Electrogenerated chemiluminescent determination of tris(2,2'-bipyridine)ruthenium ion (Ru(bpy)32+) at low levels

Deniz. Ege, William G. Becker, and Allen J. Bard

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electroactive gas, here O_2. This method was carried out by using a readily available commerical controller/rotator to vary modulation frequency. The theoretical advantage of SHM that the frequency response of modulated current is independent of the concentration of the electroactive species (D remaining constant) and of the number of electrons transferred is clearly shown for this useful case.

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Electrogenerated Chemiluminescent Determination of $Ru(bpy)_3^{2+}$ at Low Levels

Deniz Ege, William G. Becker, and Allen J. Bard*

Department of Chemistry, The University of Texas, Austin, Texas 78712

Two different chemiluminescence methods, employing the reactions between electrogenerated $Ru(bpy)_3^{3+}$ (bpy = 2,2'-bipyridine) and oxalate and between electrogenerated $Ru(bpy)_3^{+}$ and peroxydisulfate, were studied and compared. Picomolar and subpicomolar concentrations of $Ru(bpy)_3^{2+}$ were determined on the basis of electrogenerated chemiluminescence. The log–log plot of the emitted light intensity vs. $Ru(bpy)_3^{2+}$ concentration was linear over the region 10^{-13} to 10^{-7} M. Application of $Ru(bpy)_3^{2+}$ as a chemiluminescent label is suggested.

Chemiluminescent (CL) labels have been frequently used in competitive protein binding reactions to determine low concentrations of hormones, drugs, and other biologically important compounds. These labels often undergo highly sensitive and specific CL reactions and have been investigated as replacements of the potentially hazardous, less convenient, and less stable radioactive labels (*1*). Three kinds of CL labels are frequently used (*2*): (a) chemiluminescent substances such as luminol or the enzyme luciferase (*3–5*), (b) catalysts for chemiluminescent reactions (e.g., peroxidase or other oxidizing enzymes) (*6*), and (c) compounds generating products which participate in luminescence reactions (e.g., enzyme labels (*7*)). The CL reactions of labels such as luminol and related hydrazides have been widely investigated (*8*, *9*). So far, there has been no report on the use of a metal complex itself as a CL label in a competitive protein binding reaction. However, the use of [bis(salicylaldehyde)ethylenediiminato]cobalt(II) (Co(Salen)) and iron(III) 4,11,18,25-tetracarboxyphthalocyanine, (TCP–Fe(III)) have been reported as catalyst labels in CL reactions (*10*, *11*). Catalyst labels such as these, which change the rate of luminol or luciferase CL reactions, are rather nonspecific, however, since many factors and substances can perturb these reaction rates. Here we suggest the possible application of $Ru(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine) as a CL label in competitive protein binding reactions. In this study we have investigated two different CL methods of determination of $Ru(bpy)_3^{2+}$ based on electrogenerated chemiluminescence (ECL) and demonstrate that levels of $Ru(bpy)_3^{2+}$ can be detected that are consistent with the practical use of this substance as a label. The luminescence from the electronic excited state of $Ru(bpy)_3^{2+}$ is relatively insensitive to the presence of O_2, organic or inorganic impurities. The stability of $Ru(bpy)_3^{2+}$ and its intense luminescence make $Ru(bpy)_3^{2+}$ a potentially very attractive label. The CL and ECL of $Ru(bpy)_3^{2+}$ have been studied extensively in different solvents and under different conditions (*12–16*). Recently ECL resulting from the $Ru(bpy)_3^{2+}/C_2O_4^{2-}$ reaction was used for the determination of oxalate (*16*). Two different methods for generating ECL of $Ru(bpy)_3^{2+}$ have been used in this study: (a) electrochemical oxidation of $Ru(bpy)_3^{2+}$ in the presence of oxalate in an aqueous solution to form $Ru(bpy)_3^{3+}$ this reaction is followed by a chemical reduction of $Ru(bpy)_3^{2+}$ with $CO_2^{-}\cdot$, generating $Ru(bpy)_3^{+}$ (*14*)), and (b) electrochemical reduction of $Ru(bpy)_3^{2+}$ in the presence of peroxydisulfide in an acetonitrile (MeCN)/water mixture (chemical oxidation of $Ru(bpy)_3^{2+}$ follows this step to form $Ru(bpy)_3^{3+}$ upon reaction with $SO_4^{-}\cdot$ (*15*)). In both cases the light-emitting excited-state complex is formed mainly by the annihilation reaction between $Ru(bpy)_3^{+}$ and $Ru(bpy)_3^{3+}$.

EXPERIMENTAL SECTION

All chemicals used were reagent grade. $Ru(bpy)_3^{2+}Cl_2\cdot H_2O$ was obtained from Strem Chemicals (Newburyport, MA). Phosphate buffers and acetate buffers of ionic strengths 0.5 and 0.1, respectively, were used. $(NH_4)_2S_2O_8$ (MCB) was recrystallized from an EtOH/H_2O mixture. MeCN (Fisher), sodium oxalate (Allied Chemical), tetra-*n*-butylammonium fluoroborate ($TBABF_4$) (South Western Analytical Chemicals, electrometric grade), $NaH_2PO_4\cdot H_2O$ (Baker), $Na_2HPO_4\cdot 7H_2O$ (Baker), and Na_3PO_4 (Baker) were used without further purification. Solutions were prepared by using triply distilled water.

Electrochemical measurements were performed in a conventional three-electrode cell, volume 30 mL, with an optically flat bottom. The working electrode was either a glassy carbon (GC) (0.17 cm^2) or a platinum disk (0.02 cm^2) electrode; the surfaces of these electrodes were aligned parallel to the cell bottom. Platinum gauze was used as an auxiliary electrode. A saturated

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sodium chloride reference electrode (SSCE) or a silver wire quasi-reference electrode was used. Electrochemical measurements were performed with either a Princeton Applied Research (PAR) Model 175 universal programmer, a Model 173 potentiostat/galvanostat, and a Model 179 digital coulometer or an IBM Model EC 225 voltammetric analyzer. ECL intensities were measured through the bottom of the electrochemical cell with a Hamamatsu Model R 928 photomultiplier (PM) tube. Signals were recorded on and integrated by a Bascom-Turner Instruments Model 8110 electronic recorder. The working electrodes were polished by using 6-μm, 1-μm, and 0.25-μm diamond paste (Buehler) starting with the most coarse and finishing with the finest grade. The electrodes were sonicated for about 3 min in EtOH before use. Solutions were deaerated before and between measurements with solvent-saturated N_2. Filming occurred during electroreduction; this was especially pronounced with GC electrodes. Reconditioning the electrodes by dipping them into concentrated nitric acid and waiting for about 5 min between each measurement helped to remove the film. Standard solutions of $Ru(bpy)_3^{2+}$ were prepared by series dilution. Typically a solution of 1×10^{-4} M $Ru(bpy)_3^{2+}$ was prepared and diluted 100 times each time to obtain solutions of concentration 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M. The test solution was obtained by mixing 1 mL of the standard solution with 1 mL of 180 mM $S_2O_8^{2-}$ solution and 8 mL of a CH_3CN/H_2O mixture containing 0.1 M $TBABF_4$. Care was taken to ensure that the positioning of the cell over the PM tube and the placement of the electrodes within the cell was the same during each measurement. For the determination of the linearity of the light intensity as a function of $Ru(bpy)_3^{2+}$ concentration over a small range of concentrations, a microliter syringe was used to add $Ru(bpy)_3^{2+}$ stock solution to the reaction mixture in increasing amounts. The solution was mixed by bubbling N_2 after each addition of $Ru(bpy)_3^{2+}$.

RESULTS AND DISCUSSION

$Ru(bpy)_3^{2+}/C_2O_4^{2-}$ Experiments. ECL in this system arises from the oxidation of $Ru(bpy)_3^{2+}$ in the presence of oxalate in the following proposed reaction sequence (*14*):

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e \quad (1)$$

$$Ru(bpy)_3^{3+} + C_2O_4^{2-} \rightarrow Ru(bpy)_3^{2+} + C_2O_4^{-}\cdot \quad (2)$$

$$C_2O_4^{-}\cdot \rightarrow CO_2 + CO_2^{-}\cdot \quad (3)$$

$$Ru(bpy)_3^{2+} + CO_2^{-}\cdot \rightarrow Ru(bpy)_3^{+} + CO_2 \quad (4)$$

$$Ru(bpy)_3^{3+} + Ru(bpy)_3^{+} \rightarrow Ru(bpy)_3^{2+*} + Ru(bpy)_3^{2+} \quad (5)$$

Solutions of $Ru(bpy)_3^{2+}$ were prepared between 1×10^{-9} and 1×10^{-5} M concentrations. Before any ECL measurements were made, cyclic voltammograms were recorded to determine the redox potential for the $Ru(bpy)_3^{2+}/Ru(bpy)_3^{3+}$ couple. A cyclic voltammogram for a 1 mM solution of $Ru(bpy)_3^{2+}$, in phosphate buffer pH 5, in the presence of 30 mM $Na_2C_2O_4$ is shown in Figure 1A. The ECL measurements were made by stepping the potential to a value at which the electrochemical reaction was diffusion-controlled (e.g., +1.1 V vs. SSSE) for a short period of time (e.g., 5–10 s). The emission light intensity transient so obtained was integrated over the duration of the pulse. The integrated light intensities of the emission transients obtained, as a function of $Ru(bpy)_3^{2+}$ concentrations, are summarized in Table I. This system was useful only down to concentrations of about 5×10^{-8} M. At concentrations equal to and/or lower than 1×10^{-8} M $Ru(bpy)_3^{2+}$, the measured light intensity could not be differentiated from the background signal. Even in the absence of oxalate, a slight emission could be observed. This low emission did not disappear when the buffer was changed from phosphate to acetate. The origin of this emission is believed to be due to oxidation of trace impurities in the solution by the electrochemically generated $Ru(bpy)_3^{3+}$ (*14*).

The emitted light intensity decreased during successive measurements, especially when a GC electrode was used. This

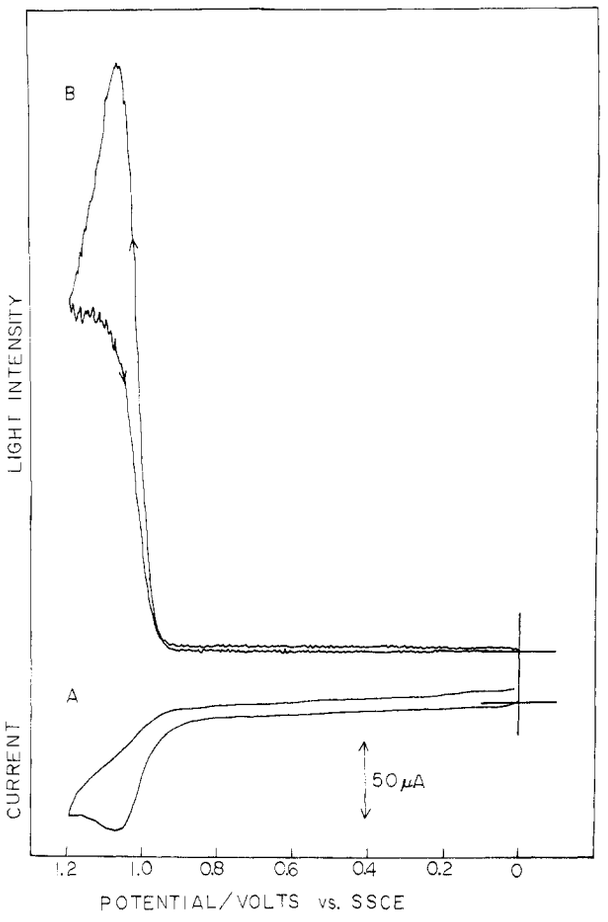

Figure 1. (A) Cyclic voltammogram for 1 mM $Ru(bpy)_3^{2+}$ in pH 5 phosphate buffer in the presence of 30 mM $Na_2C_2O_4$, at GC electrode, at a sweep rate of 50 mV s^{-1}; (B) light intensity vs. potential profile for the solution in (A).

Table I. Integrated Intensity of the Emission Transient as a Function of $Ru(bpy)_3^{2+}$ Concentration in Phosphate Buffer pH 5 in the Presence of 1 mM Oxalate, E_{app} = +1.0 - 1.4 V vs. SSCE, GCE

	integrated light intensity, arbitrary units	
concn of $Ru(bpy)_3^{2+}$/M	expt 1	expt 2
	2 ± 0.03	1 ± 0.03
1×10^{-9}	2 ± 0.6	
1×10^{-8}	1 ± 0.3	2 ± 0.05
1×10^{-7}	19 ± 6	15 ± 3
1×10^{-6}	270 ± 92	290 ± 76
1×10^{-5}	5900 ± 130	4600 ± 460

decrease is believed to be caused by formation of a passivating film on the electrode surface. The initial light intensities could be obtained again after proper treatment of the electrodes. Platinum disk electrodes gave more reproducible results than GC electrodes with a minimum of film formation. To our knowledge, passivation of electrode surfaces in the oxalate system has not been reported before. However, film formation by reduced $Ru(bpy)_3^{2+}$ species has been observed (*15*, *17*). It is known that electrochemical oxidation of $Ru(bpz)_3^{2+}$ (bpz = 2,2′-bipyrazyl) in solution results in formation of a poorly conductive film on the electrode (*18*). There was no ECL observed until a potential was applied at which $Ru(bpy)_3^{2+}$ was oxidized (Figure 1B). Although deoxygenating the solutions was not necessary to observe ECL, oxygen removal was very important in obtaining good reproducibility. As expected, a pH dependency was observed for the ECL light intensities, because $C_2O_4^{2-}$ is one of the reactive species (pK_{a_2} 5.18 at 25

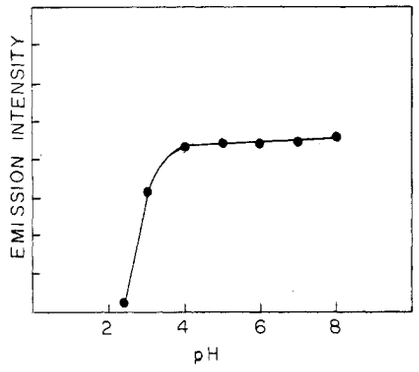

Figure 2. ECL light intensity dependence of a 0.6 mM $Ru(bpy)_3^{2+}$ solution on pH, in phosphate buffer (μ = 0.2), in the presence of 27 mM $Na_2C_2O_4$.

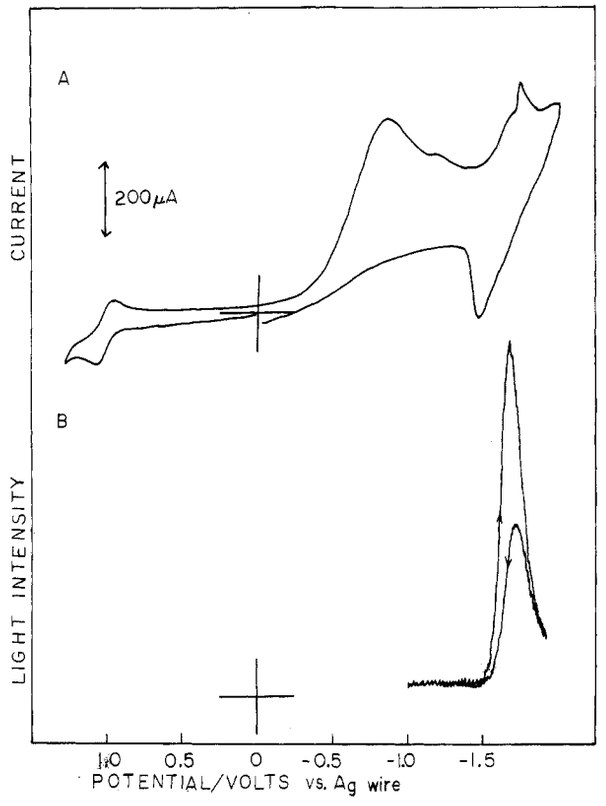

Figure 3. (A) Cyclic voltammogram for 2 mM $Ru(bpy)_3^{2+}$ in a (1:1) CH_3CN/H_2O mixture, 0.1 M $TBABF_4$ in the presence of 10 mM $Na_2S_2O_8$, at a GC electrode, at a sweep rate of 100 mV s^{-1}; (B) light intensity vs. potential profile for the solution in (A).

°C) (*19*). The measured light intensity increased with increasing pH in accord with the pK_a value reported for oxalic acid (Figure 2).

$Ru(bpy)_3^{2+}/S_2O_8^{2-}$ Experiments. ECL in this system is based on the following reaction sequence (*15*):

$$Ru(bpy)_3^{2+} + e^- \rightarrow Ru(bpy)_3^{+} \quad (6)$$

$$Ru(bpy)_3^{+} + S_2O_8^{2-} \rightarrow Ru(bpy)_3^{2+} + S_2O_8^{3-} \quad (7)$$

$$S_2O_8^{3-} \rightarrow SO_4^{2-} + SO_4^{-}\cdot \quad (8)$$

$$Ru(bpy)_3^{2+} + SO_4^{-}\cdot \rightarrow Ru(bpy)_3^{3+} + SO_4^{2-} \quad (9)$$

$$Ru(bpy)_3^{+} + Ru(bpy)_3^{3+} \rightarrow Ru(bpy)_3^{2+*} + Ru(bpy)_3^{2+} \quad (10)$$

Lower concentrations of $Ru(bpy)_3^{2+}$ were used in this system, varying between 1×10^{-13} and 1×10^{-7} M. Similar to the $Ru(bpy)_3^{2+}/C_2O_4^{2-}$ system, a cyclic voltammogram was recorded to determine the redox potential for the Ru-

Table II. Integrated ECL Intensity as a Function of $[Ru(bpy)_3^{2+}]^a$

concn of $Ru(bpy)_3^{2+}$/M	integrated ECL intensity, arbitrary units
0	1.9
1×10^{-13}	3.1
1×10^{-12}	11
1×10^{-11}	25
5×10^{-11}	43
1×10^{-10}	110
1×10^{-9}	360
1×10^{-8}	700
1×10^{-7}	7100

[a] All solutions 18 mM in $Na_2S_2O_8$ and 0.1 M $TBABF_4$ in CH_3CN:H_2O (1:1 v/v).

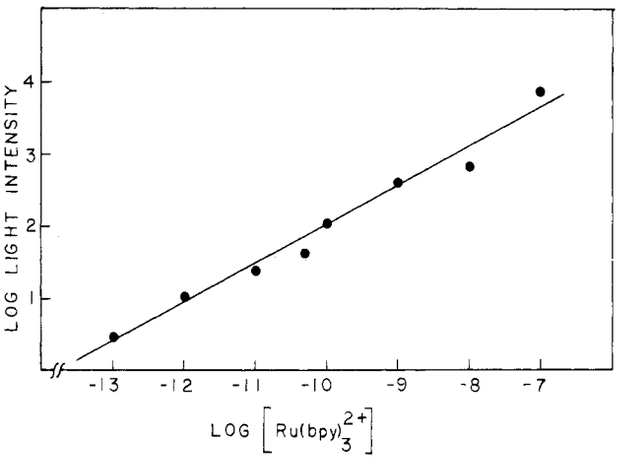

Figure 4. log–log plot of the observed light intensity vs. $Ru(bpy)_3^{2+}$ concentration.

$(bpy)_3^{2+}/Ru(bpy)_3^{+}$ couple (Figure 3A). Measurements of the ECL light intensities were carried out in a similar fashion as reported for the oxalate system. Concentrations of $Ru(bpy)_3^{2+}$ as low as 1×10^{-13} M could be detected. Deaeration of sample solutions was necessary to eliminate residual oxygen and observe any ECL, especially at very low $Ru(bpy)_3^{2+}$ concentrations. However, no deaeration was necessary at higher $Ru(bpy)_3^{2+}$ concentrations (e.g., 10^{-8} M) to detect emitted light. The log of the light intensity was linear with the log of concentration over 6 orders of magnitude with a correlation coefficient of 0.99. No emission could be detected in the absence of peroxydisulfate. The integrated light intensities are summarized in Table II as a function of $Ru(bpy)_3^{2+}$ concentration. A log–log plot of the observed light intensity vs. $Ru(bpy)_3^{2+}$ concentration is shown in Figure 4.

A decrease in the ECL light intensities was observed upon repetitive measurements with a GC electrode; this could be eliminated to a large extent by using a Pt disk electrode. Formation of a passivating film (*15*, *17*) was minimal at a Pt electrode, possibly because H_2 formation at the electrode surface had a continuous cleansing effect.

Linearity of ECL Measurements. The light intensity obtained in the $Ru(bpy)_3^{2+}/S_2O_8^{2-}$ system was linear over 6 orders of magnitude of $Ru(bpy)_3^{2+}$ concentration. To check the correlation of the signal intensity vs. concentration to a straight line, a smaller range of concentrations was used. Between 5×10^{-8} and 3.2×10^{-7} M $Ru(bpy)_3^{2+}$, the emitted light intensity was linear with a correlation coefficient of 0.999, a slope of 1.14 ± 0.5 M^{-1}, and an intercept of 1.4 ± 0.5 (light intensity units). The intercept observed can be attributed to instrumental fluctuations caused by the high sensitivity settings required to measure low light intensities (photo-

Table III. Reproducibility of Integrated Light Intensities for a 1×10^{-9} M $Ru(bpy)_3^{2+}$ solution in 18 mM $Na_2S_2O_8$ in 1:1 CH_3CN/H_2O

sample	integrated light intensity, arb units	σ^a	no. of runs
1	34[b]	9	4
2	27[b]	1	17
3	36[c]	1	9
4	21[c]	3	8
5	31[b]	5	16
6	24[b]	0.1	3

[a] Standard deviation. [b] Potential was stepped to −2.0 V vs. Ag wire, and the resulting signal was integrated for 20 s. [c] A cyclic voltammogram was run at 50 mV s^{-1} from −1.5 to −2.0 V and back to −1.5 V, and the resulting light intensity was integrated for 20 s.

multiplier tube dark current).

Reproducibility of ECL Measurements. Reproducibility of the measured light intensities was determined by performing the ECL experiments on different days or on the same day using fresh solutions. The light intensities were obtained by either recording an emission transient during a 20-s long potential step or recording the light intensity during a cyclic voltammetric run. The integrated light intensities did not vary significantly with the method employed. The results are summarized in Table III. The mean value for all the measurements was 28 with a 95% confidence limit of ±2, for a 1×10^{-9} M $Ru(bpy)_3^{2+}$ solution.

Quenching of $Ru(bpy)_3^{2+*}$ by Typical Biological Components. Samples of $Ru(bpy)_3^{2+}$ were excited by shining light at 450 nm to produce the electronic excited state $Ru(bpy)_3^{2+*}$. The resulting emission was measured in the absence and in the presence of typical biological species which may be present in a competitive protein-binding immunoassay. From these measurements, we concluded that typical biological constituents which may be used in an immunoassay do not quench $Ru(bpy)_3^{2+*}$ significantly. For example, in the absence of any biological compounds, the light intensity measured was 31 133 for 1 mM $Ru(bpy)_3^{2+}$; whereas, upon addition of phosphate buffered saline (PBS), Tween (a surfactant), and tissue liver extract, the measured light intensity was 31 516. Following photoexcitation experiments, ECL measurements were carried out on the same solutions in the presence of oxalate. The excited-state complex generated by ECL was not quenched in the presence of the added biological compounds (Figure 5). However, the potential for oxidation for $Ru(bpy)_3^{2+}$ shifted to slightly higher potentials by ca. 100 mV. The shift in the potential may be due to adsorption of some of the biological components on the electrode surface, thus changing the surface properties of the electrode.

The sensitivity of ECL measurements in the $Ru(bpy)_3^{2+}/S_2O_8^{2-}$ system is greater than the oxalate system, although their estimated coulometric efficiencies (ϕ_{coul}, photons generated per electron injected) are not significantly different. For the $Ru(bpy)_3^{2+}/S_2O_8^{2-}$ system, ϕ_{coul} was estimated to be 2.5% (*15*), whereas ϕ_{coul} for $Ru(bpy)_3^{2+}/C_2O_4^{2-}$ was reported to be 2% (*14*). The background emission observed in the oxalate system limits the sensitivity of the ECL measurements. Since there is practically no background emission detected in the peroxydisulfate, much higher sensitivities are easily achieved.

Concentrations of clinically significant analytes usually vary all the way from 10^{-1} to 10^{-12} M (*1*); in most cases, the concentration range of interest is μM–pM. For example, the levels for hepatitis viral antigen in a clinical sample vary between 10^{-10} and 10^{-12} M (*1*). Thus, $Ru(bpy)_3^{2+}$ would be a good candidate to be used as a CL label. The ECL generated by $Ru(bpy)_3^{2+}$ is very specific and quite intense. CL systems most commonly used today involve the CL reactions of luminol or

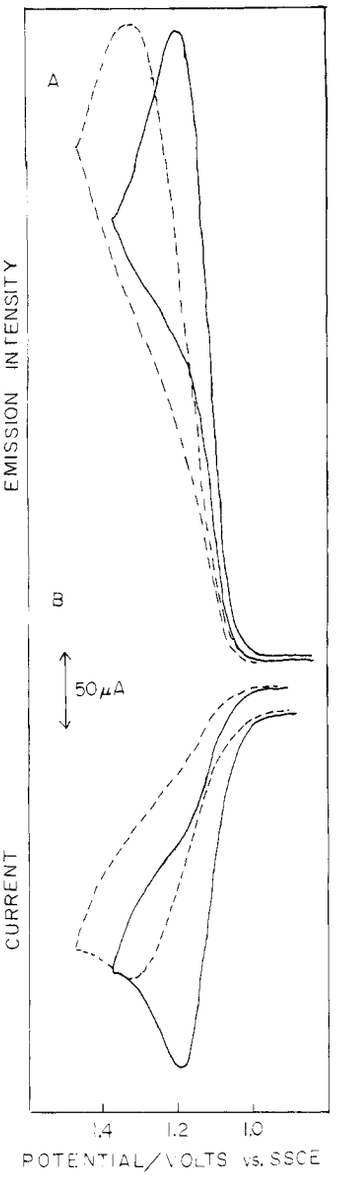

Figure 5. (A) ECL light intensity as a function of potential for 1 mM $Ru(bpy)_3^{2+}$ in the oxalate system in the absence (—) and in the presence (- - -) of liver tissue extract and ascites fluid; (B) single sweep voltammograms for the solution in (A) at a GC electrode at a sweep rate of 50 mV s^{-1}. Oxalate concentration is 30 mM.

related hydrazides. CL from luminol can be generated by oxidation by using a variety of compounds (e.g., hypochlorite, ferricyanide, persulfate, or hydrogen peroxide) at high pH (*9*). The light intensities obtained from various oxidation systems vary by more than 100-fold (*9*). Since luminol chemiluminescence is not very specific, other compounds present in biological solutions of interest may interfere with the chemiluminescence reactions. For example, the presence of proteins such as bovine serum albumin in the sample solution is reported to affect the light emission by quenching or enhancement, depending on the oxidation system employed. Other CL reactions, like enzyme-catalyzed processes that lead to formation of H_2O_2, can be coupled to CL-generating reactions in which H_2O_2 is allowed to react with CL compounds. Again these reactions lack specificity. Biological solutions consist of complex mixtures, and many of the components may interfere with the CL measurements. Several transition metal ions and complexes catalyze the luminol CL. Presence of a trace amount of a transition metal may cause an erroneous signal. ECL generated by $Ru(bpy)_3^{2+}$ has another advantage besides being specific. Repetitive measurements can be made

on the same sample, since an excess of $C_2O_4^{2-}$ or $S_2O_8^{2-}$ is used and the ECL-producing substance is generated only at the electrode surface. This also leads to an effective amplification effect. The $Ru(bpy)_3^{2+}$-label-containing species can be turned over many times during integration of an emission pulse without being depleted. Higher sensitivities can probably be obtained by increasing the working electrode area and utilizing different modulation and signal-averaging techniques. If necessary, higher specificity can be gained by observation over a restricted range of wavelengths or by time-resolved measurements.

CONCLUSIONS

The ECL method employing the $Ru(bpy)_3^{2+}/S_2O_8^{2-}$ system appears to be sensitive and specific enough to be used in competitive protein-binding reactions. Because of its specificity and high emission, $Ru(bpy)_3^{2+}$ seems to be potentially superior to other chemiluminescent labels. The measurements are fast, reproducible, and do not involve complicated instrumentation.

Registry No. $Ru(bpy)_3^{2+}$, 15158-62-0; oxalic acid, 144-62-7; peroxydisulfate, 15092-81-6.

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Potentiometric Flow-Injection Determination of Copper Complexing Inorganic Anions with a Copper Wire Indicator Electrode

Peter W. Alexander* and Paul R. Haddad

Department of Analytical Chemistry, University of New South Wales, P.O. Box 1, Kensington, New South Wales, Australia 2033

Marek Trojanowicz

Department of Chemistry, University of Warsaw, Warsaw, Poland

The potentiometric response of a metallic copper wire as an indicator electrode is measured in a flow-injection system when inorganic anions are injected into a buffered carrier stream without the addition of excess Cu^{2+}. The experimental data show good agreement with theory derived from Nernstian response due to the Cu^{2+}/Cu^0 redox couple at the solid electrode surface after anionic complex or precipitate formation. The response to pyrophosphate, tripolyphosphate, cyanide, sulfite, chloride, iodide, and thiocyanate is shown to give slopes and sensitivity dependent on the complex stoichiometry and stability at the electrode surface, with slopes varying in the range from divalent 29 mV to double monovalent 118 mV with detection of ligand quantities as low as 0.75 nmol injected.

The development of pentiometric methods of analysis based on ion selective membrane electrodes has been aimed mainly at applications for selective determinations of single species in solution. For some years, potentiometry has frequently been applied for selective detection in flow analysis by use of membrane electrodes (*1*). Recent investigations (*2–5*), however, have shown that a metallic copper electrode in flow analysis systems can be applied successfully for potentiometric detection of many different species responsive at the same electrode. The electrode potential in these cases is only partially selective and depends on the concentration of copper complexing ligands in the sample zone.

The copper electrode has been utilized as a potentiometric detector, for example, in continuous flow determination of amino acids (*2*), for detection of amino acids in HPLC (*3*), for indirect flow injection determination of cations (*4*), and for indirect determination of alkaline-earth cations in HPLC (*5*). Nonselective potentiometric response of an electrode in flow systems is therefore of potential value as a detection method in many applications of liquid chromatography.

In this study, the copper electrode is used in a flow-injection system as an indicator electrode giving direct potentiometric response to anions including phosphates, cyanide, sulfite, halides, and thiocyanate, without the addition of Cu(II) to the carrier stream. A treatment of the response characteristics

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