

Hot electron-induced time-resolved electrogenerated chemiluminescence of a europium(III) label in fully aqueous solutions

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Abstract

Time-resolved electrogenerated chemiluminescence of multidentate phenolic Eu(III) chelates were studied in aqueous solution. 2,6-bis[*N,N*-bis(carboxymethyl)-aminomethyl]-4-benzoylphenol forms a photoluminescent and electrochemiluminescent Eu(III) chelate, whereas 2,6-bis[*N,N*-bis(carboxymethyl)-aminomethyl]-4-methylphenol-chelated Eu(III) turned out to be not luminescent at all. The importance of the redox properties of both the ground and the excited states of the ligands and the central ion is shown. The former chelate shows relatively weak ECL at an oxide-covered aluminum electrode but the ECL intensity can be strongly enhanced by the addition of peroxodisulfate ions. In the presence of 1 mM peroxodisulfate ions the ECL lifetime of this chelate is 0.94 ms, thus easily allowing time-resolved detection of the chelate. This chelate can be conjugated to antibodies by thioureido linkage and used as an electrochemiluminescent label in immunoassays as a marker which displays long-lived luminescence in the red end of the optical spectrum. The present ECL is mainly based on the ligand sensitized redox excitation of the chelate by analogous pathways to those known from the studies of aromatic Tb(III) chelates but the energy transfer from the emission centers of the aluminum oxide film can also have minor contribution to the excitation of the label.

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1. Introduction

Cathodic time-resolved electrogenerated chemiluminescence (TR-ECL) of aromatic Tb(III) chelates at thin insulating film-coated electrodes provides a means for extremely sensitive detection of Tb(III), and of biologically or clinically interesting compounds, if aromatic Tb(III) chelates are used as labels in bioaffinity assays [1–7]. The high sensitivity is mainly due to the long luminescence lifetime of chelated Tb(III) and the use of time-resolved measuring techniques in connection with pulsed excitation of the chelates. The same excitation method can be used also in cases of Ru(bpy)₃²⁺ labels [8,9], metalloporphyrins [10], luminol [11] and some other organic luminophores [12].

It has been earlier suggested that tunnel emission of hot electrons is the primary step of cathodic ECL at thin insulating film-covered conducting electrodes [2–7]. Theoretically speak-

ing, the aluminum oxide film makes it possible to raise the Fermi level of aluminum above the conduction band edge of water by an applied voltage pulse, and simultaneously to create a high electric field in the oxide film. When the electric field reaches the threshold for tunnel-emission, electrons are emitted across the oxide film barrier ballistically, i.e., without a significant loss of energy, if the oxide film is ultra thin, i.e. thinner than ca. 4–6 nm [3–7]. While in case of thicker oxide films, electron tunneling is predominated by Fowler–Nordheim tunneling mechanism, where tunnel emission cannot occur through the oxide, but only to the conduction band of the oxide. After this first step, electrons are transported in the conduction band of the oxide, and then the electrons gain energy in the electric field, and parallelly lose energy in inelastic scattering. The mean free path for hot electrons in the conduction band of the oxide determines whether the net effect is further electron heating in the conduction band. Thus, the transfer of the electrons into the solution at oxide/electrolyte interface can occur from the bottom of the conduction band of the oxide or even somewhat above it [4,5,8]. Typically, the cathodic band bending is so

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strong that practically no ECL is generated at thick oxide films under Fowler–Nordheim tunneling mechanism. However, in the cases of ultra thin oxide films, electrons may reach the solution with energies well above the conduction band edge of water at the surface of the oxide film [2–11].

Eu(III) is another lanthanide(III) ion having sufficiently long luminescence lifetime for time-resolved detection when Eu(III) is suitably chelated and the detection is based on photoluminescence [13,14]. Richter and Bard [15] have described ligand-sensitized electrogenerated chemiluminescence (ECL) of europium(III) labels proposed to be usable in binding assays. The main drawback of the Eu(III) labels and the traditional electrochemical excitation methods studied by Richter and Bard for practical analysis is that fully aqueous solutions can not be used and the luminescence quantum yields of their Eu(III) labels were very poor even in non-aqueous solutions [15].

Both Tb(III) and Eu(III) ions have excellent luminescence properties, but their redox properties are quite different. Tb(III) ion is extremely difficult to one-electron reduce or oxidize ($E^0(\text{Tb}^{3+}/\text{Tb}^{2+}) = -3.7 \text{ V}$, $E^0(\text{Tb}^{4+}/\text{Tb}^{3+}) = 3.1 \text{ V}$) [16]. Eu(III) ion is one of the ions which are the most difficult to oxidize within the whole lanthanide series ($E^0(\text{Eu}^{4+}/\text{Eu}^{3+}) = 6.4 \text{ V}$) [16], but it is easiest to reduce in this series ($E^0(\text{Eu}^{3+}/\text{Eu}^{2+}) = -0.35 \text{ V}$) [16]. Aromatic Tb(III) chelates show chemiluminescence (CL) and ECL in aqueous solution either based on the ligand reduction-initiated oxidative excitation pathway (red-ox pathway) or on the ligand oxidation initiated reductive excitation pathway (ox-red pathway) [2–7]. These pathways involve one-electron reduction/oxidation of the ligand to a radical species followed by one-electron oxidation/reduction of the ligand radical. If the oxidant/reductant of the second step is strong enough the ligand is formed in its excited state. The ligand transfers its excitation energy intramolecularly to the central ion by the photophysical processes well-known from the photoluminescence studies of these chelates [13,14], and finally the central ion emits light by transitions from the lowest excited state multiplet to the ground state multiplet [13,14]. The whole process requires that (i) the triplet state of the ligand must be somewhat higher in energy than the resonance level of the lanthanide(III) ion, (ii) the triplet state of the ligand must not react with the central ion by a rapid redox reaction, and (iii) the emitting central ion must not react with the ligand [13,14,17]. These prerequisites are often easily fulfilled in the case of redox-inert Tb(III) ion, but not necessarily in case of Eu(III) ion.

The present work was carried out to find optimal conditions for the Eu(III) centered ECL and compare ECL properties of chelated Eu(III) by chelation with the ligand 2,6-bis[*N,N*-bis(carboxymethyl)-aminomethyl]-4-benzoylphenol that has been studied most extensively by us and the ligand 2,6-bis[*N,N*-bis(carboxymethyl)-aminomethyl]-4-methyl phenol that is known to give the highest TR-ECL intensity amongst all the ligands for chelation of Tb(III) so far [3,18]. The present type of ECL in aqueous solutions will be induced at insulating film-coated aluminum electrodes by high amplitude cathodic pulse polarization instead of at active metal electrodes.

2. Experimental

2.1. Reagents

$\text{K}_2\text{S}_2\text{O}_8$, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, H_2O_2 , NaNO_3 , NaBr , NaN_3 , and NaSCN were pro analysis or suprapur products of Merck. Ethanol was supplied by Oy Alko Ab and suprapur Na_2SO_4 by Merck. Hexamine cobalt(III) chloride was a product of Ventron and EuCl_3 was a product of Aldrich. The synthesis of the chelates have been described previously [18,19]. Quartz-distilled water was used in all solutions. The safety precautions required in handling of the reagents have been presented previously [8,20].

2.2. Instrumentation

The ECL measurements were made in 0.2 M boric acid buffer at pH 9.2. Boric acid buffer was used due to its unreactivity toward sulfate radicals and hydrated electrons [21]. The methods of ECL excitation and both the coulometric and potentiostatic pulse generator have been described elsewhere [5,6,15]. Aluminum electrodes were made from a nominally 99.9% pure aluminum band (Merck Art. 1057, batch K18381957 539) and were covered with natural about 2–3 nm thick oxide films [3]. Generally, the measurements were made through an interference filter having a transmission maximum of 546 nm and bandwidth of about 10 nm. The apparatus has been described earlier elsewhere [19]. However, parts of the measurements were done also using an ordinary Perkin-Elmer LS-5 spectroluminometer as a light measuring unit.

3. Results and discussion

3.1. Time-resolved ECL of the Eu(III) chelates

Those Tb(III) chelates having an aromatic phenolic backbone that we have previously studied produce high photoluminescence (PL) and cathodic TR-ECL intensity in aerated solutions free of oxygen quenching. When 2,6-bis[*N,N*-bis(carboxymethyl)-aminomethyl]-4-benzoylphenol (ligand **1**, Fig. 1) and 2,6-bis[*N,N*-bis(carboxymethyl)-aminomethyl]-4-methyl phenol-chelated (ligand **2**, Fig. 1) were used in chelation of Eu(III), it was observed that Eu(III)-**1** showed very weak TR-ECL signal (Fig. 2) in comparison to Tb(III)-**1** which shows strong metal ion centered emission [3,4] and Gd(III)-**1** which shows the triplet state emission [4] of the ligand in the present experimental conditions. The reason for this is the high energy of the lowest excited state of Gd(III) (${}^6\text{P}_{7/2}$ at 3.98 eV) [16] makes intra molecular energy transfer from the triplet state of the ligand to Gd(III) impossible, therefore the de-excitation of the chelate only occurs by ligand centered luminescence, by redox reactions or other non-radiative processes. Gadolinium(III) is even more redox inert than Tb(III), $E^0(\text{Gd}^{4+}/\text{Gd}^{3+}) = 7.9 \text{ V}$ and $E^0(\text{Gd}^{3+}/\text{Gd}^{2+}) = -3.9 \text{ V}$ [16]. Somewhat surprisingly, Eu(III)-**2** did not give any measurable ECL although Gd(III)-**2** and Tb(III)-**2** emit strongly [4] under similar conditions. When the PL efficiency of Eu(III)-**2** was studied, it was found out that

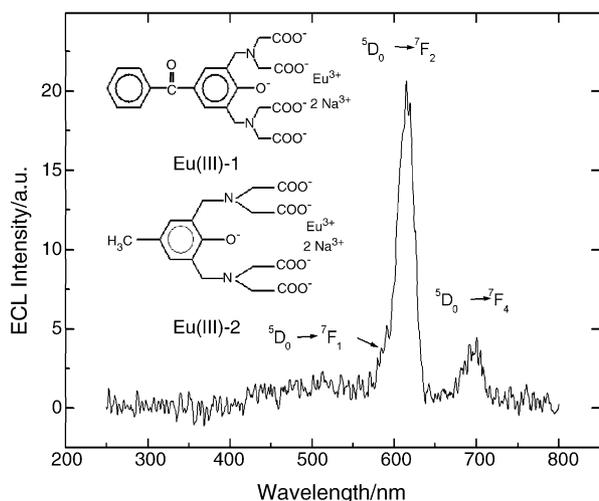


Fig. 1. The structures of the ligands used and the ECL spectra of Eu(III)-1. Conditions: oxide-covered Al-plate working electrode, Pt-wire counter electrode, coulometric pulse generator, applied pulse voltage -40 V, pulse frequency 80 Hz, pulse charge 200 μ C, 0.2 M boric acid buffer at pH 9.2 , 1.0×10^{-3} M Eu(III)-1, 3.0×10^{-3} M $K_2S_2O_8$, Perkin-Elmer LS-5, emission slit 20 nm.

Eu(III)-2 was not photoluminescent either. Thus, it seems that the gap between the triplet state energy level of the ligand **2** and energy-accepting level of Eu(III) is too large so that Eu(III)-2 could be used neither as a photoluminescent nor an electrochemiluminescent label.

Often, the use of peroxodisulfate ion as a coreactant enhances the ECL intensity of cathodic ECL at thin insulating film-coated electrodes [4,5,8]. In the present case, the addition of peroxodisulfate ions as cathodic coreactants into the solution of Eu(III)-1 resulted in a strong enhancement of ECL, and a clear Eu(III) emission could be measured even by PerkinElmer

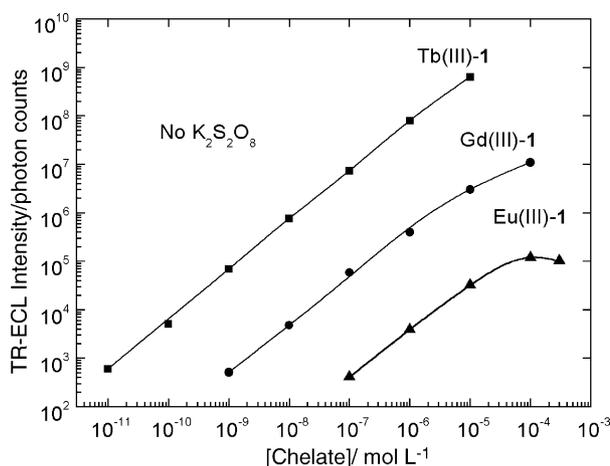


Fig. 2. Calibration curves of Tb(III)-1, Gd(III)-1 and Eu(III)-1 in the absence of peroxodisulfate ions. Conditions: Al-cup-Pt-wire cell, coulometric pulse generator, applied pulse voltage -40 V, pulse frequency 20 Hz, pulse charge 120 μ C, 10000 excitation pulses, 0.2 M boric acid buffer at pH 9.2 , 546 -nm, 405 -nm interference filters in the cases of Tb(III)-1, Gd(III)-1, respectively, and a long-pass filter with 600 -nm cut off wavelength in the case of Eu(III)-1. Delay and gate times were 50 μ s and 8.00 ms (Tb(III)-1), and 1 μ s and 1.00 ms (Gd(III)-1), and 10 μ s and 2.00 ms (Eu(III)-1).

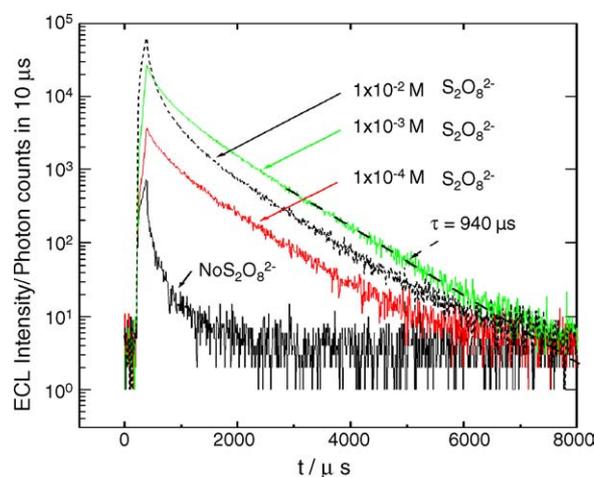


Fig. 3. ECL time spectra of Eu(III)-1 in the presence of varied concentrations of peroxodisulfate ions. Conditions: oxide-covered Al-cup working electrode, Pt-wire counter electrode, Pine Instruments RD4 potentiostat, applied pulse voltage -10 V, pulse frequency 100 Hz, pulse time 200 μ s, 10000 excitation pulses, 0.05 M sodium tetraborate buffer adjusted to pH 7.8 with sulfuric acid, 1.0×10^{-4} M Eu(III)-1. The light emission was registered by a MCS-II scaler card attached to a PC computer, with 600 -nm long pass filter.

LS5 fluorospectrometer designed only for PL measurements (Fig. 1). When the effect of peroxodisulfate concentration on the luminescence lifetime of Eu(III)-1 was studied (Fig. 3), it was observed that (i) in the absence of peroxodisulfate ions the ECL decay was very fast, possibly indicating a rapid reduction of Eu(III) to Eu(II) by the one-electron reduced ligand, or hot or hydrated electrons, (ii) an ECL lifetime equal to its PL lifetime is achieved when an appropriate balance between the reducing and oxidizing species was attained at concentration 1 mmol/L of peroxodisulfate, and (iii) when peroxodisulfate ion concentration was too high, quenching effect is again observed. Fig. 3 demonstrates that an appropriate concentration of peroxodisulfate ion increases the luminescence lifetime considerably, making the decay to be predominated by a single long-lived component with luminescence lifetime ca. 0.94 ms.

Fig. 4 displays the calibration curve of peroxodisulfate ions at fixed Eu(III)-1 concentration level (squares) and the calibration curve of Eu(III)-1 at fixed potassium peroxodisulfate concentration level (circles). Also a better calibration curve for TR-ECL of Eu(III)-1 is shown (triangles) which was measured using an electrochemiluminometer [22] modified from Wallac Arcus 1230 without any optical filtration (Fig. 4). The benefits of this instrument are that twelve separate measurements can be very rapidly carried out in standard micro titer strips, and more importantly, its photomultiplier tube (PMT) does have extremely little hysteresis after the cathodic flash originated from the oxide film, and therefore, sensitive time-resolved measurements can be carried out without any optical filtering. Addition of peroxodisulfate ions in the electrolyte induces F center luminescence [23,24] of the oxide film (Fig. 5) in the visible range that increases the blank level during cathodic pulse. Thus, when peroxodisulfate is used as a coreactant in the present method utilizing Al/Al₂O₃ electrodes, a PMT is needed, which can tolerate relatively high light fluxes onto its cathode during the cathodic pulse without

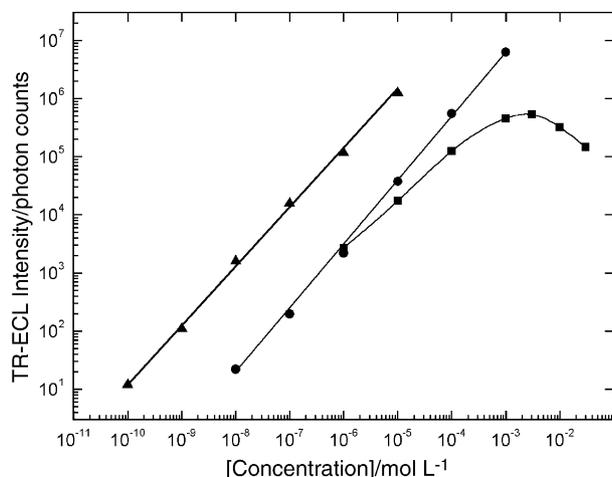


Fig. 4. Calibration curves of (a) Eu(III)-1 in the presence of 3×10^{-3} M $K_2S_2O_8$ (●), (b) $K_2S_2O_8$ in the presence of 1×10^{-4} M Eu(III)-1 (■), (c) Eu(III)-1 in the presence of 1×10^{-3} M $K_2S_2O_8$ as measured without optical filtration using a semiautomatic electrochemiluminometer modified from Wallac Arcus 1230 (▲). Conditions (a and b): oxide-covered Al-cup working electrode, Pt-wire counter electrode, coulostatic pulse generator, applied pulse voltage -40 V, pulse frequency 20 Hz, pulse charge $120 \mu\text{C}$, 0.2 M boric acid buffer at pH 9.2, long-pass filter with 600-nm cut off wavelength, delay and gate times were $50 \mu\text{s}$ and 2 ms; (c) 12-spike Al-rake electrode, pulse time $240 \mu\text{s}$, pulse voltage -10 V, delay time $10 \mu\text{s}$ and gate time 2.5 ms, 0.05 M $Na_2B_4O_7$ buffer adjusted to pH 7.8 with sulphuric acid, 1×10^{-3} M $K_2S_2O_8$.

giving “after pulses”, or alternatively an electronic switching system that lowers the PMT voltage during the cathodic excitation pulses.

3.2. Tunnel emission of hot electrons

We have previously proposed [2–8] that in cases where hot electrons are entering aqueous electrolyte solution with energies above the conduction band edge of water (Fig. 5), hot electrons may either act as extremely strong reductants at the oxide/electrolyte interface or they may be thermalized and solvated to produce hydrated electrons (1):

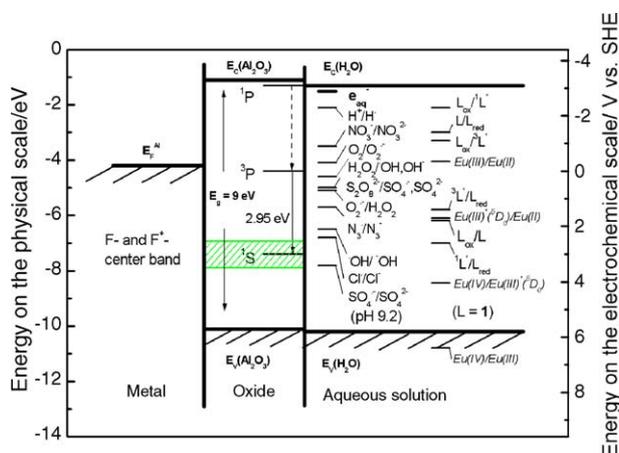


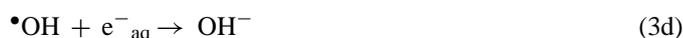
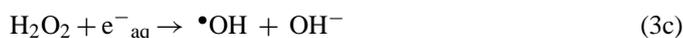
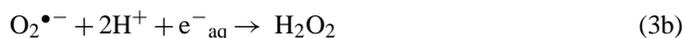
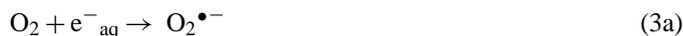
Fig. 5. The energy diagram of the present ECL system in flat band conditions [16,17,23].

When hydrated electrons ($E^0 = -2.9$ V) [21] are produced in the presence of peroxodisulfate ions, an extremely strong oxidant, sulfate radical (with $E^0(\text{SO}_4^{\bullet-}/\text{SO}_4^{2-}) = 3.4$ V) [27], is produced at near diffusion controlled rate ($k_2 = 1.2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$) [21]:



Sulfate radical acts mainly as a one-electron oxidant with a strong tendency towards reactions with aromatic compounds and it has a very low reactivity with water ($k(\text{SO}_4^{\bullet-} + \text{H}_2\text{O}) < 60 \text{ L mol}^{-1} \text{ s}^{-1}$) [28].

If the sample solution contains dissolved oxygen, also a series of oxyradicals and hydrogen peroxide are also rapidly formed (3a)–(3d):



with the second-order rate constants $k_{3a} = 1.9 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$, $k_{3b} = 1.3 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$, $k_{3c} = 1.2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ and $k_{3d} = 3.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$, respectively [21].

It has been suggested previously also in case of n -Si/SiO₂ electrodes [5,7], that the anion vacancies and E_1' electron centers (one electron trapped in an anion vacancy) can act as stronger oxidants than hydroxyl radicals but as weaker oxidants than sulfate radical at pH 9, at which the ECL measurements were performed. One electron trapped in an anion vacancy in aluminum oxide is called as F+ center (an analog of E_1' center in silicon dioxide) and the energy level of F+ centers in aluminum oxide allows to propose that F+ centers and anion vacancies can also act as oxidants at the Al_2O_3 /electrolyte interface (Fig. 5) and oxidize hydroxide ions or surface hydroxyl groups of the oxide film to hydroxyl radicals which can mediate oxidations beyond the tunneling distance from the electrode. Therefore, the hydroxyl radicals may be produced also in the absence of dissolved molecular oxygen at oxide-covered aluminum electrodes by supplying an appropriate anodic currents between cathodic excitation pulses to empty the filled electron traps, i.e., to regenerate the oxidant [5,7].

In addition, many organic luminophores, $\text{Ru}(\text{bpy})_3^{2+}$ as well as $\text{Tb}(\text{III})$ -1 show considerable higher ECL intensity in the presence of cathodically produced sulfate radicals than in the presence of hydroxyl radicals [2–11,25,26]. Therefore, the addition of moderate concentrations of peroxodisulfate in the sample solutions is favored, although peroxodisulfate consumes reducing equivalents, e.g. hydrated electrons, in the system.

3.2.1. Effect of free radical scavengers

Effects of some hydrated electron and sulfate radical scavengers were studied (Fig. 6). Some relevant reaction rate constants are collected in Table 1. If fast hydrated electron scavengers, such as $\text{Co}(\text{NH}_3)_6^{3+}$ complexes, are added in the present

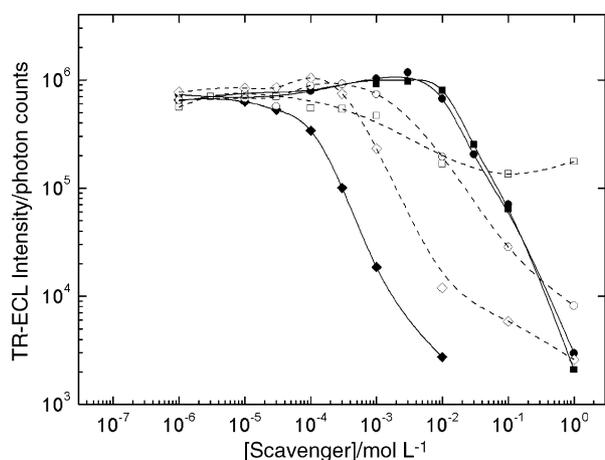
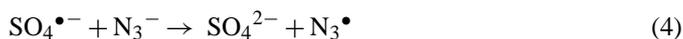


Fig. 6. Quenching effects of added hydrated electron scavengers (NO_3^- , ■), (H_2O_2 , ●), ($\text{Co}(\text{NH}_3)_6^{3+}$, ◆) and sulfate radical scavengers (Cl^- , ·), (Br^- , ○), (N_3^- , ◇). Conditions: as in Fig. 4 except that the concentrations of Eu(III)-1 and peroxodisulfate ion were 1×10^{-4} and 1×10^{-3} mol/L, respectively.

Eu(III)-1- $\text{S}_2\text{O}_8^{2-}$ ECL system, strong quenching effect on the ECL is observed (Fig. 6). Hydrogen peroxide is a close analog of peroxodisulfate ion: it also produces a very strong oxidant after one-electron reduction. However, the product, hydroxyl radical, differs from sulfate radical in many respects, e.g., it has a strong tendency to addition and hydrogen abstraction reactions, and the oxidizing power of hydroxyl radical is pH dependent [21]. When hydrogen peroxide is added in the present ECL system, a strong quenching effect at its high concentrations is observed, but at its intermediate concentrations a slight ECL-enhancing effect exists.

If fast sulfate radical scavengers (which are unreactive towards hydrated electrons) are added in the system, the ECL can also be strongly quenched. If sulfate radical is scavenged by azide ion, a secondary radical N_3^\bullet with $E^0 = 1.33$ V versus SHE [29] is produced:

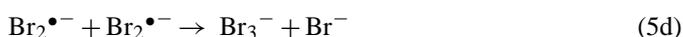
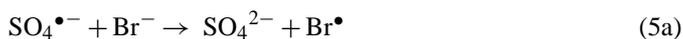


Although the azide radical is an efficient one-electron oxidant, in the present case it is clear that the “conversion” of sulfate radicals to azide radicals drastically decreases ECL intensity (Fig. 6). An almost equal effect is observed in the presence of

Table 1
Second-order reaction rate constants for hydrated electron and sulfate radical scavengers and some aromatic moieties [21,28]

Scavenger	$k(e_{\text{aq}}^- + \text{Sca})$ ($\times 10^6 \text{ L mol}^{-1} \text{ s}^{-1}$)	$k(\text{SO}_4^{\bullet-} + \text{Sca})$ ($\times 10^6 \text{ L mol}^{-1} \text{ s}^{-1}$)
$\text{S}_2\text{O}_8^{2-}$	12000	
NO_3^-	9700	
H_2O_2	11000	
$\text{Co}(\text{NH}_3)_6^{3+}$	87000	
Benzophenone	1000	
Benzene		300
Tyrosine		300
Chloride		200
Bromide		3500
Azide		3000

bromide ions, which produces bromine atoms, dibromine radical ions and molecular bromine as scavenging products [30]:



The secondary radical formed is mainly bromine atom at low bromide ion concentrations, and mainly dibromine radical ion at higher concentration. According to the small ECL enhancing effect observed at intermediate concentrations of bromide ions, the prevailing scavenging product, bromine atom with $E^0 = 1.92$ V versus SHE [29] is at least as equally good an oxidant for the present ECL system as the sulfate radical. However, dibromine radical ion with $E^0 = 1.62$ V versus SHE [29] prevailing at higher bromide ion concentration is clearly not an efficient oxidant for the ECL generation.

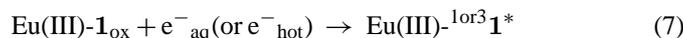
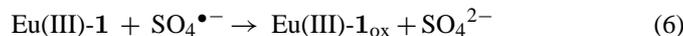
Chloride ion produces an analogous series of scavenging products to bromide ion, but the initial step of oxidation by sulfate radical occurs at a lower rate. The main difference is that the reaction products, chlorine atoms, with $E^0 = 2.22$ V versus SHE [29] are stronger oxidants than the analogous bromine atoms. In the present conditions chlorine atom is ca. 0.3 V stronger oxidant than hydroxyl radical, i.e., it can relatively rapidly oxidize hydroxide ions. For some luminophores which show strong chemiluminescence (CL) in the presence of hydroxyl radicals, the conversion of sulfate radicals to chlorine atoms has an enhancing effect on CL intensity [30]. This was mainly attributed to the ca. 2 orders of magnitude faster oxidation of hydroxide ions by chlorine atoms than by sulfate radicals. In the present case, the secondary and tertiary radicals produced by addition of chloride ions are clearly not beneficial for ECL of Eu(III)-1.

All in all, none of the oxidizing secondary radicals formed is markedly better oxidant for the present system than sulfate radical and an efficient oxidant for the present system has to have standard reduction potential preferably at least 1.9 V versus SHE or higher. According to experiments with sulfate radical scavengers the efficient ECL production of the present chelate needs an oxidant with standard reduction potential of about 1.9 V versus SHE or higher, and none of the secondary radicals generated from the halide and pseudo halide series precursors works better than the sulfate radical.

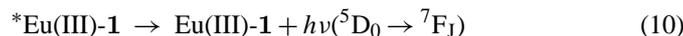
3.3. Mechanism of the ECL

Tb(III) chelates produce ECL and CL mainly via the above-mentioned ox-red pathway but probably also simultaneously via the red-ox pathway [2–5,25,26]. The comproportionation pathway commonly observed in non-aqueous solvents for aromatic luminophores is generally not important in aqueous solutions due to the very limited lifetime of aromatic cation and anion radicals [26]. The redox and optical properties of the ligand 1 were adopted from a previous publication [17], as well as, the energetics of the Al/Al₂O₃ electrode [23] and Eu(III) [16] which are collected in Fig. 5.

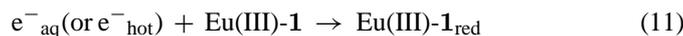
The ox-red [3,4] excitation pathway is valid also in the present case, because Eu(III) can not be oxidized in aqueous solutions [31], not even by sulfate radical. Sulfate radical oxidizes rapidly even benzene ($k = 2.4 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$) [28], and it is known to oxidize phenoxides at near diffusion controlled rate [28]. Therefore, ligand **1** containing phenoxide moiety is rapidly oxidized by sulfate radical [2,26,31] (6), which is followed by a rapid reduction of the oxidized ligand radical $\mathbf{1}_{\text{ox}}$ by either a hydrated or hot electron (7). Hydrated electron is well known not to follow Marcus electron transfer theory and it reacts exceedingly fast also in reduction reactions having exceedingly negative Gibbs free energy change [21]:



Step (7) is sufficiently energetic for singlet state excitation of the ligand (Fig. 4), but it is known that in analogous reactions with aromatics in solid state, both excited triplet and singlet states are formed, i.e., so-called recombination phosphorescence and fluorescence occurs in the radioluminescence of aromatic compounds [32]. If singlet state excitation occurs, an intersystem crossing follows (8). After this energy is transferred from the triplet state of ligand to the resonance level $^5\text{D}_0$ of Eu(III) at 2.1 eV (9) which finally emits light (10):

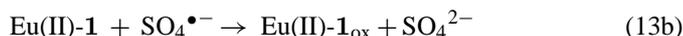
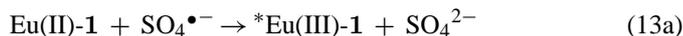


However, a ligand reduction-initiated red-ox excitation pathway can not be exactly similar to that in the case of Tb(III)-**1** chelate [2,3] due to the easy reducibility of Eu(III) ion [16]. Of course, the ligand can be very fastly reduced by hydrated electron in all [3,4] cases of Tb(III), Gd(III) and Eu(III) chelates (the second-order reaction rate constant of the reduction of benzophenone by hydrated electron is reported to be $1.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$) [21]. However, it is highly probable that the one-electron reduced ligand form $\mathbf{1}_{\text{red}}$ immediately reduces the central ion in the case of Eu(III). Also a direct reduction of Eu(III) should be considered, because it is known that a hydrated Eu(III) ion is reduced by hydrated electron at near diffusion controlled rate ($k = 6.1 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$) [33]. However, it is known that the chelation of Eu(III) e.g. with EDTA decreases the reduction rate of the central ion by one-order of magnitude [34]. Therefore, it is probable that Eu(III) is reduced in the present case through the ligand **1** (11)–(12) but a direct reduction by hydrated or even by presolvated hot electron is also possible in principle:



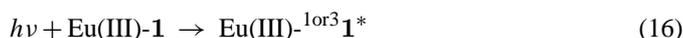
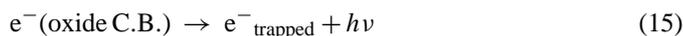
After step (12), cathodically generated sulfate radical may directly oxidize Eu(II) ion to Eu(III) in its excited state (13a) followed by (8) or it may one-electron oxidize the ligand (13b). The

former possibility is an energy-sufficient route for direct excitation of Eu(III) in its higher excited states (Fig. 5), but it requires electron transfer over a rather high energy gap which might indicate quite a slow reaction if Marcus electron transfer theory is obeyed here and the inverted region exists. Reaction (13b) followed by an intramolecular electron transfer from Eu(II) to $\mathbf{1}_{\text{ox}}$ (14) is not energy sufficient for triplet state excitation of the ligand nor for the $^5\text{D}_0$ excitation of the central ion (Fig. 5) and, hence, can result in only ground state products:



The high-field conditions in the oxide film necessary for hot electron emission into aqueous electrolytes can induce also solid state electroluminescence inside the oxide film, and also F-center luminescence at the surface of the oxide film as discussed previously [23,24]. The significance of these processes probably depends strongly on the thickness of the oxide film and its purity. F-center luminescence emission peaks at 420 nm (2.95 eV) giving possibility to energy transfer to Eu(III) at close proximity of the surface, and the high-energy tail of the emission band may induce even weak Eu(III)-**1** excitation via the ligand.

During the cathodic pulse, a small fraction of the tunnel emitted electrons are assumed to lose so much energy in inelastic scattering that they fall to the bottom of the aluminum oxide conduction band and some of them get trapped in the band gap showing transitions known from F-center photoluminescence (15), and possibly also transitions to other states inside the band gap [23,24]. Here, e^-_{trapped} denotes electrons trapped in anion vacancies, F^+ -centers and impurity centers. In principle, these intrinsic emission centers can transfer energy to the chelates radiatively or non-radiatively, after which chelates can emit their metal centered transitions by reactions (9)–(10):



In addition, another energy transfer pathway may have some contribution in the observed ECL emission. In this pathway, a sulfate radical is first reduced by hot or hydrated electron resulting in an excited sulfate ion which then might transfer its energy to the chelate. However, it is suggested that the ox-red excitation pathway is the predominant pathway for generation of excited chelates in the present experiments, and the energy transfer pathways are having only minor significance.

3.4. Analytical applicability of Eu(III)-1 ECL

Eu(III)-**1** can be linked with antibodies and other proteins, e.g. by a thioureido group as described elsewhere [1,18], and during the synthesis of the label, the central ion can be freely chosen from the lanthanide series. The luminescence lifetime of Eu(III)-**1** is shorter than that of Tb(III)-**1** and the ECL intensity is weaker. However, Eu(III)-**1** is a second choice of ECL

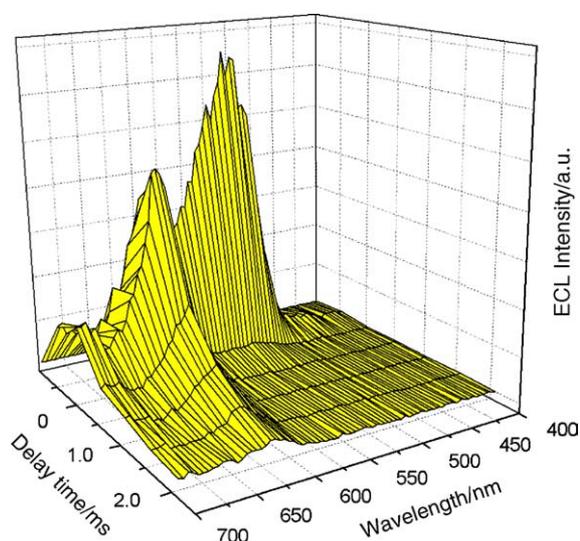


Fig. 7. Simultaneous excitation of FITC and Eu(III)-1-NCS. Conditions as in Fig. 4 except $K_2S_2O_8$ concentration was 1×10^{-3} mol/L. Time-resolved spectra were measured with an instrument having relatively good sensitivity but poor resolution [35].

labels with long luminescence lifetime at tunnel emission electrodes (Figs. 3 and 4) together with some metalloporphyrins [10]. In addition to these labels, a wide variety of short-lived ECL-emitting organic luminophores can also be efficiently excited in similar conditions [8,12,19]. Thus, in these systems it is possible to obtain inner standardization or multiparameter analysis with multiple labels, not only on the basis of wavelength discrimination but also on the basis of time-resolution, or by combining these resolution methods.

Simultaneous excitation of e.g. a well-known luminescent label fluorescein isothiocyanate and Eu(III)-1 isothiocyanate is therefore possible (Fig. 7). Fig. 7 shows that fluorescein isothiocyanate (or its hydrolysed form) emits a short-lived ECL emission at ca. 520 nm, where Eu(III)-1 does not emit, and the overlap of the tail of fluorescein emission band with the emission lines of Eu(III)-1 label does not matter, when the latter label is detected with time-resolved photon counting. Fluorescein is known to react at near diffusion controlled rate with hydrated electrons and hydroxyl radicals at the pH of our measurements ($k(e^-_{aq} + \text{fluorescein}) = 1.4 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$; $k(\bullet\text{OH} + \text{fluorescein}) = 1.2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$) [21], but the rate constant of oxidation by sulfate radical is unknown. We have previously shown, that even labels emitting in UV range can be efficiently excited at several thin insulating film-coated electrode types [25,26]. In addition, a variety of other organic luminophores emitting in the visible range are available for use as labels in connection with the present excitation method [10,11,25,35].

4. Conclusions

The present ECL method allows parallel excitation of some organic labels, Ru(bpy) $_3^{2+}$ -based labels, and aromatic Eu(III) and Tb(III) chelates. Organic luminophores generally show a short-lived singlet state emission, but lanthanide chelates show

long-lived ligand sensitized emission of the central ion, or in the cases of Gd(III) chelates, a relatively long-lived triplet state emission of the ligand, which allows the create multiparameter bioaffinity assays. Thus, novel types of immunoassays and DNA-probe assays can be developed in which a combination of time-resolution and wavelength discrimination can be used in the detection of the labels. The highest sensitivity-demanding parameter must be preferably labelled with Tb(III) chelates, and the parameters where less sensitivity is required should be labelled with organic luminophores [10,11,25,35], transition metal chelate labels [8,9] or with the presently studied Eu(III) label. The presently studied ligand is also interesting because, if both Tb(III) and Eu(III) ions are chelated with this ligand, (i) the emission of both can be triggered if peroxydisulfate is added as a coreactant and only the emission of Tb(III) if azide is added as a coreactant. This might be very useful in some novel applications not necessarily dealing with analytical chemistry.

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