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Development and application of a ruthenium(II) complex-based photoluminescent and electrochemiluminescent dual-signaling probe for nitric oxide

Wenzhu Zhang *, Jingmei Zhang, Hailei Zhang, Liyan Cao, Run Zhang, Zhiqiang Ye, Jingli Yuan *

State Key Laboratory of Fine Chemicals, School of Chemistry, Dalian University of Technology, Dalian 116024, PR China

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ABSTRACT

A ruthenium(II) complex, $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ (bpy: 2,2'-bipyridine; DA-phen: 5,6-diamino-1,10-phenanthroline), has been developed as a photoluminescent (PL) and electrochemiluminescent (ECL) dual-signaling probe for the highly sensitive and selective detection of nitric oxide (NO) in aqueous and biological samples. Due to the presence of electron transfer process from diamino group to the excited-state of the Ru(II) complex, the PL and ECL intensities of the probe are very weak. After the probe was reacted with NO in physiological pH aqueous media under aerobic conditions to afford its triazole derivative, $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ (TA-phen: 5,6-triazole-1,10-phenanthroline), the electron transfer process was inhibited, so that the PL and ECL efficiency of the Ru(II) complex was remarkably increased. The PL and ECL responses of the probe to NO in physiological pH media are highly sensitive with the detection limits at low micromolar concentration level, and highly specific without the interferences of other reactive oxygen/nitrogen species (ROS/RNS) and metal ions. Moreover, the probe has good cell-membrane permeability, and can be rapidly transferred into living cells for trapping the intracellular NO molecules. These features enabled the probe to be successfully used for the monitoring of the endogenous NO production in living biological cell and tissue samples with PL and ECL dual-modes.

1. Introduction

In the last few years, the use of transition metal complexes as luminescent chemosensors and cell imaging probes has attracted much attention due to their abundant photophysical, photochemical, and electrochemical properties [1–5]. In particular, Ru(II) complexes with polypyridyl ligands are enjoying the increasing interest in the fields of luminescent bioprobes, dye-sensitized solar cells (DSSC), molecular catalysts, and so on [6–8]. As one of useful luminescent reporters, Ru(II)-polypyridyl complexes featuring distinctive optical properties contribute significantly to the molecular recognition and sensing systems for bioactive molecules, metal cations and anions [9–11]. In addition to their application to photoluminescence detection technique, Ru(II) complexes also show good applicability as electrochemical biosensing reagents with advantages of high stability, sensitivity and selectivity, and lower environmental sensitivity compared to organic fluorophores [12].

As luminophores, it has been known that the emission behaviors of Ru(II)-polypyridyl complexes can be modulated by the ligand modification. With the intramolecular photoinduced electron transfer (PET) mechanism to corrupt the excited-state, we have succeeded in designing and synthesizing several photoluminescent (PL) and electrochemiluminescent (ECL) probes for bioactive molecules using tris(2,2'-bipyridine)Ru(II) complex as a signaling moiety [13–15]. Compared to the organic dye-based probes, these probes possess some unique advantages, such as visible-light excitation and emission with large Stokes shifts, high photo- and chemical stabilities, low cytotoxicity, good water-solubility, and high PL and ECL response efficiency.

On the other hand, as an intra- and inter-cellular messenger molecule, nitric oxide (NO) transfers signal in the cardiovascular and nervous systems to regulate the immune balance in animal systems [16,17], and in plant systems, NO is implicated in many key physiological processes, including growth regulation, cell differentiation, stomatal closure, phytoalexin accumulation, and plant responses against a variety of abiotic stress factors, such as wounding, salinity, drought, and hypoxia [18,19]. Due to the outstanding importance of NO in biological processes, during the past years, several methods based on fluorescent [20–22], chemiluminescent [23], electron paramagnetic resonance spectroscopy [24–26], and electrochemical [27] techniques have been established for the NO detection. Among these methods, the fluorescence sensing technique with the use of NO-specifically-responsive probes is considered

* Corresponding authors. Tel./fax: +86 411 84986041.
E-mail addresses: wenzhuzhang@yahoo.com.cn (W. Zhang), jingliyuan@yahoo.com.cn (J. Yuan).

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to be one of the most promising methods due to its high sensitivity, selectivity, and experimental feasibility.

Comprehensively considering the abundant PL and ECL properties of Ru(II) complexes, in this work, a Ru(II) complex bearing two 2,2'-bipyridine (bipy) ligands and one 5,6-diamino-modified 1,10-phenanthroline (DA-phen) ligand, $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$, was proposed to be a new PL and ECL dual-signaling probe for the detection of NO in living biological samples. As a PL probe, $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ could be anticipated to be weakly luminescent due to the presence of photoinduced electron transfer (PET) process from electron-rich *o*-diamino group (electron donor) to the excited-state of the Ru(II)-polypyridyl complex core (electron acceptor) [13]. However, if $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ is reacted with N_2O_3 to afford its triazole derivative $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$, the PET process would be corrupted, thus the luminescence of the Ru(II)-polypyridyl complex could be restored. Based on this mechanism, Ghosh et al. recently described the application of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for the PL detection of NO_2^- in a 1 M HCl solution (N_2O_3 was generated by the decomposition of NO_2^- in the strongly acidic solution) [28].

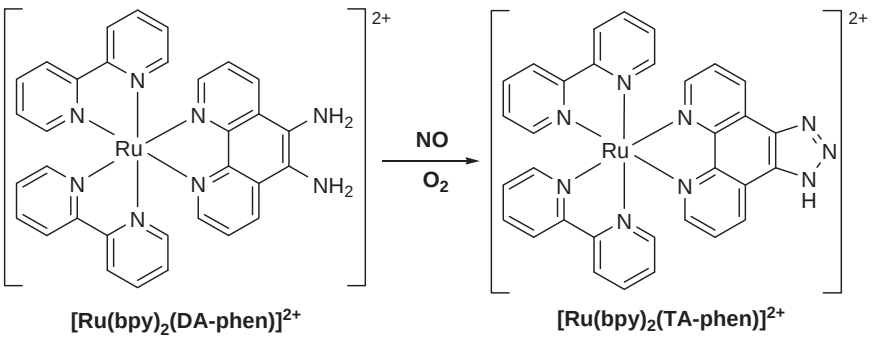
Our idea that $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ could be a potential PL and ECL dual-signaling probe for the detection of NO in living biological samples is based on the following considerations: (1) NO could be rapidly oxidized to N_2O_3 in physiological pH aqueous media under aerobic conditions, while NO_2^- is highly stable without decomposition under the same conditions; (2) $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ could also react with N_2O_3 in the physiological pH solution to form $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$; (3) both the PL and ECL efficiency of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ would be lower due to the excited-state-quenching induced by the electron transfer process, while that of $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ would be higher; (4) $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ could be well cell-membrane permeable due to its cationic property, which enables it to be used for the intracellular NO detection. Fortunately, our characterization results reveal that the PL and ECL responses of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ towards NO are highly sensitive and selective without interferences of NO_2^- and other reactive oxygen/nitrogen species (ROS/RNS) in the physiological pH aqueous media. On the basis of these characters, $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ was successfully used as a probe for the detection of NO in living plant cell and tissue samples both with the PL and ECL modes. Scheme 1 shows the structure of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ and its reaction with NO under aerobic conditions.

2. Experimental

2.1. Materials and physical measurements

1,10-Phenanthroline, tri-*n*-propylamine (TPrA), and 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (c-PTIO) were purchased from Sigma-Aldrich. cis-$Ru(bpy)_2Cl_2 \cdot 2H_2O$ was synthesized by using the literature method [29]. The cultured tobacco cells (*Nicotiana tabacum var. sam sun* NN) were obtained from Dalian Institute of Chemical Physics, Chinese Academy of Sciences. Fresh onion inner-layer epidermal peel samples were prepared in our laboratory. NO gas (purity, 99.95%) was purchased from Guangming Research and Design Institute of Chemical Industry Corporation, Dalian. The saturated NO solution was prepared by bubbling NO gas (passing through a degassed KOH solution to remove trace impurities) for 3 h into an argon-deoxidized 0.1 M phosphate buffer of pH 7.4. The NO concentration in the solution was determined to be 2.2 mM by using the Griess method [30]. NaONOO solution was prepared by reacting 0.6 M $NaNO_2$ with 0.7 M H_2O_2 in a quenched-flow reactor. After the solution was treated with MnO_2 to eliminate excess H_2O_2, the $ONOO^-$ concentration was determined by measuring the absorbance at 302 nm with a molar extinction coefficient of 1670 M^{-1} cm^{-1} [31]. Hydroxyl radical (·OH) was generated in the Fenton system from $(NH_4)_2Fe(SO_4)_2$ and H_2O_2 [32]. Superoxide anion radical (O_2^-) was generated from the xanthine–xanthine oxidase system [33]. Singlet oxygen ($^1O_2$) was generated from the HOCl–H_2O_2 system [34]. The freshly prepared aqueous solutions of NaOCl, $NaNO_2$ and KNO_3 were used as ClO^-, NO_2^- and NO_3^- sources, respectively. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

NMR spectra were recorded on a Bruker Avance spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C). ESI-MS spectra were measured on a HP 1100 LC/MSD MS spectrometer. Elemental analysis was carried out on a Vario-EL CHN analyzer. Absorption spectra were recorded on an Agilent Cary 300 UV–vis spectrometer. PL spectra were measured on a Perkin-Elmer LS 50B luminescence spectrometer with excitation and emission slits of 10 nm. Emission lifetimes of the Ru(II) complexes were measured on an ISS-Chronos multifrequency cross-correlation phase and modulation lifetime spectrometer (ISS Inc., Champaign, IL). PL imaging measurements were carried out on a Nikon TE2000-E luminescence microscope. The microscope, equipped with a 100 W mercury lamp, a Nikon B-2A filters (excitation filter, 450–490 nm; dichroic mirror, 505 nm; emission filter, >520 nm) and a color CCD camera system (RET-2000R-F-CLR-12-C, Qimaging Ltd.), was used for the PL imaging measurements with an exposure time of 10 s. All the ECL measurements were carried out on an ECL instrument system (MPI-A, Remex Electronics Instrument Ltd. Co.) using a small quartz ECL cell at room temperature. The glassy carbon (3.0 mm in diameter) electrode and KCl saturated Ag/AgCl electrode were used as working electrode and reference electrode, respectively, and a platinum wire (0.3 mm in diameter) was used as the auxiliary electrode. Before measurements, the glassy carbon working electrode was soaked in 10% HNO_3 in an ultrasonic water bath for 5 min, polished by an Al_2O_3 slurry, and thoroughly rinsed with deionized water for 5 min. The voltage of the photomultiplier tube was set at 900 V in the detection process while collecting the ECL signals.



Scheme 1. Structure of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ and its reaction with NO under aerobic conditions.

2.2. Syntheses of the ruthenium(II) complexes

The reaction pathway for the synthesis of $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ and $[Ru(bpy)_2(TA\text{-}phen)](PF_6)_2$ is shown in Scheme S1. After DA-phen was synthesized by using the literature method [35], the two Ru(II) complexes were synthesized according to the literature methods [13,28]. Characterization results for $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$, ^{1}H NMR ($CD_3CN$): δ=4.75 (d, 4H), 7.22 (m, 2H), 7.41–7.62 (m, 6H), 7.82(m, 4H), 7.97 (m, 2H), 8.08 (m, 2H), 8.50 (m, 6H); ^{13}C NMR (CD_3CN): δ=124.05, 124.15,124.45, 124.57, 124.99, 127.35, 127.46, 129.49, 137.49, 137.64, 142.33, 148.04, 151.74, 151.86, 157.02, 157.26; Elemental analysis calcd (%): C 41.30, H 2.92, N 12.04; found (%): C 41.66, H 2.95, N 12.24; ESI-MS (m/z): 769.0 ($[M–PF_6]^+$), 312.1 ($[M–2PF_6]^{2+}$). Characterization results for $[Ru(bpy)_2(TA\text{-}phen)](PF_6)_2$, ^{1}H NMR ($CD_3CN$): δ=7.23 (t, 2H), 7.44 (t, 2H), 7.61 (d, J=4.0 Hz, 2H), 7.78–7.83 (m, 4H), 7.98–8.12 (m, 5H), 8.49–8.54 (m, 4H), 9.01 (brs, 2H); ^{13}C NMR (CD_3CN): δ=124.23, 124.30, 126.99, 127.46, 127.60, 137.85, 137.96, 151.93, 152.08, 156.95, 157.17; Elemental analysis calcd (%): C 41.35, H 3.05, N 13.15; found (%): C 41.01, H 2.95, N 13.51; ESI-MS (m/z): 780.1 ($[M–PF_6]^+$), 317.4 ($[M–2PF_6]^{2+}$).

2.3. PL and ECL responses of $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ towards NO

The PL and ECL responses of $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ towards NO were measured at room temperature in 0.1 M phosphate buffer of pH 7.4 and 0.1 M phosphate buffer of pH 7.4 containing 10 mM of TPrA, respectively. After different volumes of the saturated NO solution were added into a solution of $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ (final concentration of the complex, 10 μM), the solutions were stirred for 30 min, and then directly subjected to the PL and ECL measurements on the Perkin-Elmer LS 50B luminescence spectrometer and the MPI-A ECL instrument, respectively.

2.4. Reactions of $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ with different ROS/RNS and metal ions

All the reactions were carried out in 0.1 M phosphate buffer of pH 7.4 or 10 mM TPrA-0.1 M phosphate buffer of pH 7.4 with the same concentration of $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ (10 μM). After reacted with NO (60 μM for PL and 30 μM for ECL), other ROS/RNS (100 μM) or the mixed metal ions (containing Na^+, K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}, 100 μM for each) for 30 min at room temperature, respectively, the PL or ECL intensities of the solutions were determined.

2.5. PL imaging of the NO production in plant cell and tissue samples

Fresh tobacco cells cultured in the MX medium [36] were harvested by filtration of the cell suspension through a 200-mesh nickel screen and rinsed twice with the culture medium. After the cells were incubated in 0.1 M phosphate buffer of pH 7.4 containing 0.2 mM $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ at room temperature for 0.5 or 1.0 h, the cells were washed 3 times with the phosphate buffer, and then subjected to the PL imaging measurement on the Nikon TE2000-E luminescence microscope. To confirm that the PL signals from the cells were originated from the reaction product of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ with the endogenous NO produced in the cells, a control experiment was carried out by incubating tobacco cells in 0.1 M phosphate buffer of pH 7.4 containing a NO scavenger, c-PTIO (0.2 mM), for 1.0 h at room temperature to remove the intracellular NO. After washing, the cells were incubated in the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$-containing medium for 0.5–1.0 h, and then subjected to the PL imaging measurement.

The same procedures as described above were also used for the PL imaging measurement of the onion inner-layer epidermal peel sample.

2.6. ECL detection of the NO production in plant cells

Fresh tobacco cells (1.0 g) suspended in 15 mL of 0.1 M phosphate buffer of pH 7.4 were divided into 5 parts (3.0 mL per part). Each part was incubated with 50 mM $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ in the phosphate buffer at room temperature with slow shaking. At different incubation times (0, 15, 30, 45, and 60 min), the cells were separated by centrifugation for 5 min at 10,000 rpm, washed 3 times with the phosphate buffer, and then added into 3.0 mL of 10 mM TPrA-0.1 M phosphate buffer of pH 7.4 for the ECL measurement.

3. Results and discussion

3.1. Spectral properties of the Ru(II) complexes

The UV–vis absorption spectra of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ and $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ were measured in 0.1 M phosphate buffer of pH 7.4. As shown in Fig. S1, the two complexes displayed typical absorption spectra of the Ru(II)-polypyridyl complexes. The absorption at ~285 nm is attributed to the π–π* transition of the ligands, while the absorptions with the peaks at 448 nm and 455 nm for $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ and $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ are caused by the metal-to-ligand charge transfer (MLCT) transition. There is no remarkable difference between the two spectra, indicating that the emission behavior change from $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ is indeed modulated by the electron transfer mechanism.

The PL properties of the two Ru(II) complexes in 0.1 M phosphate buffer of pH 7.4 were measured to quantitatively compare their PL behaviors. The results are summarized in Table 1. Although $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ and $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ exhibit the similar patterns on their excitation and emission spectra, compared to weakly luminescent $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$, $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ emits remarkably strong luminescence with a 28.4-fold increase in luminescence intensity ($\varepsilon \times \phi$). This result is remarkably higher than that of our previously reported Ru(II) complex-based NO probe $[Ru(bpy)_2(dabpy)]^{2+}$ (which shows 12.4-fold increase in $\varepsilon \times \phi$ after reacted with NO) [13], suggesting that $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ could be a more useful turn-on type PL probe with a higher signal-to-background ratio for the luminescence detection of NO in physiological pH aqueous media. The emission lifetimes of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ and $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ in the phosphate buffer were determined to be 644 ns and 854 ns, respectively, which indicate that the PL emissions of the two Ru(II) complexes are all attributed to the long-lived ^{3}MLCT-based phosphorescence transitions, but the efficient PET from diamino group to the Ru(II) center in $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ could shorten the excited-state lifetime of the complex.

Table 1
PL properties of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ and $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$ in 0.1 M phosphate buffer of pH 7.4 at room temperature.

Complex	$\lambda_{ex,max}$ (nm)	$\varepsilon_{455\ nm}$ ($cm^{-1}\ M^{-1}$)	$\lambda_{em,max}$ (nm)	ϕ[a] (%)	τ (ns)
$[Ru(bpy)_2(DA\text{-}phen)]^{2+}$	448	1.43×10^4	613	0.12	644
$[Ru(bpy)_2(TA\text{-}phen)]^{2+}$	455	1.68×10^4	613	2.90	854

[a] Luminescence quantum yields were measured using $[Ru(bpy)_3]Cl_2$ (ϕ=2.8%) [37] as a standard.

3.2. PL response of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ towards NO

Because the reaction of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ with NO under aerobic conditions is a time-dependent process, and which could affect the practical applicability of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for probing NO, at first, the PL response kinetics of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to different concentrations of NO were investigated in 0.1 M phosphate buffer of pH 7.4. As shown in Fig. S2, in the absence of NO, the PL intensity of the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ solution was low and unchanged with the increase of the reaction time. After reacted with different concentrations of NO, the PL intensity of the solution instantly increased to reach a steady level within 20 s, and then kept at the steady level under the continuous excitation. These results indicate that the aerobic oxidation reaction of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ with NO in physiological pH aqueous media is very fast, which enables $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to rapidly probe NO in living biological samples to avoid the effects of other undesirable reactions.

To quantitatively evaluate the PL response of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to NO, the PL titration experiments were carried out by adding different volumes of the saturated NO solution (2.2 mM) into the solution of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (total volume, 3.0 mL; final concentration of the complex, 10 μM) in 0.1 M phosphate buffer of at pH 7.4. As shown in Fig. 1A, upon additions of different volumes of the saturated NO solution, the PL intensity of the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ solution was sensitively increased with the increase of the NO concentration. The dose-dependent luminescence enhancement showed a good linear relationship in the NO concentration range of 0–45 μM (Fig. 1B). The detection limit for NO, calculated as the concentration corresponding to triple standard deviations of the background signal, is 0.37 μM, indicating that $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ can be used as a PL probe for the quantitative detection of NO at a low micromolar concentration level.

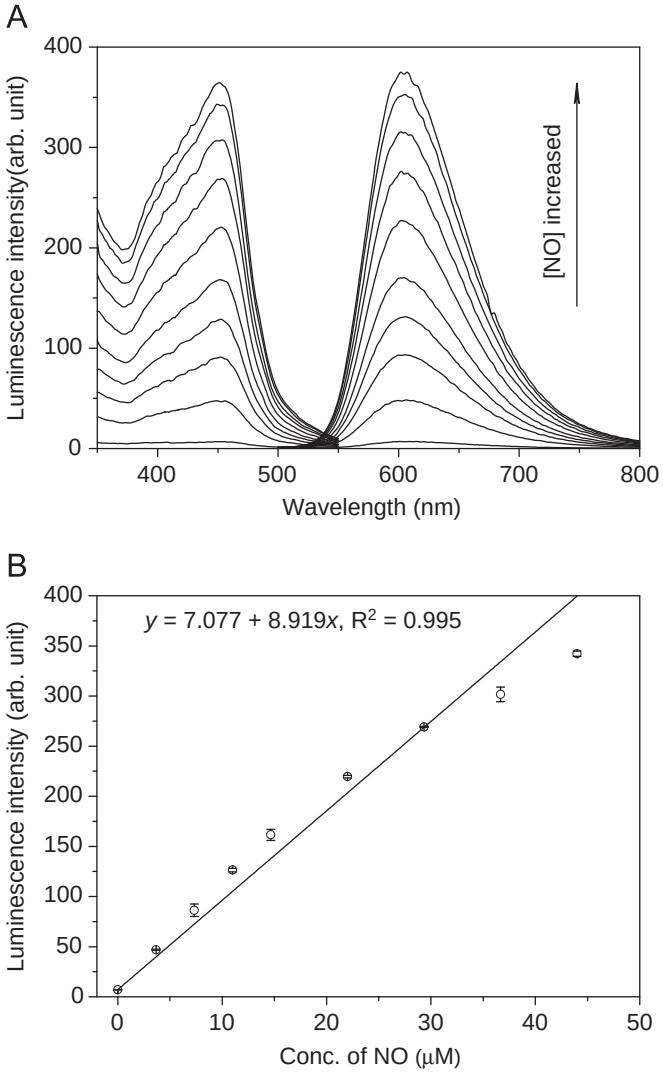


Fig. 1. (A) Excitation and emission spectra of the products of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ reacted with different volumes (0, 5, 10, 15, 20, 30, 40, 50, 60, 70 μL) of the saturated NO solution (2.2 mM) at room temperature in 0.1 M phosphate buffer of pH 7.4 (total volume, 3.0 mL; final concentration of the complex, 10 μM). (B) Calibration curve for the PL detection of NO.

Since diverse ROS/RNS and metal ions often co-exist with NO in the biological systems, to evaluate the PL response specificity of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to NO, the PL intensity changes of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (10 μM in 0.1 M phosphate buffer of pH 7.4) upon reactions with different ROS/RNS (NO, 60 μM; the others, 100 μM) and the mixture of Na^+, K^+, Mg^{2+}, Ca^{2+} and Zn^{2+} (100 μM for each) were determined. As shown in Fig. 2, compared to the remarkable luminescence enhancement of the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$–NO reaction, the PL intensity of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ did not show significant responses to NO_2^-, $ONOO^-$, ClO^-, ·OH, H_2O_2, O_2^-, NO_3^-, $^1O_2$ and the mixed metal ions. These results reveal that the PL response of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to NO is highly specific in physiological pH aqueous media without interferences of other ROS/RNS and metal ions.

3.3. ECL response of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ towards NO

To confirm whether the ECL intensity of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ also responds to the NO concentration changes, the ECL intensities of the products of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (10 μM) reacted with different concentrations of NO in 0.1 M phosphate buffer of pH 7.4 containing 10 mM of TPrA were determined. As shown in Fig. 3A, two ECL peaks appeared when the cyclic potential was scanned from 0.2 V to 1.8 V and then backed from 1.8 V to 0.2 V, respectively. The first main peak during the oxidation process of the Ru(II) complex with a maximum at ~1.4 V is a typical ECL emission from the excited-state of the Ru(II) complex, $[RuL_3]^{2+*}$, that produced by the reduction of $[RuL_3]^{3+}$ with the TPrA· radicals ($[RuL_3]^{3+}+TPrA \rightarrow [RuL_3]^{2+*}$) [38,39], while the second side peak appeared during the reduction process of the Ru(II) complex can

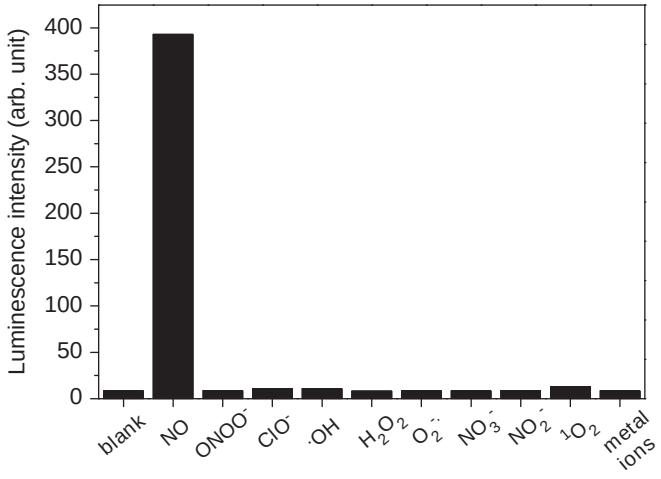


Fig. 2. PL intensity changes of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (10 μM in 0.1 M phosphate buffer of pH 7.4) upon reactions with different ROS/RNS and metal ions (NO: 60 μM; $ONOO^-$: 100 μM NaONOO; ClO^-: 100 μM NaOCl;·OH: 100 μM H_2O_2+100 μM Fe^{2+}; H_2O_2: 100 μM; O_2^-: 100 μM xanthine +100 μM xanthine oxidase; NO_3^-: 100 μM $NaNO_3$; NO_2^-: 100 μM $NaNO_2$; $^1O_2$: 100 μM H_2O_2+100 μM NaOCl; metal ions: the mixture of Na^+, K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}, 100 μM for each).

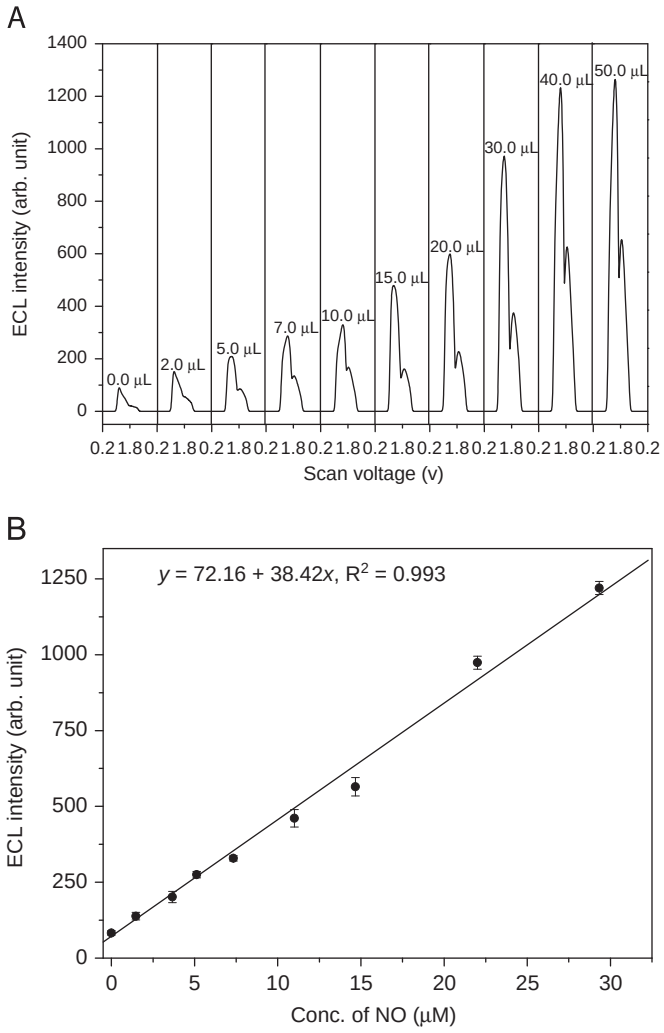


Fig. 3. (A) ECL intensity responses of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to the additions of different volumes (0–50 μL) of the saturated NO solution (2.2 mM) at room temperature in 10 mM TPrA-0.1 M phosphate buffer of pH 7.4 (total volume, 3.0 mL; final concentration of the complex, 10 μM). The voltage of cyclic voltammetry was set up from 0.2 to 1.8 V, and scan rate of ECL was 100 mV s^{-1}. (B) Calibration curve for the ECL detection of NO.

be attributed to the emission from the excited-state of the Ru (I) complex, $[RuL_3]^{+*}$, that produced by the reduction of $[RuL_3]^{2+}$ with the TPrA· radicals ($[RuL_3]^{2+}$+TPrA→$[RuL_3]^{+*}$) [39,40]. However, despite of the ECL behavior of the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$–NO reaction solution, it should be noteworthy that the ECL intensity of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ itself was very low, while it was sensitively increased after the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$–NO reaction. By plotting the ECL intensity versus the NO concentration, a good linear calibration curve with a dynamic range of 0–40 μM and a detection limit of 0.29 μM for the ECL detection of NO was obtained (Fig. 3B). This result indicates that $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ can be also used as a ECL probe for the quantitative detection of NO at a low micromolar concentration level.

The ECL response specificity of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to NO was also investigated by determining the ECL intensities of the products of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (10 μM) reacted with different ROS/RNS and the mixed metal ions in 0.1 M phosphate buffer of pH 7.4 containing 10 mM of TPrA. Similar to the PL results, the ECL intensity of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ could not be enhanced by various ROS/RNS and metal ions except NO (Fig. 4), demonstrating that the ECL response of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ to NO in physiological pH aqueous media is also highly specific.

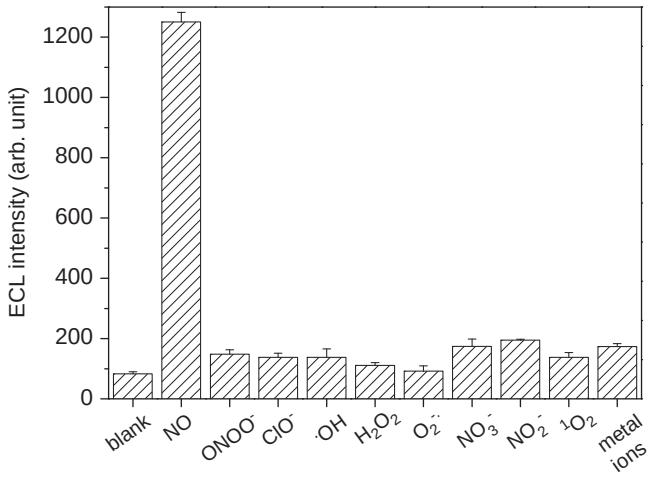


Fig. 4. ECL intensity changes of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (10 μM in 10 mM TPrA-0.1 M phosphate buffer of pH 7.4) upon reactions with different ROS/RNS and metal ions (NO: 30 μM; the others: the same as in Fig. 2).

3.4. Application of $[Ru(bpy)^2(DA\text{-}phen)]^{2+}$ for the detection of NO in biological samples

In order to evaluate the applicability of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for the detection of NO in biological samples, at first, the PL imaging experiments of the endogenous NO production in living tobacco cells and in onion inner-layer epidermal peels were carried out by using $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ as a probe. Fig. 5 shows the bright-field and PL images of tobacco cells before and after incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (0.2 mM) for 30 min and 60 min at room temperature. Compared to the weakly blue images of the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$-untreated cells (Fig. 5A), strong red PL signals were clearly observed from the 30 min or 60 min $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$-treated cells (Fig. 5B and D). However, when the cells were treated with a NO scavenger, c-PTIO, and then incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$, the red PL signals from the cells disappeared (Fig. 5C and E). These results demonstrate that $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ can be rapidly transferred into the tobacco cells for specifically reacting with the intracellular NO molecules to give the red PL signals of $[Ru(bpy)_2(TA\text{-}phen)]^{2+}$.

Fig. 6 shows the bright-field and PL images of onion inner-layer epidermal peels before and after incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (0.2 mM) for 30 min in the absence and presence of c-PTIO at room temperature. Similar to the imaging result of tobacco cells, strong red PL signals from the onion tissue cells were observed in the absence of c-PTIO, while no any PL signals could be observed from ones in the presence of c-PTIO, indicating that $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ can be also used for the PL imaging of the NO production in living plant tissue samples. In addition, compared to the previously reported NO probe $[Ru(bpy)_2(dabpy)]^{2+}$, $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ has a higher signal-to-background ratio for responding NO, which allows the concentration changes of intracellular NO in the samples to be clearly observed in a shorter time (using $[(Ru(bpy)_2(dabpy)]^{2+}$) as a probe, ~1 h is necessary [13]).

To evaluate the applicability of $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for the ECL detection of NO in living plant cell samples, the ECL intensity changes of the tobacco cells incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ at different incubation times were recorded. As shown in Fig. S3, accompanied by the increase of incubation time, the ECL intensity of the cells was gradually increased with a good linear relationship versus the incubation time from 0 to 45 min. This result indicates that the production of endogenous NO in tobacco cells is a uniform and continuous process, and which can be monitored by recording the ECL intensity changes of the $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$-loaded

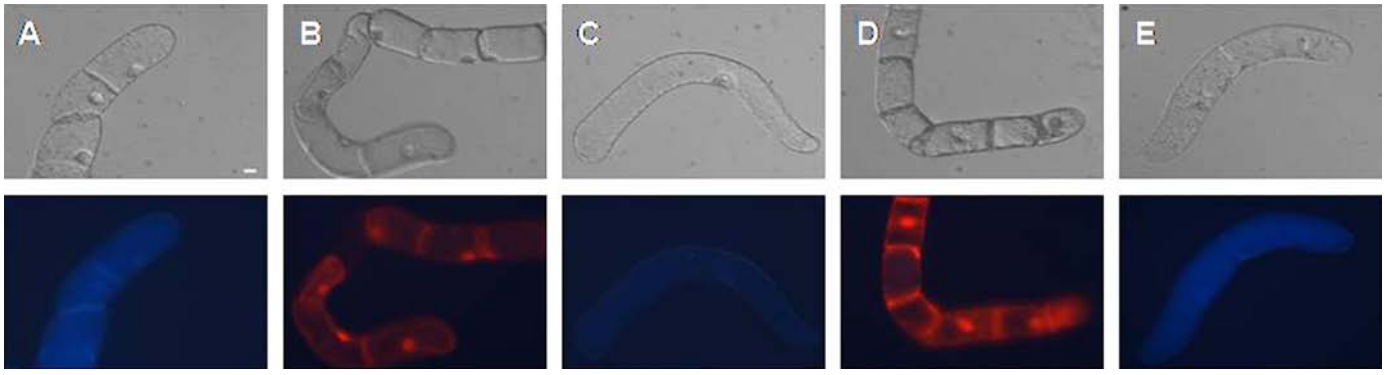


Fig. 5. Bright-field (top) and PL (bottom) images of tobacco cells before and after incubated with 0.2 mM $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ in 0.1 M phosphate buffer of pH 7.4. (A) The $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$-untreated cells; (B) the cells were incubated with $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ for 30 min; (C) the cells were treated with c-PTIO (0.2 mM) for 1 h, and then incubated with $[Ru(bpy)_2(DA\text{-}phen)](PF_6)_2$ for 30 min; (D) the cells were incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for 60 min; (E) the cells were treated with c-PTIO (0.2 mM) for 1 h, and then incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for 60 min. Scale bar: 10 μm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article).

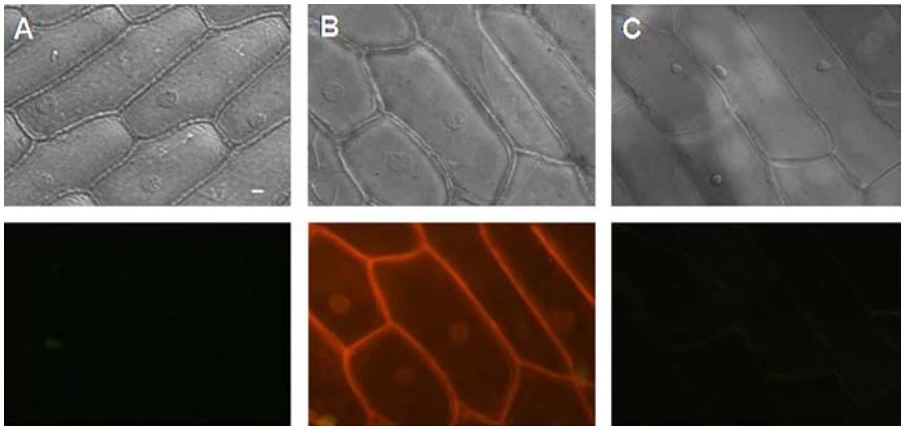


Fig. 6. Bright-field (top) and PL (bottom) images of onion inner-layer epidermal peel samples before and after incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ (0.2 mM) in 0.1 M phosphate buffer of pH 7.4. (A) The sample before incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$; (B) the sample was incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for 30 min; (C) the sample was treated with c-PTIO (0.2 mM) for 1 h, and then incubated with $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$ for 30 min. Scale bar: 10 μm.

cells. Because of the lack of a suitable ECL imaging instrument, the ECL imaging experiment of the NO production in tobacco cells was not carried out.

4. Conclusion

In this work, a Ru(II) complex bearing two 2,2'-bipyridine ligands and one 5,6-diamino-1,10-phenanthroline ligand, $[Ru(bpy)_2(DA\text{-}phen)]^{2+}$, has been demonstrated to be a useful PL and ECL dual-signaling probe for the detection of NO. This probe showed good cell-membrane permeability with highly sensitive and selective PL/ECL responses towards NO, which enabled it to be successfully used for the detection of the endogenous NO production in living biological samples with PL and ECL dual-modes. The new probe, with fine PL and ECL response capacities to NO, provides a unique method for monitoring the amount and spatial distribution of NO produced in living biological cell and tissue samples, which would be a useful tool for investigating the biological functions of NO in living systems.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.064.

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