1H), 7.58 (d, J = 8.1 Hz, 1H), 7.55–7.50 (m, 2H), 7.18–7.16 (m, 1H), 6.48 (d, J = 2.6, 2H), 6.42 (s, 1H), 6.41 (s, 1H), 6.16 (dd, J = 8.9, 2.6 Hz, 2H), 4.95 (s, 2H), 4.52 (s, 2H), 4.31 (s, 5H), 3.28 (q, J = 7.0 Hz, 8H), 1.10 (t, J = 7.0 Hz, 12H). 13C NMR (100 MHz, CDCl3, TMS): δ = 168.5, 167.9, 153.8, 153.3, 148.6, 139.5, 133.8, 130.7, 123.8, 124.4, 123.1, 110.3, 109.0, 107.0, 96.8, 71.2, 70.0, 68.1, 66.1, 53.3, 44.2, 12.7. HR-MS: m/z [M]+: calcd. for C44H43FeN5O3: 745.2715; found: 746.2786 ([M+H]+).

4.3. Crystal Structure Determination. Orange single crystals were developed through slow evaporation of a solution of FR-P in CH2Cl2/MeOH (1:1, v/v) at 0−4 °C. The selected single crystal of FR-P was mounted on the glass fiber. The intensity data were measured at 293 K on an Agilent SuperNova CCD-based diffractometer (Cu Kα radiation, λ = 1.54184 Å). Empirical absorption corrections were used with SQUEEZE. The structure was solved by direct methods and difference Fourier syntheses and refined by the full-matrix least-squares technique on F2 with SHELXS-9753 and SHELXL-97.54 All nonhydrogen atoms were refined using anisotropic displacement parameters. Hydrogen atoms linked to refined atoms were placed in geometrically idealized positions and refined by a riding model with C−H = 0.93, 0.97, and 0.96 Å for aromatic, methylene, and methyl H, respectively, Uiso(H) = 1.5Ueq(C) for methyl H, and Uiso(H) = 1.2Ueq(C) for all other H atoms. Crystallographic data for FR-P have been deposited with the Cambridge Crystallography Data Centre (CCDC No. 1978652).

4.4. UV−Vis/Fluorescence and Electrochemistry Tests. A stock solution of FR-P (0.5 mM) was prepared in DMF, and the stock solutions of all metal salts (5.0 mM) were prepared in distilled water. The solution of FR-P was then diluted to 50 and 25 μM with H2O/DMF (9:1, v/v) solvents for UV−vis and fluorescence studies, respectively. For fluorescence tests, the excitation was provided at 563 nm, while the emission was collected from 576 to 800 nm. The fluorescence intensity at 590 nm was used to assess the performance of the proposed assay strategy. Both excitation and emission slits applied in the assays are 5 nm. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were carried out using a solution of FR-P (0.5 mM) in MeCN/CH2Cl2 (9:1, v/v) with 0.1 M n-Bu4NCIO4 as the supporting electrolyte and Hg/Hg2Cl2 as the reference electrode, along with platinum working and auxiliary electrodes. DPV measurements were performed with a 50 ms pulse width and a scan rate of 100 mV s⁻¹.

4.5. Cell Culture and Fluorescence Imaging. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) along with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The sample was excited at 561 nm, and the emission spectrum collection ranges from 570 to 650 nm.

Before the experiments, HeLa cells were washed with phosphate-buffered saline (PBS) buffer and then incubated with 50 μM FR-P in PBS/DMF (2:1, v/v) at 37 °C for 30 min. At last, 100 μM Hg2+ was added for the amplification reaction. The process was carried out at 37 °C for 10 min. Cell imaging was then performed after washing cells with PBS.

4.6. Theoretical Calculations. All ground-state optimizations were performed with the density functional theory (DFT) using the Gaussian 09W program. All geometry optimizations were made with tight convergence criteria in the gas phase by utilizing the B3LYP level, with the 6-31G** basis set for C, H, N, and O atoms and the LANL2DZ basis set for Fe and Hg atoms.55,56

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02197.
FT-IR, NMR, HR-MS, and UV—vis absorbance spectra; fluorescence emission; CV; DPV spectra; crystal structural data; time response; theoretical calculation data; Job’s plot of changes in absorbance at 567 nm; selected bond lengths and angles; fluorescent intensity changes; DFT-optimized structures; Cartesian coordinates; and crystallographic data.

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**Notes**

The authors declare no competing financial interest.

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