Electrogenerated Chemiluminescence 69: The Tris(2,2′-bipyridine)ruthenium(II), (Ru(bpy)₃²⁺)/Tri-n-propylamine (TPrA) System Revisited—A New Route Involving TPrA⁺ Cation Radicals

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Abstract: The reaction occurring on electrooxidation of Ru(bpy)₃²⁺ (bpy = 2,2′-bipyridine) and tri-n-propylamine (TPrA) leads to the production of Ru(bpy)₃²⁺⁺ and light emission. The accepted mechanism of this widely used reaction involves the reaction of Ru(bpy)₃³⁺ and a reduced species derived from the free radical of the TPrA. However, this mechanism does not account for many of the observed features of this reaction. A new route involving the intermediacy of TPrA cation radicals (TPrA⁺) in the generation of Ru(bpy)₃²⁺⁺ was established, based on results of scanning electrochemical microscopy (SECM)-electrogenerated chemiluminescence (ECL) experiments, as well as cyclic voltammetry simulations. A half-life of ~0.2 ms was estimated for TPrA⁺ in neutral aqueous solution. Direct evidence for TPrA⁺ in this medium was obtained via flow cell electron spin resonance (ESR) experiments at 20°C. The ESR spectra of the TPrA⁺ species consisted of a relatively intense and sharp septet with a splitting of ~20 G and a g value of 2.0038.

Introduction

Noffsinger and Danielson¹ first reported the chemiluminescence of Ru(bpy)₃³⁺ with aliphatic amines. Following this study, Leland and Powell reported the electrogenerated chemiluminescence (ECL) of the Ru(bpy)₃²⁺ (bpy = 2,2′-bipyridine) with tri-n-propylamine (TPrA) as a coreactant.² Since then, a wide range of ECL analytical applications involving Ru(bpy)₃²⁺ or its derivatives as labels have been reported.³ The Ru(bpy)₃²⁺ (or its derivatives) with TPrA exhibit the highest ECL efficiency, and this system forms the basis of commercial systems for immunoassay and DNA analysis.²,³,⁴ The ECL intensity for this system is proportional to the concentration of both Ru(bpy)₃²⁺ and TPrA species²,⁴−⁶ and also depends on the solution pH and the electrode material.¹,²,⁵ The ECL mechanism of this reaction has been investigated by many workers¹,²,⁴,⁵,⁷ and follows the now familiar coreactant mechanism⁸ where oxidation of TPrA generates a strongly reducing species. This oxidation can be via a “catalytic route” where electrogenerated Ru(bpy)₃³⁺ reacts with TPrA as well as by direct reaction of TPrA at the electrode described by both Scheme 1 and Scheme 2: [where TPrA⁺ = (CH₃CH₂CH₂)₂N⁺, TPrAH⁺ = Pr₂NH⁺, TPrA⁺ = Pr₃NC=CHCH₂CH₃, P₁ = Pr₂N⁺C≡HCH₂CH₃, and P₂ = Pr₂NH + CH₃CH₂-CHO]. The “catalytic” route involving homogeneous oxidation of TPrA with Ru(bpy)₃³⁺ is shown in Scheme 3. The contribution of this process to the overall ECL intensity depends on the Ru(bpy)₃²⁺⁺ concentration and is small when relatively low concentrations of Ru(bpy)₃²⁺⁺ are used.⁵

The mechanisms proposed above, however, do not account for a number of observations made for this ECL system. The strong dependence of ECL efficiency on electrode material\(^5\) and surfactants\(^6\)–\(^12\) strongly supports the importance of the direct oxidation of TPrA. However, two ECL waves occur at a glassy carbon and a gold electrode, with the first ECL wave in a potential range less positive than that for the oxidation of Ru(bpy)\(_3\)\(^{2+}\) (whereas the second wave occurs at potentials for Ru(bpy)\(_3\)\(^{2+}\) oxidation). Both waves were associated with the emission of Ru(bpy)\(_3\)\(^{2+}\) to Ru(bpy)\(_3\)\(^{2+}\). The necessary oxidant to produce ECL at this first wave is not apparent. Moreover, in the commercial Origen analyzer (IGEN International, Inc., Gaithersburg, MD), the Ru(bpy)\(_3\)\(^{2+}\)-tagged species in an immunosassay are immobilized on 2.8 \(\mu\)m diameter magnetic beads that are brought to an electrode by a magnetic field. Direct oxidation of Ru(bpy)\(_3\)\(^{2+}\) on the beads would only occur for those within electron tunneling distance from the electrode, \(~1\) to 2 nm, so most of the labels on the bead would not contribute to the ECL response. However, the high sensitivity of the technique indicates that most of the labels on the beads participate in the reaction, so that some other reactions involving the TPrA coreactant must generate precursors that can form Ru(bpy)\(_3\)\(^{2+}\).

Clearly, the present ECL mechanisms proposed above (Schemes 1–3) cannot explain these results. Moreover, there has been no convincing explanation of the relative efficiencies of related coreactants, e.g., Et\(_3\)N vs TPrA, based on the mechanism that only involves the free radical reductant as an intermediate (e.g., correlating the reducing power of the radical with observed ECL emission efficiency). Studies that attempting to correlate coreactant efficiency with electron donating/withdrawing power of the amine have been reported previously.\(^{13}\)

An understanding of the mechanism of the Ru(bpy)\(_3\)\(^{2+}\)/TPrA system is important in designing and selecting new coreactants and in improving the sensitivity and reproducibility of the ECL system. In this paper, a new route involving TPrA corecatalytic reduction for the generation of the excited-state Ru(bpy)\(_3\)\(^{2+}\) is presented. This mechanism is supported by a scanning electrochemical microscopy (SECM)–ECL experiment, cyclic voltammetry (CV), and CV digital simulations, and by the direct detection by electron spin resonance (ESR) showing that the TPrA \(^{2+}\) cation radical is relatively stable under conditions similar to those used in the ECL experiments, i.e., in aqueous solutions at neutral pH.

### Experimental Section

**Chemicals.** Tris(2,2\(^’\)-bipyridyl)ruthenium(II) dichloride hexahydrate (Ru(bpy)\(_2\)Cl\(_2\)-6H\(_2\)O), tri-n-propylamine (TPrA, 99.4\% ), and Freny’s salt ([KSO\(_3\)\(_2\)]NO\(_3\)) from Aldrich (Milwaukee, WI), (3-aminopropyl)-trimethylsilane ([CH\(_3\)O\(_2\)]Si(CH\(_2\))\(_3\)NH\(_2\), \(\geq 97\%\)) and lithium perchlorate (LiClO\(_4\), \(> 99\%\)) from Fluka (Milwaukee,WI), potassium permanganate (KMN\(_2\O_4\)), sodium phosphate dibasic dehydrate (Na\(_2\)HPO\(_4\)\(_7\)H\(_2\)O, \(98.0\%\)), and phosphoric acid (H\(_3\)PO\(_4\), 85\%) from Fisher (Fairlawn, NJ), potassium phosphate monobasic (KH\(_2\)PO\(_4\), 99.6\%) from J. T. Baker (Phillipsburg, NJ), tris(hydroxymethyl)aminomethane (Tris, ultrapure) from Life Technologies (Rockville, MD), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma Ultra) and 1-methylimidazolide from Sigma (St. Louis, MO), hydrochloric acid (HCl, GR) from EM (Gibbstown, NJ), tolune (A. R.) from Mallinckrodt (Hazelwood, MO), and Et\(_2\)O (200-proof) from Aaper Alcohol (Shelbyville, KY) were used without further purification. Bis(2,2\(^’\)-bipyridine)-4,4\(^’\)-dicarboxy-2,2\(^’\)-bipyridineruthenium(II) hexa-fluoro-phosphates {Ru(bpy)\(_3\)[bpy(COOH)]\(_2\)[PF\(_6\)]\(_2\)} were prepared by following a procedure in the literature.\(^{14}\) Unless otherwise stated, all solutions were freshly prepared with deionized water (Milli Q, Millipore).

**Immobilization of Ru(bpy)\(_3\)[bpy(COOH)]\(_2\)^{2+} on an ITO Electrode.** A clean, dried indium tin oxide (ITO) electrode (resistance, 30–60 ohm/square; Delta Technologies, Stillwater, MN) with dimensions of \(\sim1 \times 1\) cm was immersed in a 5\% of (CH\(_3\)O)\(_2\)Si(CH\(_3\))\(_2\)NH\(_2\) tolune solution and kept in a desiccator for 24 h. During this process, (CH\(_3\)O)\(_2\)Si(CH\(_3\))\(_2\)NH\(_2\) becomes immobilized by formation of ITO/O–Si(CH\(_3\))\(_2\)NH\(_2\) bonds.\(^{15}\) The electrode was then washed with Et\(_2\)O and transferred into a 0.10 M 1-methylimidazolide/HCl buffer solution (pH = 7) containing \(\sim10\) mM Ru(bpy)\(_3\)[bpy(COOH)]\(_2\)\(^{2+}\) and 10 mM EDAC. After a 45 min incubation at \(70^\circ\)C, the ITO electrode was washed thoroughly with Et\(_2\)O and then water. By this treatment, a layer of Ru(bpy)\(_3\)[bpy(COOH)]\(_2\)\(^{2+}\) was covalently attached to the aminosilane formed previously on the ITO, to produce ITO/O–Si(CH\(_3\))\(_2\)NH\(_2\)–[CO–(bpy)[bpy(COOH)]][bpy]Ru(II) (designated as ITO/OlSiRu\(_3\)) electrode was subsequently immersed in a 0.10 M Tris/HCl buffer (pH = 8) and maintained in the dark until further use.

**Electrochemical and ECL Measurements.** Fast scan CV was performed with the model 660 electrochemical work station (CH Instruments, Austin, TX). A conventional three-electrode cell was used, with Pt wire as the counter electrode and an Ag/AgCl/KCl(sat.) as the reference electrode. The working electrodes used for the examination of TPrA redox behavior at fast scan rates were of different materials [Pt, Au, carbon fiber, and glassy carbon (GC)] and different dimensions (3 mm, 100 \(\mu\)m, 25 \(\mu\)m, 5 \(\mu\)m diameter Pt; 2 mm diameter Au; 3 mm diameter GC and 10 \(\mu\)m diameter carbon fiber). The ECL along with the CV signals were measured simultaneously with a home-built potentiostat combined with a photomultiplier tube (PMT, Hamamatsu R4220p, Japan) installed under the electrochemical cell. A voltage of \(-750\) V was supplied to the PMT with a high-voltage power supply series 225 (Bertan High Voltage Corp., Hicksville, NY). A 3 mm diameter GC working electrode, and the counter and reference electrodes the same as those used for fast scan CV were used.

The SEC-MEC experiments were taken with a CHI 900 SEC system (CH Instruments, Austin, TX) combined with the ECL instrument described. Figure 1 shows a schematic diagram of the setup. The working electrode (tip), was a 1.5 mm diameter hemispherical Au, and the ITO/OlSiRu\(_3\) (“modified ITO”) served as the substrate. The direct oxidation of covalently attached Ru(bpy)[bpy(COOH)]\(_2\)\(^{2+}\) species at...
the ITO electrode was avoided by holding the electrode at an open circuit. A Pt wire and an Ag/AgCl/KCl (sat.) electrode were also used as the counter and reference electrodes, respectively.

The simulation of the cyclic voltamogram of TPrA oxidation in aqueous solution was carried out by using the simulation package DigiSim V 3.03 (Bioanalytical Systems Inc., West Lafayette, IN).

ESR Experiments. The cation radical of TPrA, TPrA$^{+}$, was generated by flowing 0.10 M TPrA (pH 7, adjusted by HNO$_3$) and an 0.03 M Ru(bpy)$_3$$^{3+}$ solution through a quartz flat cell$^{16}$ via a two-jet mixing chamber (Wilmad, Prod. No. WG-801-Q, Buena, NJ). The Ru(bpy)$_3$$^{3+}$ solution was freshly prepared by bubbling Cl$_2$ gas, generated via the reaction of solid KMnO$_4$ and concentrated HCl, directly into a 0.030 M Ru(bpy)$_3$.Cl$_2$ solution. A clear color change from reddish orange to dark greenish blue was observed upon the complete oxidation of Ru(bpy)$_3$$^{3+}$ to Ru(bpy)$_3$$^{4+}$.

X-band ESR spectra were recorded on an ER-300 ESR spectrometer (IBM Instruments Inc.) with a modulation frequency of 100 kHz and microwave power at 12.6 mW. The swept field and the $g$ value were calibrated externally using a 5.0 mM aqueous Femy’s salt solution.$^{17,18}$ The quartz flat cell was installed in a TESLA rectangular cavity. Overall, solution flow rates of 2–5 mL/s were employed, and the relative flow rates for the TPrA and Ru(bpy)$_3$$^{3+}$ solutions were adjusted so that an orange color appeared at the outlet of the flow cell.

The spectra were simulated using PEST Wimsin Software$^{19}$ (National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC).

All experiments were conducted at a temperature of 20 ± 2 °C, unless otherwise stated.

Results and Discussion

Electrochemical and ECL Behavior of the Ru(bpy)$_3$$^{2+}$/TPrA System. An earlier report$^{5}$ showed that at low concentrations of Ru(bpy)$_3$$^{2+}$ ($\sim$μM) and 10–100 mM TPrA in aqueous 0.15 M phosphate buffer, pH 7.5, the ECL emission vs potential curves displayed two broad waves. Similar behavior was also observed in the presence of 0.10 M TPrA when a much lower concentration of Ru(bpy)$_3$$^{2+}$, 1.0 nM. (Figure 2a) and a different buffer solution, 0.10 M Tris/0.10 M LiClO$_4$, pH = 8, were used. (Figure 2). The initial ECL signal started at potentials where the direct oxidation of TPrA at the GC electrode occurs (Figure 2a,b), and reached a first maximum at a potential of about 0.90 V vs Ag/AgCl, about 50 mV less positive than the peak potential for TPrA oxidation, and well before Ru(bpy)$_3$$^{2+}$ oxidation. The second ECL signal has a peak potential value of 1.14 V vs Ag/AgCl in the potential region of the direct oxidation of Ru(bpy)$_3$$^{2+}$ at a GC electrode. For comparison, Figure 2 also includes the ECL signal profile obtained with the Ru(bpy)$_3$$^{2+}$ concentration of 1.0 μM (Figure 2c). The corresponding TPrA oxidation CV is not included because it is essentially the same as that in Figure 2b. The relative ECL intensity from the first wave is significant, particularly in the 1.0 nM Ru(bpy)$_3$$^{2+}$ solution, and thus, the bulk of the ECL signal obtained in this system with low concentrations of analytes, as in immunoassays and DNA probes with Ru(bpy)$_3$$^{2+}$ as an ECL label, probably originates from the first ECL wave.

Note that even with a high concentration of Ru(bpy)$_3$$^{2+}$ ($\sim$mM), the ECL signal first appeared in a potential range less positive than that for the oxidation of Ru(bpy)$_3$$^{2+}$. However, the initial ECL signal is relatively small compared to the large

![Figure 2](image)

**Figure 2.** (a) ECL and (b) cyclic voltammogram of 1.0 nM Ru(bpy)$_3$$^{2+}$ in the presence of 0.10 M TPrA with 0.10 M Tris/0.10 M LiClO$_4$ buffer (pH = 8) at a 3 mm diameter glassy carbon electrode at a scan rate of 50 mV/s. (c) As (a) but with 1.0 μM Ru(bpy)$_3$$^{2+}$. The ECL intensity scale is given for (c) and should be multiplied by 100 for (a).

![Figure 3](image)

**Figure 3.** Linear sweep voltammograms and their corresponding ECL signals for 1 μM Ru(bpy)$_3$$^{2+}$ and 50 mM TPrA in pH 8.5 PBS at a 3 mm diameter glassy carbon electrode at different scan rates. Inset, plot of the first and the second ECL signals versus $\nu^{1/2}$.

ECL signal generated via Schemes 1 and 2. Note also that the ECL signal observed under current conditions is not due to the reaction of Ru(bpy)$_3$$^{2+}$ with hydroxide in solution as reported previously$^{20}$ because no ECL signal was seen in the absence of TPrA when the electrode potential was scanned from 0 to 1.4 V vs Ag/AgCl for the oxidation of Ru(bpy)$_3$$^{2+}$ in a buffer solution of 0.10 M Tris/0.10 M LiClO$_4$, pH = 8.

Figure 3 shows linear sweep voltammograms and the corresponding ECL signals obtained from 1 μM Ru(bpy)$_3$$^{2+}$ and 50 mM TPrA in 0.20 M phosphate buffer solution (PBS, pH 7.5) at a GC electrode at different potential scan rates (υ). A linear relationship between the ECL peak intensity $(i_{ECL})$ for both the first and the second ECL waves and $\nu^{1/2}$ was observed (Figure 3, inset), consistent with the linear relationship between the peak current for the oxidation of TPrA and $\nu^{1/2}$. These results

References:

suggest that under current experimental conditions, both the first and the second ECL signals are directly related to the oxidation of TPrA.

The effect of both TPrA and Ru(bpy)$_3^{2+}$ concentrations on the first ECL peak intensity ($i_{\text{p1,ECL}}$) was also investigated. As shown in Figure 4a, in 1 μM Ru(bpy)$_3^{2+}$ (0.20 M PBS, pH 7.5), the $i_{\text{p1,ECL}}$ is linearly proportional to the concentration of TPrA. No ECL signal was detected in the absence of either TPrA or Ru(bpy)$_3^{2+}$, so the first ECL signal must be associated with a reaction between the species generated from TPrA and Ru(bpy)$_3^{2+}$. Figure 4b shows the $i_{\text{p1,ECL}}$ changes as a function of Ru(bpy)$_3^{2+}$ concentration when TPrA concentration kept at a constant value of 100 mM (0.20 M PBS, pH 8.5). In the range of 10 μM to 50 μM of Ru(bpy)$_3^{2+}$, $i_{\text{p1,ECL}}$ steadily rises with an increase of Ru(bpy)$_3^{2+}$ concentration. Beyond 50 μM, $i_{\text{p1,ECL}}$ reaches a plateau. In contrast, the ECL intensity of the second wave was always proportional to the concentration of Ru(bpy)$_3^{2+}$, for concentrations up to 1 mM. Figure 4c shows both the first and second ECL responses when the Ru(bpy)$_3^{2+}$ concentration was in the “plateau” range of 50 μM to 1.0 mM. These results clearly indicate that the mechanism for the first ECL signal is different from the second one. As demonstrated later, the intensity of the first ECL signal is determined by the quantity of TPrA derived radicals (cation and free) generated from the oxidation of TPrA at the electrode. In a relative excess of Ru(bpy)$_3^{2+}$ solution, the $i_{\text{p1,ECL}}$ is governed by the concentration of TPrA (Figure 4a). On the other hand, when the reaction produces excess TPrA radicals relative to the concentration of Ru(bpy)$_3^{2+}$, the $i_{\text{p2,ECL}}$ will be controlled by the concentration of Ru(bpy)$_3^{2+}$. When the Ru(bpy)$_3^{2+}$ concentration is equivalent to or greater than the concentration of radicals formed from TPrA oxidation, the $i_{\text{p2,ECL}}$ will be independent of the concentration of Ru(bpy)$_3^{2+}$. Figure 4c exhibits these two features simultaneously. Studies on the relationship between $i_{\text{p1,ECL}}$ and TPrA concentration have been reported previously.$^{2,5,6}$

**SECM–ECL Experiments.** To prove that oxidation of TPrA generates an ECL signal without direct oxidation of Ru(bpy)$_3^{2+}$, experiments with the instrumental setup shown in Figure 1 were carried out. In this experiment, Ru(bpy)$_3[\text{bpy(COOH)}_2]^{2+}$, whose ECL behavior is very similar to that for Ru(bpy)$_3^{2+}$ in the presence of TPrA,$^{12}$ was covalently immobilized on an ITO electrode. During the course of the SECM–ECL experiment, this modified ITO electrode was at an open circuit potential and served as the substrate. The reaction medium used was 10.0 mM TPrA solution in 0.10 M Tris/0.10 M LiClO$_4$ buffer (pH = 8). Note that the tip in this experiment was considerably larger than those usually employed in SECM$^{21}$ to generate a sufficient flux of TPrA radicals to obtain an observable emission signal. However, this electrode still showed a decreasing SECM current response on approach to a substrate that blocked diffusion of reactant to the tip (Figure 5). The distance between the tip and the substrate ($d$) was estimated from this approach curve. By holding the tip potential at 0.85 V vs Ag/AgCl, where TPrA oxidation occurs, a gradual decrease in tip current was initially observed as the tip slowly approached the substrate (~0.05 μm/s at the last stage). The zero distance was indicated by a sudden increase in the tip current when contact is made between the tip and ITO. The tip was withdrawn from the substrate a given distance and cycled between 0 and 1.0 V vs Ag/AgCl at $v$ = 50 mV/s, and the current and any ECL signal generated in the electrochemical cell were monitored. A typical experimental result for $d = -1.92$ μm is given in Figure 6.

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Upon oxidation of TPrA at 0.80 V vs Ag/AgCl, an ECL signal appears and tracks the tip current during potential cycling. A pair of prewaves located at 0.5 V vs Ag/AgCl on the CV were frequently observed during the oxidation of TPrA, and may be attributed to the gold surface oxide formation and reduction.\textsuperscript{5} The ECL signal could be detected only within a very small distance (< 5–6 \mu m); the maximum intensity as a function of the tip distance is shown in Figure 7. At short distance, the ECL intensity decreased almost exponentially with the tip distance, indicating that the amount of dissolved Ru(bpy)\textsubscript{2}[(bpy(COOH)\textsubscript{2})\textsuperscript{2+}] species from the substrate in the solution phase (if any) is negligible under the experimental conditions. This experiment clearly demonstrates that by simply oxidizing TPrA intermediates are formed that can cause excitation of Ru(bpy)\textsubscript{3}\textsuperscript{2+} and is a direct confirmation of the ability to form excited states at all positions of a 2.8 \mu m bead on an electrode surface.

The electrochemical oxidation of TPrA under various conditions has been extensively studied and the following mechanism for reactions occurring in aqueous solution is generally accepted (Scheme 4):\textsuperscript{4,22–26} (where TPrA\textsuperscript{++}, TPrA, P1, and P2 species have the same significance as in Scheme 2).

Deprotonated TPrA molecules first undergo one-electron oxidation to form TPrA cation radicals (TPrA\textsuperscript{++}), which rapidly deprotonate to form TPrA free radicals (TPrA\textsuperscript{+}). These oxidize further by losing one electron to produce the iminium ion (P1), which subsequently hydrolyzes to products, P2.

On the basis of previous study,\textsuperscript{5} we believe that the ECL signal generated under the present conditions is the emission of immobilized Ru(bpy)\textsubscript{2}[(bpy(COOH)\textsubscript{2})\textsuperscript{2+}], simplified as “Ru(bpy)\textsubscript{3}\textsuperscript{2+}” in the following discussions because Ru(bpy)\textsubscript{2}[(bpy(COOH)\textsubscript{2})\textsuperscript{2+}] and Ru(bpy)\textsubscript{2}\textsuperscript{2+} species exhibit very similar electrochemical\textsuperscript{27} and ECL\textsuperscript{12} behavior. According to Scheme 4, the direct oxidation of TPrA at the tip of SECM – ECL experiments leads to the formation of reducing radicals (TPrA\textsuperscript{+}, E° ~ 0.83 to 0.95 V vs Ag/AgCl)\textsuperscript{28} that can diffuse away from the tip and react with immobilized Ru(bpy)\textsubscript{3}\textsuperscript{2+} to generate Ru(bpy)\textsubscript{3}\textsuperscript{+}. To produce Ru(bpy)\textsubscript{3}\textsuperscript{+}, some oxidant is required to remove an electron from Ru(bpy)\textsubscript{3}\textsuperscript{2+}. The most likely oxidant candidate in this system is the cation radical TPrA\textsuperscript{++}, E° ~ 1.7 V vs Ag/AgCl\textsuperscript{28}. However, the deprotonation process of TPrA\textsuperscript{++} to TPrA\textsuperscript{+} has usually been taken to be an extremely rapid process, resulting in an insufficient flux of TPrA\textsuperscript{++} from the tip to produce measurable ECL. We considered the possible production of Ru(bpy)\textsuperscript{3+} from reaction of Ru(bpy)\textsubscript{2}\textsuperscript{2+} with TPrA\textsuperscript{++}, but the redox potential of the TPrA\textsuperscript{++}/TPrA couple is not sufficient for this reaction (see references 22–26).

References:


(28) Miao et al.)
Scheme 5, 29 where the potentials have been converted to Ag/AgCl by taking $E^{0}_{\text{Ag/AgCl}} = 0.20$ V vs NHE.30

Scheme 6 summarizes a new route for the generation of ECL within the potential range before direct oxidation of $\text{Ru(bpy)}_3^{2+}$ at the electrode, involving formation of excited state on reaction of TPrA$^+$ with $\text{Ru(bpy)}_3^+$ (formed by reaction of $\text{Ru(bpy)}_3^{2+}$ with TPrA$^*$).

If the maximum distance at which light is observed, $\sim 6 \text{ m}$, represents the distance that TPrA$^+$ can diffuse before deprotonation, given a typical diffusion coefficient of $5 \times 10^{-6}$ cm$^2$/s, the half-life, $\tau_{1/2}$, of the cation radical would be $\sim 0.2 \text{ ms}$ (See the next section for details). A similar lifetime of TPrA$^+$ can also be inferred from the data in ref 6.

**Fast Scan CV and Digital Simulations.** With $\tau_{1/2} \approx 0.2 \text{ ms}$, one would estimate that TPrA$^+$ could be detected by CV, if $v > 130 \text{ V/s}$ ($-RT/F\tau_{1/2})$.30 Fast scan CV experiments were thus carried out to detect TPrA$^+$ species after TPrA oxidation at the electrode by scan reversal. A wide range of TPrA aqueous solutions ($\sim 0.010$ to $0.10 \text{ M}$) with different supporting electrolytes and different pHs (from $0.2 \text{ M H}_2\text{SO}_4$ to pH $\approx 10$), different materials of the working electrode (GC, carbon fiber, Au, Pt) with different dimensions (from $5 \text{ mm}$ to $3 \text{ mm}$ diameters), and different scan rates between $20 \text{ mV/s}$ and $1000 \text{ V/s}$ were used. However, no wave associated with the reduction of TPrA$^+$ on the reverse scan of the CVs was ever found. Scan rates faster than $1000 \text{ V/s}$ and CV with background subtraction were also tried without success, since in these cases, significant charging currents and probably limitations from the rate of the heterogeneous electron transfer from TPrA interfered.

The failure to detect the cation radical can be ascribed to the fact that the oxidation of TPrA is an ECE process and that at potentials where TPrA$^+$ is reduced, the radical TPrA is oxidized, so that a cathodic current is at least partially compensated by an anodic one. To investigate this effect, CV digital simulations following Scheme 4 were carried out assuming different values for the heterogeneous electron-transfer rate constants $k_s$, $k_f$, and $k_b$ values of eq 4.3. Other parameters were either adopted from the literature or assigned to reasonable values. Figure 8a shows a simulated ECL of TPrA oxidation in pH 8 solution at a scan rate of 900 V/s. (b) to (e) The concentration profiles for TPrA$^+$ and TPrA$^*$ at a specific points, A, B, C, and D, shown in (a). Parameters used in the simulation were (see Scheme 4 for further information): $E^{0}_{\text{TPrA}^+/\text{TPrA}} = 0.88$ V vs Ag/AgCl, $k_s$ (for eqs 4.2 & 4.4) = $7 \times 10^6$ s$^{-1}$, $k_{f,TPrA^-}$ = $10 \text{ mM}$. All species were assumed to have a diffusion coefficient of $5 \times 10^{-6}$ cm$^2$/s except for $\text{H}^+$ ($D_{\text{H}^+} = 5 \times 10^{-5}$ cm$^2$/s).

CV of TPrA oxidation in pH 8 solution at a scan rate of 900 V/s, with the parameters shown in the figure caption. The cross over on the reverse scan, where the anodic current is larger on the reverse scan than it is on the forward scan current (at $\sim 0.96$ V vs Ag/AgCl), can be attributed to the greater contribution of TPrA$^*$ to the measured current. The concentration profiles (c vs x) for both TPrA$^+$ and TPrA$^*$ at specific potential values are shown in Figure 8b–e. Upon the oxidation of TPrA, a significant amount of TPrA$^+$ cation radical is formed at the electrode surface (Figure 8b), which further dissociated to TPrA$^*$.
Because the free radical is a very strong reducing agent and can be oxidized immediately at the electrode, its surface concentration equalling zero is expected. During the potential scan period of A to B (Figure 8b–c), due to the continuous conversion of TPRA+ to TPRA•, as well as diffusion to the bulk solution, the surface concentration of TPRA+ starts to decrease, while the overall amount of TPRA• in the electrode surface region increases. After potential B, substantial reduction of TPRA• to TPRA is expected, which results in a cathodic current and the surface concentration of TPRA+ species decreases dramatically (Figure 8d). Note that even in this potential region, the overall current at the electrode is always anodic, because the current contribution from the oxidation of TPRA• is larger. Therefore, the absence of a reduction wave following the oxidation of TPRA with fast scan CV does not indicate that TPRA+ is so unstable that it cannot participate in the ECL reaction.

These digital simulations were also carried out to evaluate the lifetime of TPRA+ based on the data obtained from the previous SECM–ECL experiment in 10 mM TPRA (pH 8). Figure 9 shows the simulated concentration distribution for TPRA+ formed at the tip as a function of distance from the tip. This curve was recorded at the potential A on the CV (inset of Figure 9), at which a maximum surface concentration and a maximum diffusion distance of TPRA+ (Figure 9) are needed to diffuse to the substrate and react with immobilized Ru(bpy)32+. By comparing the data shown in Figure 9 with those in Figure 7, about 3.6% of the initial TPRA+ (or 71 nM, at x = 6 µm, Figure 9) are needed to diffuse to the substrate and react with immobilized Ru(bpy)32+ to generate an ECL signal.

With the assumption that the TPRA+ deprotonation reaction (eq 4.3) is a first-order process, the half-life (τ1/2) of TPRA+ is τ1/2 = 0.693/kf, where kf is the forward rate constant of eq 4.3, ~3500 s⁻¹ based on the digital simulation. Thus, τ1/2 ~ 0.2 ms for TPRA+ cation radicals can be estimated.

ESR of TPRA+ in Aqueous Solution. With the above estimated lifetime, it should be possible to detect TPRA+ by ESR. Tertiary amine radical cations are generally believed to be short-lived intermediates in amine oxidation because of rapid deprotonation at the α-carbon to produce α-aminoalkyl free radicals. However, previous studies also have shown that trimethylamine radical cation has a similar lifetime as that estimated here for TPRA+. Under some experimental conditions, such as strongly acidic solution and formation by ionizing radiation, the ESR of several small aliphatic amines cation radicals has been reported.

To detect the TPRA+ cation radicals, an ESR quartz flat flow cell was used. Such cells have proven effective in producing a steady-state concentration of many reactive intermediates within the microwave cavity of the ESR instrument. Freshly prepared ~0.03 M Ru(bpy)33+ and 0.10 M TPRA (pH 7) aqueous solutions were flowed into the mixing chamber at a rate of 2 to 5 mL/s and the ESR spectrum of the product in the quartz cell obtained. The major advantages of using Ru(bpy)33+ as the oxidant are that the reaction between Ru(bpy)33+ and TPRA is rapid at neutral pH and it is a one-electron-transfer process. Many other oxidants, such as Ce4+, KMnO4, KBrO3, and Cl2, were found to be unsuitable for TPRA oxidation because either they required the use of strongly acidic solutions, where TPRA oxidation was slow, the oxidant itself or its reduction products gave strong ESR background, or the redox reaction was not fast enough. Figure 10 shows the experimentally measured (dotted line) as well as the simulated (solid line) ESR spectra of TPRA+ generated in the flow cell at 20 °C. The spectrum consists of a relatively intense and sharp septet with a splitting of ~20 G due to the six equivalent α-hydrogens. On the basis of the simulation, hyperfine coupling constants of 19.87 and 20.05 G for 14N (1N, I = 1) and α-1H (6H, I = 1/2) were evaluated. The experimental ESR spectrum had a g-value of 2.0038. Recently, Liu et al. have reported the structure and dynamics of TPRA+ cation radicals generated in solid AlPO4 by ionizing radiation. Our solution-phase ESR spectrum is consistent with their solid-state ESR spectrum at 300 K.

During the ESR experiments, Ru(bpy)33+ and Ru(bpy)32+ species can also produce signals. However, they have no effect on the ESR spectrum of TPRA+ because the g-values of these two species are very different than that of TPRA+. No ESR signal associated with TPRA• was observed, probably because they were oxidized by excess oxidant in the solution. Note that

the expected ESR spectrum of the carbon-centered TPrA\(^+\) would be very different than the one observed.

**Conclusions**

The ECL emission as a function of potential for the \(\text{Ru(bpy)}_3^{2+}/\text{TPrA}\) system consists of two waves. The first occurs with the direct oxidation of TPrA at the electrode, and the second where \(\text{Ru(bpy)}_3^{2+}\) is oxidized. In dilute \(\text{Ru(bpy)}_3^{2+}\) solutions (< ~μM) containing mM TPrA, the intensity of the first ECL wave is significant and can be larger than that for the second ECL wave. A new route for the generation of \(\text{Ru(bpy)}_3^{2+}\) at potentials of the first wave is proposed, where TPrA\(^+\) formed during TPrA oxidation is a sufficiently stable intermediate that can oxidize \(\text{Ru(bpy)}_3^+\) (formed from the reduction of \(\text{Ru(bpy)}_3^{2+}\) by TPrA\(^+\) free radical) to give \(\text{Ru(bpy)}_3^{2+}\). On the basis of SECM–ECL experiments and digital simulation, a half-life of ~0.2 ms was estimated for TPrA\(^+\) in aqueous solution. An ESR spectrum of TPrA\(^+\) in aqueous solution was obtained via flow mixing experiments. Fast scan CV experiments and simulations revealed that the absence of reduction wave for TPrA\(^+\) on the reverse scan can be attributed to the cancellation of the component of current from the reduction of TPrA\(^+\) by the anodic current contribution from the oxidation of TPrA\(^+\). These results also explain why selection of highly efficient coreactants is difficult for determination of low concentrations of Ru-label. The coreactant must form both oxidant (e.g., TPrA\(^+\)) and reductant (e.g., TPrA\(^+\)) with appropriate redox potentials and lifetimes. Thus, the deprotonation rate of TPrA\(^+\) must be just right to build up the needed concentrations of both species.

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