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Colorimetric and luminescent dual-signaling responsive probing of thiols by a ruthenium(II)-azo complex

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Abstract

A dinuclear ruthenium(II) complex linked via a reducible azo group \([\text{Ru}(\text{bpy})_2(\text{azobpy})\text{Ru}(\text{bpy})_2]^2\text{Cl}_4\) (Ru2azo, bpy = 2,2'-bipyridine, azobpy = 4,4'-azobis(2,2'-bipyridine)) was adopted as a probe for thiols. Results showed that Ru2azo could selectively and effectively react with biological thiols (such as cysteine, homocysteine and glutathione) with a 10⁻⁷ M detection limit. After it reacted with thiols, the original gray color of Ru2azo solution immediately turned yellow and the luminescence significantly enhanced, showing “naked-eye” colorimetric and “off-on” luminescent dual-signaling response for thiols. Mechanism studies demonstrated that Ru2azo reacted with thiols undergoing a two-electron transfer process, forming the azo⁺⁻ anion product.

Article info

Keywords:
Ruthenium(II) complex
Azo
Biological thiols
Chemosensor
Redox reaction

1. Introduction

Among all the amino acids, cysteine (Cys) combined with homocysteine (Hcy) and reduced glutathione (GSH) compose the primary biological thiols which are responsible for the redox homeostasis for their participation in the process of reversible redox reactions. Biological thiols can protect the cells from oxidative stress by trapping free radicals that induce DNA and RNA damage. The abnormal level of those thiols has been proven to be directly linked to a series of diseases, such as hematopoiesis decrease, muscle and fat loss, and psoriasis, and Hcy is also a risk factor for cardiovascular [2] and Alzheimer’s disease [3,4].

The design of highly selective and sensitive reporters for detecting biological thiols levels has attracted much attention in the past decades. A variety of analytical methods [5–16] have been developed and many compounds [17–25] have been designed to detect thiol levels. Very recently, Yoon et al. have reviewed the recent progress in chemosensors for detection of thiols [26,27]. Among all these chemosensors, most of them are single signaling responsive [17–22,28–31], either luminescent responsive or colorimetric responsive, or else. Only a few of them are dual signaling responsive [23,32–34]. To develop the thiol molecular probe, some thiol-specifically acceptors, such as aldehyde groups [22], disulfide bond [24], maleimide [28] and sulfonate ester [34], are often used as the thiol-recognition moieties for the structural design of the probes. In recent years, more and more modified gold nanoparticles are designed as thiol probes, considering the strong bind affinity of sulfide atom with gold [9,17,30].

Ru(II) polypyridyl complexes, due to their intense polarized luminescence, large Stokes shifts, high chemical and photo-stability, low energy absorption and relatively long lifetimes, have emerged as novel and promising probes in the recognition and detection of anions [35–37], metal ions [38,39], small molecules [34,40,41] and biomacromolecules [42–44]. However, study involving the probing of biothiols is rare. Recently, Yuan [22] and Chen [45] have developed some Ru(II) complexes containing aldehyde group that show good luminescent selectivity towards Cys and Hcy, but the detection limits for Cys or Hcy were only at the micro molar level, which hinder their further application. Yuan [34] and Zhao [46] improved the detection sensitivity for biothiols by using Ru(II) complex as the luminophore which prior protected by 2,4-dinitrobenzenesulfonyl (DNBS), however, such a probe will releases an environmental toxic and health-harmful gas SO₂ after the degradation of sulfonate ester. Even worse, DNBS was found to induce colonic damage in animal and human body [47,48]. In this work, we present a molecular probe \([\text{Ru}(\text{bpy})_2(\text{azobpy})\text{Ru}(\text{bpy})_2]^2\text{Cl}_4\) (Ru2azo, bpy = 2,2'-bipyridine, azobpy = 4,4'-azobis(2,2'-bipyridine), Scheme 1) [49,50] with specific selectivity towards thiols, on which the redox-active azo group will react with biological redundants Cys, Hcy and GSH, resulting in naked-eyed colorimetric and “off-on” luminescent dual response for biothiols.

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2. Experimental

2.1. Materials and measurements

The Ru(bpy)2Cl2 [51] and azobpy ligand [52] were synthesized according to the literature methods. Unless otherwise stated, all the amino acids and other reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments.

Microanalysis (C, H and N) was carried out with a Perkin-Elmer 240Q elemental analyzer. Infrared spectra were recorded on a Bruker VECTOR22 spectrometer in KBr pellets over a range of 400–4000 cm\(^{-1}\). \(^1\)H NMR spectra were recorded on Varian-300 spectrometer. All chemical shifts are given relative to tetramethylsilane (TMS). Electrospray mass spectra (ES-MS) were recorded on a LCQ system (Finnigan MAT, USA). The electronic absorption spectra were recorded using Perkin Elmer Lambda850 spectrometer. Emission spectra were recorded on a Perkin-Elmer L55 spectrofluorophotometer. The pH measurements were carried out in the Sartorius PB-10 pH-meter. Cyclic voltammetry measurements were performed on a CHI 660A Electrochemical Workstation. All samples dissolved in CH\(_2\)CN were purged with Ar prior to measurements and 0.1 M tetrabutylammonium perchlorate (TBAP) was used as a supporting electrolyte. A standard three-electrode system comprising a glassy carbon working electrode, Pt-wire auxiliary electrode and a saturated calomel reference electrode (SCE) was used. The ESR spectra were measured using a Bruker e-scan ESR spectrometer. The electrolysis experiments were carried out in deaerated CH\(_2\)CN containing 0.1 M TBAP as supporting electrolyte under certain electrolysis potential using DJS-292B potentiostation (Shanghai Shengke Instrument Equipment Co. Ltd (China)).

2.2. Synthesis

The dinuclear Ru(II) complex was synthesized according to the previously reported method [52] with some modification. A mixture of Ru(bpy)\(_2\)Cl\(_2\) (0.1 g, 0.21 mmol) and azobpy ligand (0.034 g, 0.1 mmol) was suspended in 20 mL EtOH/H\(_2\)O (1:1, v/v) and heated at 85 °C under Ar. After 8 h reflux, the mixture was evaporated under reduced pressure, and the crude product was purified by column chromatography on alumina with CH\(_2\)Cl\(_2\)-EtOH (10:1, v/v) as eluent. Yield: 77 mg, 60%. Anal. Calcd. for C\(_{60}\)H\(_{46}\)N\(_{14}\)Cl\(_4\)Ru\(_2\): C 55.13%, H 3.55%, N 15.00%. Found: C 55.01%, H 3.47%, N 15.15%. MS (ESI) \(m/z\): 291.0 ([M]^+) and 400.1 ([M + Cl]^+). \(^1\)H NMR (300 MHz, DMSO-d6): \(\delta\) 9.06 (s, 2H), 8.84 (d, J = 8.4 Hz, 2H), 8.62 (d, J = 8.1 Hz, 8H), 7.95 (m, 10H), 7.82 (d, J = 6.3 Hz, 2H), 7.66 (d, J = 5.4 Hz, 2H), 7.60 (d, J = 7.2 Hz, 2H), 7.56 (d, J = 5.4 Hz, 2H), 7.50 (d, J = 4.8 Hz, 6H), 7.40–7.29 (m, 10H).

2.3. UV–visible (UV-\(\pi\)) spectral responses of Ru2azo toward thiols

The UV–vis spectral responses of Ru2azo toward different amino acids were measured in a 10 mM HEPES (pH 7.5 at room temperature. When adding different concentrations of Cys, Hcy or GSH into Ru2azo (10 \(\mu\)M) solution, the mixture was stirred for 5 min and then the absorption spectra were acquired on the Perkin Elmer Lambda850 spectrometer.

2.4. Luminescent responses of Ru2azo toward thiols

The luminescent responses of Ru2azo toward different amino acids were measured in a 10 mM HEPES buffer of pH 7.5 at room temperature. When adding different concentrations of Cys, Hcy or GSH into Ru2azo (10 \(\mu\)M) solution, the mixture was stirred for 5 min and then the luminescent spectra were acquired on the Perkin-Elmer L55 spectrofluorophotometer.

3. Results and discussion

3.1. pH effect

To evaluate the effects of pH on the stability of Ru2azo, the electronic absorption spectra of Ru2azo were measured at different pH conditions ranging from 1.97 to 12.0. To minimize the errors produced by the baseline shift under different pH, we used the ratio of absorbance in 562 nm via in 439 nm as an index for estimating the stability of complex. The value of \(A_{562\text{ nm}}/A_{439\text{ nm}}\) was changed little from pH 1.97 to 12.0 (as shown in Fig. S1), demonstrating that the Ru2azo was stable in weakly acidic, neutral and weakly basic conditions. This result basically indicated that Ru2azo can work well as a probe for biological thiols such as Cys, Hcy and GSH among all the amino acids.

3.2. UV–vis spectral responses of thiols

Fig. 1a showed a ratio of \(A_{562\text{ nm}}/A_{439\text{ nm}}\) upon addition of different amino acids, and Fig. 2a gave photographs directly illustrating the colorimetric response of complex upon additions of Cys, Hcy and GSH among different amino acids. The UV–vis spectrum of Ru2azo was recorded in HEPES buffer solution (10 mM, pH 7.5) in the absence or presence of 20 equiv. of different amino acids (shown in Fig. S2). No detectable change in absorption spectra was observed upon the addition of amino acids including Ala, Arg, Asp, Asn, Glu, Gln, Gly, His, Leu, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. However, upon addition of Cys, the absorbance band at 562 nm of Ru2azo (attributed to low energy MLCT (d→π* azobpy π*)) significantly decreased, and the hyperchromic effect and red shift of absorption at 439 nm (attributed to MLCT (d→π* bpy π*)) were also observed. Similar cases were observed upon addition of Hcy and GSH, while no change in absorption happened upon addition of cystine (Cys) which was as oxidative form of Cys and linked by disulfide bond. At the same time, the color of the complex solution changed immediately from gray to yellow after adding Cys, Hcy or GSH. The colorimetric effect could be directly observed by naked eyes. Among all the amino acids, Ru2azo was significantly responsive of thiol-containing amino acids, acting as a “naked-eye” probe for thiols. These results demonstrated that complex was characteristic of high selectivity toward thiols over other proteinogenic amino acids.

To further investigate the interaction of Ru2azo and thiols, an absorption titration experiment was carried out. Fig. 3 displayed the changes in absorption spectra of complex (10 \(\mu\)M) in the presence of different concentrations of Cys in the HEPES buffer. Upon addition of Cys, the absorbance at 562 nm decreased gradually, and the absorption band at 439 nm increased and exhibited a red-shift to
When the azo group was reduced to the electron transfer (PET) process, which have been proven by density functional calculations [52]. The decrease of A562 nm via the concentration of Cys increased (Fig. 5a) and the luminescence intensity remarkably increased (Fig. 5b). These competition experiments demonstrated that all the other 19 proteinogenic amino acids and Cyst have little interference with the sensitive detection of thiols by Ru2azo in the colorimetric and luminescent response.

3.4. Competition experiments

It is a challenge for a probe to achieve high selectivity over a complex background of potentially competing species. Therefore, the competition experiments were also carried out. Ru2azo was mixed with 20 equiv. Ala, Arg, Asn, Asp, Cyst, Gln, Glu, Gly, His, Lys, Leu, Ile, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val, and its absorption and luminescence spectra were measured to be found no difference with complex solution alone. However, by adding 2 equiv. Cys to the above mixture, the absorption of the complex solution was evidently changed (Fig. 5a) and the luminescence intensity remarkably increased (Fig. 5b). These competition experiments demonstrated that all the other 19 proteinogenic amino acids and Cyst have little interference with the sensitive detection of thiols by Ru2azo in the colorimetric and luminescent response.

3.5. Detection limit of thiols

The detection limits of thiols such as Cys, Hcy and GSH were determined based on the luminescence titration with a three time signal-noise ratio. Under titration of different concentrations of thiols, a good linear relationship between the luminescence intensity and the thiol concentration could be obtained in the 0–10 μM (R > 0.998) (Fig. 6), and the detection limits of thiols were calculated as 2.29 × 10⁻⁷ M for Cys, 2.27 × 10⁻⁷ M for Hcy and 2.42 × 10⁻⁷ M for GSH, respectively (Table 1). Compared to the previously reported Ru(II) based Cys/Hcy probes, such as [Ru(CHO-bpy)₃]²⁺ (1.41 × 10⁻⁷ M for Cys, 1.19 × 10⁻⁶ M for Hcy) [22], [Ru(bpy)₂(L2)]²⁺ (5 × 10⁻⁶ M for Cys, 4 × 10⁻⁶ M for Hcy) [45], [Ru(bpy)₂(L1)]²⁺ (4 × 10⁻⁶ M for Cys, 2 × 10⁻⁶ M for Hcy) [45], [Ru(bpy)₂(L2)]²⁺ (2 × 10⁻⁶ M for Cys, 5 × 10⁻⁷ M for Hcy) [45] and [Ru(bpy)₂(L2)]²⁺ (1 × 10⁻⁶ M for Cys, 3 × 10⁻⁷ M for Hcy) [45], Ru2azo was more significant for the quantitative determination of thiol concentrations.

3.6. Mechanism

According to the frontal conclusions, we initially speculated that the azo group in Ru2azo was directly involved in the thiol-recognition reaction and was reduced by thiols undergoing a two-electron transfer mechanism. To confirm this hypothesis, cyclic voltammograms of Ru2azo before or after addition of Cys were carried out, and product of Ru2azo reacted with Cys was isolated and further characterized by IR spectra, ¹H NMR, ESR and ESI-MS. As shown in Fig. 7, cyclic voltammograms for a CH₃CN solution Ru2azo (100 μM) showed two reversible reduction waves at the potential of −0.30 V (φazo/₀/₁) and −0.73 V (φazo/₁/₀)−, corresponding to consecutive one-electron reductions. Upon addition of 1 equiv Cys, reduction waves of azo⁻¹/₂− disappeared, and the current of φazo/₀/₁− was found to decrease. While adding 2 equiv. Cys, both reduction waves disappeared. The result confirmed that the azo group was the thiol-recognition moiety in Ru2azo.
Thiols are one kind of important reductant in biological system and can be oxidized into RSSR form. In this case, Cys was tested with Ru$_2$azo and the oxidative Cyss form was confirmed by the ES-MS spectra (Fig. S5). After reacting with Ru$_2$azo, the anion peak in m/z = 120.1 (corresponded to [Cys]$^-$) and m/z = 240.9 (corresponded to [2Cys + H$^+$]$^-$) disappeared, and a new anion peak in m/z = 239.1 was clearly observed, which could be corresponded to [Cyss + H$^+$]$^-$.

As to the oxidant, the azo group could be completely reduced into amino group (process I, four-electron and four-proton transfer) or partially reduced into hydrazo group (process II, two-electron and two-proton transfer), azo anion (process III, two-electron transfer) or azo radical (process IV, one-electron transfer), as seen in Scheme 2. The IR spectra exhibited no absorption peaks at 3200–3400 cm$^{-1}$ which should correspond to the amine group or imino group absorption (Fig. S6). And what’s more, there was no observation of proton signal of N–H in the $^1$H NMR data. However, the proton nearby the azo moiety significantly shifted to the high field (from 9.05 to 7.22 for Ha$_3$, and from 7.68 to 6.80 for Ha$_5$, respectively, as shown in Fig. S7), indicating a destruction of electronic screening effect of the azo group. These data could be exclusion of amino or hydrazo group in the isolated product. Further, the ESR data displayed no radical signal (Fig. S8), and this result eliminated the azo radical product.

The exclusion of amino group, hydrazo group and azo radical demonstrated that the only product of complex reacted with thiols was...
the azo anion. The ES-MS results showed evidence of the existence of the azo anion. The isolated product displayed a trivalent ion peak in m/z = 388, which could be contributed as \([M+2e^-+H^+]^{3+}\), and a divalent ion peak in m/z = 583 was also observed, which could be contributed as \([M+2e^-]^{2+}\) (Fig. S9). The azo anion was further confirmed by the electrolysis experiments. From the cyclic voltammetric experiment, we got the two reversible redox potentials of azo group: 

-0.30 V for \(\text{azo}_0/\text{azo}^-\)

-0.73 V for \(\text{azo}^-/\text{azo}^{2-}\), respectively. Thus we electrolyzed the isolated product using different potential and then measured its electronic absorption (Fig. S10). Little change was found in the absorption spectrum when Ru\(_2\)azo was electrolyzed under +0.5 V. However, when electrolyzed under +1.0 V, the absorbance at 562 nm significantly increased. Although its absorption spectrum could not be the same of Ru\(_2\)azo due to the adsorption of electrolyzed product in the electrodes and diffusion in the mixture solution, the increasing 562 nm band in absorption spectrum could still strongly support that the isolated product was reduced into azo\(_{2-}\) form.

4. Conclusions

Among all the twenty proteinogenic amino acids, Cys is the primary biological thiols, combined with Hcy and GSH, which are responsible for the redox homeostasis for their participation in the process of reversible redox reactions. Herein, a dinuclear Ru(II) complex Ru\(_2\)azo was successfully developed to recognize and detect the thiols. The new probe exhibited a “naked-eye” colorimetric and “off-on” luminescent dual-signaling response after mixing with biological thiols with a 10\(^{-7}\) mol/L detection limit. Mechanism studies demonstrated that Ru\(_2\)azo reacted with thiols undergoing a two-electron transfer process and finally produced a two-electron reduction product azo\(_{2-}\) anion. Compared to the previously reported thiol probes, Ru\(_2\)azo exhibited highly selectivity, fast dual-signaling response and excellent sensitivity in complicate background. Information obtained from the present study should be of value in further recognizing and detecting biothiols, as well as providing a new strategy for the rational design of Ru(II) polypyridyl complexes as probes for various biological molecules.

Abbreviations

- bpy 2,2′-bipyridine
- Hcy homocysteine
- GSH glutathione
- DNBS 2,4-dinitrobenzenesulfonyl
- azobpy 4,4″-azobis(2,2′-bipyridine)
- HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- Cys cystine
- CHO-bpy 4-methyl-2,2′-bipyridyl-4′-carboxaldehyde
- dmb 4,4′-dimethyl-2,2′-bipyridine
- L1 4-methyl-2,2′-bipyridine-4′-carboxaldehyde

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Fig. 5. Competitive reaction of Ru\(_2\)azo-Cys with different amino acids recorded in absorption spectra (a) and luminescence spectra (b). Ru\(_2\)azo first reacted with 20 equiv. different amino acids (gray bars) and then 2 equiv. Cys were added into the mixture (red bars). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. The calibration curves for luminescence detection of Cys (a), Hcy (b) and GSH (c) using 10 \(\mu\)M Ru\(_2\)azo.
References