

# Hot electron-induced cathodic electrochemiluminescence of rhodamine B at disposable oxide-coated aluminum electrodes

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## Abstract

Rhodamine B (RhB) exhibits strong cathodic electrogenerated chemiluminescence (ECL) in aqueous solutions during high-amplitude pulse polarization at thin oxide film-coated aluminum electrodes. This method allows the detection of RhB below nanomolar concentration level and provides linear calibration plots spanning over several orders of magnitude of concentration. In addition, a relatively long ECL lifetime of RhB provides a basis for time-resolved detection. Thus, widely used RhB-based labels can also be suggested to be usable as electrochemiluminescent labels in fully aqueous solutions in bioaffinity assays such as in immunoassays and DNA-probing assays. Support was obtained for the chemiluminescence generation mechanism to be essentially the same as that of radiochemiluminescence in aqueous solution.

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## 1. Introduction

Rhodamine compounds are a group of xanthene dyes that have been widely used in analytical chemistry e.g. as an active medium of dye lasers [1,2] and in bioanalytical chemistry [3,4]. The photophysical properties of rhodamines in solution have been extensively studied [5] and amongst these compounds, rhodamine B (RhB) dye is excited in the visible part of the spectrum (at about 560 nm), has a large molar extinction coefficient ( $88,000 \text{ cm}^{-1} \text{ M}^{-1}$ ), and a photoluminescence (PL) quantum yield close to unity. In addition, it is quite photostable (e.g., as compared to fluorescein) [6].

In some applications, these rhodamine compounds have been used as chemiluminescent reagents although in many cases (e.g. RhB) they were only considered as a sensitizer for application in other chemiluminescent systems [7–9].

The chemiluminescence characteristics based on oxidation of some xanthene dyes have been studied in aqueous solutions [10–12], however, electrochemiluminescence (ECL) of RhB in an aqueous solution has not yet been studied.

It has earlier been suggested that oxide-coated aluminum electrodes [13–18] and silicon electrodes [19,20] can be used to inject hot electrons into an aqueous solution and induce chemiluminescence not attainable by the means of the traditional electrochemistry at active metal electrodes [13–20].

We have also previously demonstrated that the best known electrochemiluminescent labels, such as, luminol, *N*-(6-aminohexyl)-*N*-ethyl isoluminol (AHEI), *N*-(6-aminobutyl)-*N*-ethyl isoluminol (ABEI) and  $\text{Ru}(\text{bpy})_3^{2+}$ -based labels, normally in use in the better established anodic ECL applications can also be efficiently excited with our cathodic pulse method [21,22]. However, the best labels for our method seem to be Tb(III) chelates which allow very low detection limits by time-resolved ECL detection [18,23]. When luminophores exhibit relatively long-lived luminescence,

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time-resolved luminescence detection makes it possible to separate the label specific emission from the short-lived solid-state electroluminescence background signal originated from the oxide film by measuring the luminescence after a certain time has elapsed from the end of excitation pulse. This offers an overall increase in detection ability by considerably improving the S/N ratio. So far, in addition to aromatic Tb(III) chelates Pt(II) porphyrins have been known to be usable as electrochemiluminescent labels for time-resolved measurements using our techniques [24].

Typically, the most important ECL excitation routes of the above-mentioned luminophores have been observed to be reduction-initiated oxidative excitation (red–ox) or oxidation-initiated reductive excitation (ox–red) pathways in which the luminophores are excited in successive one-electron steps by the species cathodically generated at the tunnel emission electrodes [15,17–23].

Radiochemiluminescence (RCL) is still one of the least studied forms of luminescence [25–28] but RhB was already quite a long time ago observed to produce RCL under steady X-ray irradiation in an aqueous solution [29,30]. RCL of RhB was later studied using pulse radiolysis with 2- $\mu$ s, 4-MeV electron pulses [31]. During and also after the electron pulse, Pruetz and Sommermeyer [32] observed the existence of both one-electron reduced and oxidized radical forms of RhB (RhB<sub>red</sub><sup>•</sup> and RhB<sub>ox</sub><sup>•</sup>), which were produced by the primary species of the radiolysis of water, i.e. hydrated electrons and hydroxyl radicals, respectively. The oxidation of RhB<sub>red</sub><sup>•</sup> by hydroxyl radical and reduction of RhB<sub>ox</sub><sup>•</sup> by hydrated electron were assumed to be the sources of the excited RhB<sup>\*</sup>. The excitation by electron pulses appeared to be approximately 1000 times more efficient than the RCL induced by 8.5 and 24 keV [31] X-rays with the same concentration of dye. The RCL lifetime after the electron pulse was very short but could be made somewhat longer by addition of some hydroxyl radical scavengers from halide series. At pH 10 the absolute quantum yield for reaction



was determined to be 0.018 [32].

We have previously proposed that hot electrons are injected into the conduction band of water during a high-amplitude pulse polarization of thin insulating film-coated cathodes, which probably also results in the generation of hydrated electrons from these presolvated hot electrons by thermalization and solvation. Subsequently, hydrated electrons or presolvated hot electrons can generate highly oxidizing species from suitable precursors, such as peroxodisulfate ions, hydrogen peroxide or molecular oxygen [13–18,21,22]. Thus, simultaneously highly reducing and highly oxidizing conditions can be created in the vicinity of the electrode surface. The hot electrons have been suggested to tunnel through a thin insulator film in direct field-assisted regime (around 3–5 nm thick oxide films) into the conduction band of water. After thermalization and solva-

tion processes [15,17,18], the energetic electrons result in hydrated electrons ( $E^{\circ} = -2.9$  V versus SHE) [33] capable of inducing chemiluminescence from various luminophores and coreactants. Typical for hydrated electron is that it does not follow Marcus electron transfer theory but reduces extremely fast not only in reactions having a moderately negative Gibbs free energy change but also in reactions with an extremely negative free energy change [33]. Thus, simultaneous and parallel reductions of species having very different redox properties can be carried out by hydrated electrons.

In our preliminary tests, we have observed that many fluorescent labels not mentioned above and originally designed for photoluminescence purposes can be electrically excited in our cells, but however, some of the fluorescent luminophores do not give any detectable ECL emission. Thus, RhB should be an excellent probe to shed more light on the mechanisms of our cathodic pulse method: (i) if no ECL of RhB would be generated, we surely would not have both the hydrated electrons and hydroxyl radicals as reducing and oxidizing mediators in our cells and (ii) if the cathodic ECL would exist, the scavenging of hydroxyl radicals and/or hydrated electrons should have a strong quenching effect on the CL and (iii) the ECL should be observable also a short time after the cathodic pulse [32], if hydrated electrons and hydroxyl radicals would exist as redox mediators in the cell.

The purpose of the present work was to study the possibly existing cathodic ECL of RhB at oxide-covered aluminum electrode and the detectability of RhB on this basis.

## 2. Experimental

The ECL measurement were carried out by using single photon counting with an instrument consisting of a Hamamatsu R 1527 photomultiplier, Stanford Research Systems SR-440 preamplifier, SR-400 gated photon counter and Nucleus MCS-II multiscaler card. Similar measurement system have been described in detail elsewhere [14,34,35]. The difference from previous instrumentation was that SR-400 gated photon counter and nucleus MCS-II multiscaler card were attached to two computers, respectively. This configuration of apparatus allowed time-resolved measurements and simultaneous measurements of light emitted during cathodic and intermittent zero voltage during one measurement. The cell used here is a two-electrode system, consisting of a sample holder made of Teflon, a fine Pt-wire counter electrode and a disposable aluminum working electrode. Aluminum electrodes from usually 99.9% pure aluminum band, 0.3 mm thick (Merk Art. 1057, batch 720 K22720857) were covered by a 2–3 nm thick natural oxide film, and were cut into 15 mm  $\times$  15 mm pieces. The effective area of the electrode in this cell was 63.6 mm<sup>2</sup>. A laboratory-made coulostatic pulse generator [36] was applied to generate cathodic pulses and the pulse generator was adjusted to yield cathodic pulses with 120  $\mu$ C of charge, –45 V of voltage at a frequency of 20 Hz.

The ECL spectra were measured using an Al sheet working electrode with a Pt wire counter electrode between two PTFE layers in a 1 cm polystyrene cuvette, and LS-50B luminescence spectrometer (Perkin-Elmer) was employed for light emission measurement.

Most measurements were made in 0.05 mol/L sodium tetraborate buffer at pH 9.2 except where specially mentioned. This buffer is known to be quite unreactive with hydrated electrons and hydroxyl radicals, as well as with sulfate radicals [33]. NaI, NaBr,  $\text{NaB}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ,  $\text{NaN}_3$ ,  $\text{K}_2\text{S}_2\text{O}_8$ ,  $\text{NaNO}_3$ ,  $\text{NaNO}_2$ ,  $\text{H}_2\text{O}_2$  were pro analysi products of Merck.  $\text{CH}_3\text{CH}_2\text{OH}$  was pro analysi product of Primalco. Rhodamine B was pro analysi product of Sigma.

In cases of anodically oxidized electrodes used, the electrodes were anodized in neutral 0.50 M ammonium borate buffer, first, galvanostatically up to the forming voltage with current density of  $0.20 \text{ mA cm}^{-2}$  and then potentiostatically until the current density decayed below  $10 \mu\text{A cm}^{-2}$ . The thickness was calculated by using the anodization ratio value  $1.4 \text{ nm V}^{-1}$  [37,38].

### 3. Results and discussion

#### 3.1. Spectra of RhB

The ECL spectrum of RhB induced on oxide-coated aluminum electrodes and fluorescence spectra are illustrated in Fig. 1. The shape of the ECL spectrum of RhB is similar to its fluorescence emission spectrum with emission maxima at 575 nm. Thus, it seems reasonable that the emission in both cases occurs from the same excited state. The inset in Fig. 1 shows the molecular structure of RhB.

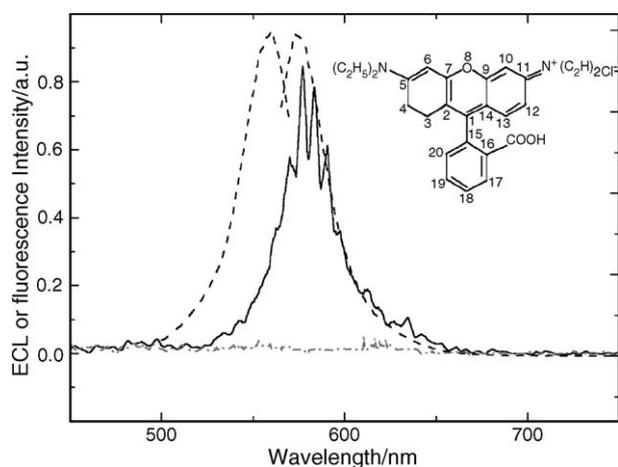


Fig. 1. Uncorrected ECL (solid line) and fluorescence (dashed lines) spectra of rhodamine B. The ECL blank is given as dark grey dashed line. Conditions: scanning speed  $240 \text{ nm min}^{-1}$  with Perkin-Elmer LS-50B spectrometer, slit widths 10 nm, coulostatic pulse generator: pulse voltage  $-45 \text{ V}$ , pulse frequency 80 Hz, pulse charge  $120 \mu\text{C}$ , aluminum strip cathode, platinum wire anode. Molecular structure of RhB is given in the inset.

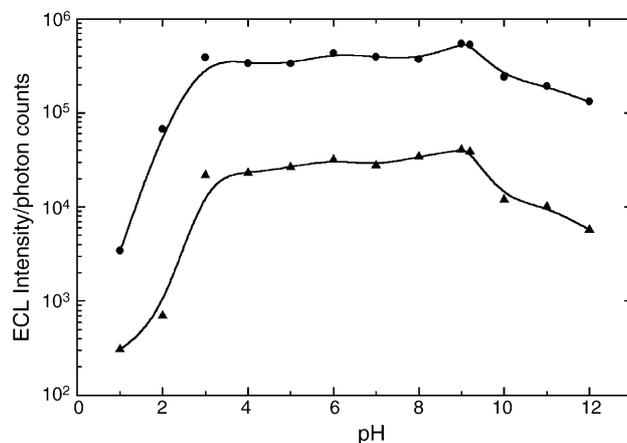


Fig. 2. Effect of pH on ECL: (▲) cathodic ECL during the pulse; (●) time-resolved ECL. Conditions:  $1.0 \times 10^{-6} \text{ M}$  RhB in  $0.1 \text{ M Na}_2\text{SO}_4$  and  $30 \text{ mM Na}_2\text{B}_4\text{O}_7$  supporting electrolyte solution,  $0.01 \text{ M NaN}_3$ . Solutions were adjusted to the desired pH with sulfuric acid or sodium hydroxide. Pulse voltage  $-45 \text{ V}$ , pulse charge  $120 \mu\text{C}$ , pulse frequency 20 Hz. TR-ECL signals were measured by delay  $0 \mu\text{s}$ , gate time  $200 \mu\text{s}$ . ECL and TR-ECL intensities were integrated over 1000 excitation cycles. All signals were measured through a 600 nm interference filter with half-width of the transmission band ca. 40 nm.

#### 3.2. Effect of pH on ECL

The ECL dependence of RhB on pH in air-saturated tetraborate buffer solution is presented in Fig. 2. The ECL intensity of RhB was relatively unchanged in a wide pH range from 3 to 9 and the maximum ECL intensity was obtained in weakly alkaline solution around pH 9. The ECL intensity decreases steeply when pH is below 3. Hydrated electron is fast converted to its conjugated acid, a hydrogen atom, in relatively high acidic solutions ( $k(e_{\text{aq}}^- + \text{H}^+) = 2.3 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ ) [33]. Therefore, it is proposed that the claimed hydrated or presolvated hot electron is consumed in this way from the light generating pathways (see also later the tests with the other hydrated electron scavengers). In addition, the carboxylate group of RhB is protonated when the pH is lower than about 3 [39]. However, we suggest that the main reason for the rapid decrease in ECL intensity is due to the dissolution of oxide film upon the aluminum electrode under so acidic conditions [38]. Therefore, the overall effect of pH being less than 3 is a rapid decrease in ECL of RhB.

On the other hand, when pH exceeds 11, the ECL intensity of RhB is gradually decreased as well. The explanation is mainly related to the damage of the oxide film in highly alkaline solution [40]. It is worth of noting that the pH range of maximum ECL intensity of RhB coincides with the physiological pH range. Thus, the studied system has potential in immunoassay applications by using derivatives of RhB as electrochemiluminescent labels.

#### 3.3. Effect of oxide film thickness on ECL

The naturally existing oxide film on aluminum can be easily made thicker by anodic oxidation [37]. The anodic oxide

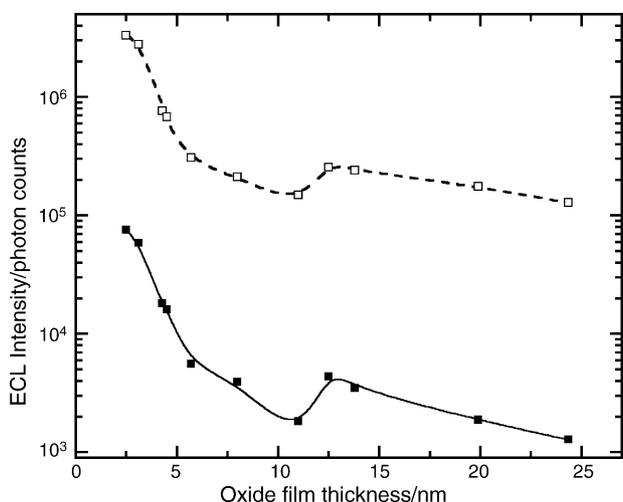


Fig. 3. Effect of oxide film thickness on ECL: (□) cathodic ECL; (■) time-resolved-ECL. Conditions:  $1.0 \times 10^{-6}$  M RhB in 0.05 M  $\text{Na}_2\text{B}_4\text{O}_7$  buffer solution. Aluminum electrodes with varied oxide thickness were used as disposable working electrodes. Other conditions are the same as in the Fig. 2

films are usually regarded as a mixture of amorphous and  $\gamma\text{-Al}_2\text{O}_3$  [38]. The effect of oxide film thickness on ECL intensity of RhB is presented in Fig. 3. The ECL intensity decreases rapidly with the increase in oxide film thickness over about 3 nm.

In principle, emitted hot electrons are transferred to electrolyte solution by direct tunnel emission in the case of ultra thin oxide films (thickness < ca. 4 nm) [41]. In this case, electrons directly tunnel from an already high energy level through the barrier to an equal energy level and no considerable loss of electron energy occurs. Therefore, electrons with energy above the conduction band edge of water can become thermalized and hydrated, and the ECL is obtained. ECL is considerably decreased with the increase in the oxide film thickness. With some other luminophores and in the cases of oxide film thickness exceeding about 5 nm, the ECL has been observed to decrease exponentially with the increase in oxide film thickness, due to Fowler–Nordheim (F–N) tunneling being the predominant tunneling mechanism [17,18,41]. Under this regime, the electrons are first tunneled into the conduction band of oxide and then heterogeneously transferred to the solution species from the bottom of the conduction band or from somewhat above it at the oxide/solution interface. During the transport under high electric field in the conduction band of the oxide the electrons are partly gaining energy by the field and partly losing it by inelastic scattering. Thus, probably an analogous but an unknown distribution of energy of electrons to that reported for  $\text{SiO}_2$  by Di Maria and Cartier [41] is formed in  $\text{Al}_2\text{O}_3$ , and finally, not all of the electrons are transferred from the bottom of the conduction band to solution but also with energies somewhat above the conduction band edge.

We have earlier observed with some other luminophores that are very difficult to reduce [15,18,23] that the heteroge-

neously transferred electrons are not sufficiently energetic to induce the ECL phenomena due to the cathodic band bending. In these cases, it was considered that it was not energetically possible that hydrated electrons would have been formed from the electrons transferred from the bottom of the conduction band to solution. Therefore, the existence of either presolvated electrons in the conduction band of water or fully hydrated electrons was assumed to be the prerequisite of the ECL excitation of those luminophores.

Again here, the ECL is very strong only under direct field assisted tunneling regime dominating in cases of ultra thin oxide films (Fig. 3.) However, it is interesting to notice that in the present experiments the ECL intensity started to increase slightly after the initial very steep decrease and was then again decreased in a mildly sloping manner with the continuous increase in oxide film thickness (Fig. 3). This was the first time for us that we observed considerable ECL on aluminum electrodes coated by such thick oxide films. We propose that this behavior under F–N tunneling regime is based on the mechanism in which RhB is excited by the above-mentioned red–ox excitation pathway. However, RhB is a relatively easily reduced luminophore (formal reduction potential  $-0.52$  V versus SHE at pH 7 [42]). Hence, the reducing electrons need not be hydrated electrons but the heterogeneously transferred electrons from the conduction band of aluminum oxide even under band bending conditions might be sufficiently energetic to start the red–ox excitation route.

#### 3.4. Effect of free radical scavengers on ECL

If the generation of hydrated electrons were the primary step of the excitation pathway of present RhB ECL system as proposed above, hydrated electron scavengers would have a striking effect on the ECL intensity. Thus, we studied the effects of different kinds of hydrated electron scavengers and the results are shown in Fig. 4. The quenching effect of  $\text{Co}(\text{NH}_3)_6^{3+}$  is the strongest amongst all tested scavengers. This complex is about 10-fold stronger quencher than nitrate ion and nitrite ion at same concentration. The effects can be explained by the second-order reaction rate constants of these scavengers in reaction with hydrated electron.  $k(\text{e}_{\text{aq}}^- + \text{Co}(\text{NH}_3)_6^{3+}) = 8.7 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$  is about 10 times higher than  $k(\text{e}_{\text{aq}}^- + \text{NO}_3^-) = 9.7 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$  or  $k(\text{e}_{\text{aq}}^- + \text{NO}_2^-) = 4.1 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$  [33]. Although the rate constants  $k(\text{e}_{\text{aq}}^- + \text{NO}_3^-)$  and  $k(\text{e}_{\text{aq}}^- + \text{NO}_2^-)$  are smaller than that of RhB ( $k(\text{RhB} + \text{e}_{\text{aq}}^-) = 3 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ ), the reactions between scavengers and hydrated electrons will predominate when the concentrations of the scavengers are much higher than that of RhB in the system. Quenching of the RhB ECL was observed when concentrations of nitrate and nitrite ions exceeded  $10^{-4}$  M. Nitrite ions have a slightly stronger quenching effect on ECL than nitrate ions do. The reason might be that nitrite also scavenges hydroxyl radical [ $E^\circ(\text{NO}_2^\bullet/\text{NO}_2^-) = 1.02 \text{ V}$  [43],  $k(\bullet\text{OH} + \text{NO}_2^-) = 1.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ ] [33] and hence it

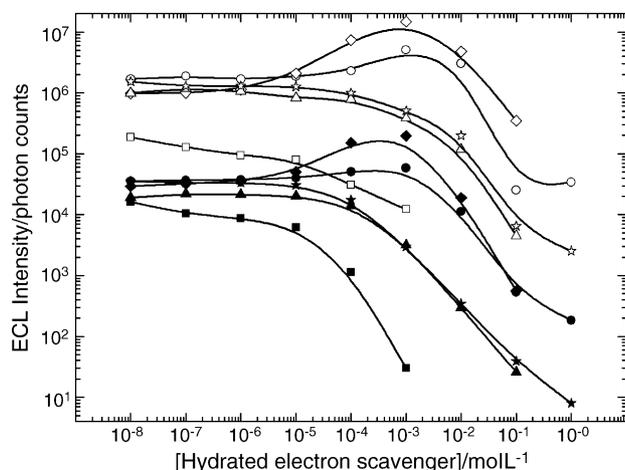


Fig. 4. Effect of electron scavengers on the ECL: ( $\diamond$ ) effect of  $\text{K}_2\text{S}_2\text{O}_8$  on TR-ECL; ( $\blacklozenge$ ) effect of  $\text{K}_2\text{S}_2\text{O}_8$  on cathodic ECL; ( $\circ$ ) effect of  $\text{H}_2\text{O}_2$  on TR-ECL; ( $\bullet$ ) effect of  $\text{H}_2\text{O}_2$  on cathodic ECL; ( $\square$ ) effect of  $\text{Co}(\text{NH}_3)_6^{3+}$  on TR-ECL; ( $\blacksquare$ ) effect of  $\text{Co}(\text{NH}_3)_6^{3+}$  on cathodic ECL; ( $\triangle$ ) effect of  $\text{NaNO}_2$  on TR-ECL; ( $\blacktriangle$ ) effect of  $\text{NaNO}_2$  on cathodic ECL; ( $\star$ ) effect of  $\text{NaNO}_3$  on TR-ECL; ( $\blackstar$ ) effect of  $\text{NaNO}_3$  on cathodic ECL. Conditions:  $1.0 \times 10^{-6}$  M RhB, 0.05 M  $\text{Na}_2\text{B}_4\text{O}_7$  at pH 9.2 buffer solution, otherwise the same as in Fig. 2.

removes both hydrated electron and hydroxyl radical from the light generating pathways. In addition, the quenching of the present ECL by  $\text{NO}_3^-$  and  $\text{NO}_2^-$  are analogous to their effects to Tb(III) chelate, luminol and metalloporphyrins ECL systems [17,22–24].

On the other hand,  $\text{Co}(\text{NH}_3)_6^{3+}$  and nitrate ion are very unreactive towards hydrogen atom ( $k(\text{H}^\bullet + \text{Co}(\text{NH}_3)_6^{3+}) < 9 \times 10^4 \text{ L mol}^{-1} \text{ s}^{-1}$ ,  $k(\text{H}^\bullet + \text{NO}_3^-) = 1.4 \times 10^6 \text{ L mol}^{-1} \text{ s}^{-1}$ ) [33]. The significant quenching of ECL by  $\text{Co}(\text{NH}_3)_6^{3+}$  and nitrate ion supports the hydrated electron being the primary species of ECL excitation routes rather than hydrogen atoms suggested for the role of reducing mediators quite a long time ago [34].

Peroxodisulfate ions and hydrogen peroxide enhanced the ECL signal from  $10^{-6}$  M, the most up to  $10^{-3}$  M, after which an increase in concentration of the electron scavenger resulted in quenching of ECL signal. The rate constant  $k(\text{e}_{\text{aq}}^- + \text{S}_2\text{O}_8^{2-}) = 2.3 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$  [33] is a bit less than  $k(\text{e}_{\text{aq}}^- + \text{RhB}) = 3 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$  [31]. Thus, peroxodisulfate ions produce a sulfate radical by consuming hydrated electrons. The sulfate radical is a strong one-electron oxidant ( $E^\circ = 3.4 \text{ V}$  versus SHE [44]) capable of oxidizing a number of aromatic compounds. It reacts very sluggishly with water and has much less tendency for other type of reaction modes than hydroxyl radical in addition to one-electron oxidation [45]. Because the ECL-enhancing effect of peroxodisulfate ions is stronger than that of hydrogen peroxide or molecular oxygen one might assume that it would be beneficial to use peroxodisulfate as a coreactant in the present ECL system. However, it is also likely to induce a higher blank signal, because the intrinsic cathodic ECL of thin aluminum oxide films is enhanced by the presence of peroxydisulfate ion. Hydrogen peroxide does not enhance the

cathodic intrinsic electroluminescence (IEL) so much under the same conditions [14].

On the other hand, the excess concentration of  $\text{S}_2\text{O}_8^{2-}$  ions ( $>10^{-3}$  M) quenched the present ECL due to the too efficient removal of hydrated electrons from light generating pathways and by spoiling the balance between the reducing and oxidizing species in the system. In addition, a high local concentration of sulfate radical also consumes it back to peroxodisulfate ion ( $k(\text{SO}_4^{\bullet-} + \text{SO}_4^{\bullet-}) = 5.1 \times 10^8 \text{ L mol}^{-1} \text{ s}^{-1}$ ) [45]. Thus, the overall effect is the decrease in both sulfate radical concentration and hydrated electron concentration.

Hydrogen peroxide enhances RhB ECL analogously to peroxodisulfate ions by producing hydroxyl radical upon one-electron reduction ( $k(\text{H}_2\text{O}_2 + \text{e}_{\text{aq}}^-) = 1.2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ ) [33] but more mildly (Fig. 4). Again, above  $10^{-3}$  M hydrogen peroxide quenched the ECL signal by the same reasons as in the case of peroxodisulfate ion. In addition, hydroxyl radical has a tendency to react with aromatic molecules by other reaction modes than that of one-electron oxidation [33].

Hydroxyl radical can be also generated by fast one-electron oxidation steps from dissolved molecular oxygen with following second-order rate constants  $k(\text{O}_2 + \text{e}_{\text{aq}}^-) = 1.9 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ ;  $k(\text{O}_2^{\bullet-} + 2\text{H}^+ + \text{e}_{\text{aq}}^-) = 1.3 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ ;  $k(\text{H}_2\text{O}_2 + \text{e}_{\text{aq}}^-) = 1.2 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$  [33].

In addition, it has been suggested that  $\text{F}^+$ -centre and anion vacancies existing in the oxide film could act as the primary oxidants capable of oxidizing hydroxide ions to hydroxyl radicals in alkaline aqueous solution by following reaction [18,19]:



where  $\text{F}^+$  denotes one-electron trapped in an oxygen ion vacancy in aluminum oxide and a F-centre means two electrons trapped in an oxygen ion vacancy. In addition, the presence of dissolved oxygen in aqueous solution can also produce hydroxyl radicals ( $E^\circ(\text{OH}^\bullet/\text{OH}^-) = 2.2 \text{ V}$  at pH 9) [46].

Therefore, if the above-mentioned pathways would be producing hydroxyl radical in the present system in the absence of added hydrogen peroxide, hydroxyl radical scavengers should also have a strong effect on the ECL intensity of RhB. Thus, we studied the effects of different types of hydroxyl radical scavengers, such as halide or pseudo-halide ions and ethanol on the ECL of RhB in alkaline solution. The results are shown in Fig. 5.

It was observed that bromide and azide ions weakly enhanced ECL intensity of RhB from  $10^{-5}$  to  $10^{-2}$  M, however, iodide ions and ethanol quenched the ECL of RhB when concentration of scavengers were higher than  $10^{-5}$  M. The effect of halides and some pseudo halides ( $\text{X}^-$ ) on the ECL intensity can be explained by following reactions:



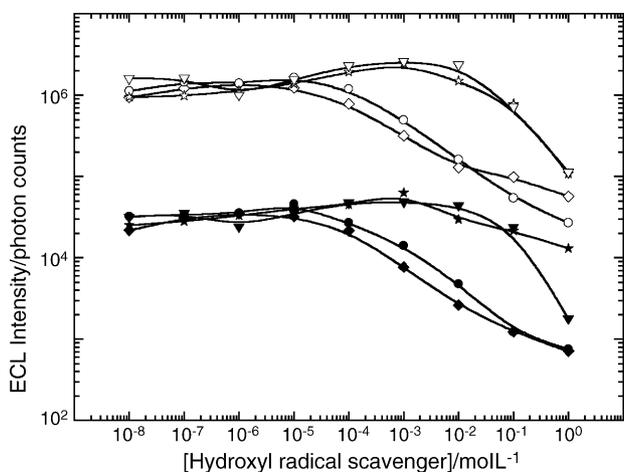
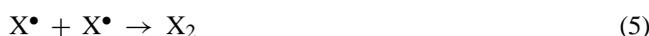


Fig. 5. Effect of different hole scavengers on the ECL intensity of RhB ( $1 \times 10^{-6}$  M): ( $\diamond$ ) TR-ECL, ethanol; ( $\blacklozenge$ ) cathodic ECL ethanol; ( $\circ$ ) TR-ECL, NaI; ( $\bullet$ ) cathodic ECL, NaI; ( $\nabla$ ) TR-ECL,  $\text{NaN}_3$ ; ( $\blacktriangledown$ ) cathodic ECL,  $\text{NaN}_3$ ; ( $\star$ ) TR-ECL, NaBr; ( $\blackstar$ ) cathodic ECL, NaBr. Conditions as in Fig. 5.



Except ethanol, these hydroxyl radical scavengers produce a series of secondary oxidizing radicals by one-electron oxidation ( $E^\circ(\text{Br}^\bullet/\text{Br}^-) = 1.92$  V,  $E^\circ(\text{I}^\bullet/\text{I}^-) = 1.33$  V and  $E^\circ(\text{N}_3^\bullet/\text{N}_3^-) = 1.33$  V [43]). Ethanol “converts” hydroxyl radicals to reducing equivalents by hydrogen abstraction reaction ( $E^\circ(\text{C}_2\text{H}_5\text{OH}/\text{C}_2\text{H}_4\text{O}^{\bullet-}) = -1.2$  V) [47,48]. The rate constants for hydroxyl radical reacting with bromide, iodide, azide and ethanol are:  $1 \times 10^{10}$ ,  $1.1 \times 10^{10}$ ,  $1.2 \times 10^{10}$  and  $1.9 \times 10^9$   $\text{L mol}^{-1} \text{s}^{-1}$ , respectively [33]. These rate constants are almost as high as that of RhB reacting with hydroxyl radical ( $k(\text{OH}^\bullet + \text{RhB}) = 9 \times 10^9$ – $2.5 \times 10^{10}$   $\text{L mol}^{-1} \text{s}^{-1}$ ) [38,49].

The ECL enhancement by bromide is almost similar to that of azide ion in the present system. Increase in concentration of bromide ions or azide ions results in the formation of  $\text{Br}^\bullet$  ( $E^\circ(\text{Br}^\bullet/\text{Br}^-) = 1.92$  V [43]) and  $\text{Br}_2^{\bullet-}$  ( $E^\circ(\text{Br}_2^{\bullet-}/2\text{Br}^-) = 1.62$  V [43]) or  $\text{N}_3^\bullet$  ( $E^\circ(\text{N}_3^\bullet/\text{N}_3^-) = 1.3$  V [43]) by reacting with hydroxyl radicals. Both  $\text{Br}_2^{\bullet-}$  and  $\text{N}_3^\bullet$  seem to be sufficiently oxidizing for the present ECL system. However, continuous increase in concentration of bromide or azide results in formation of  $\text{Br}_3^-$  ( $E^\circ(\text{Br}_2/\text{Br}_2^{\bullet-}) = 0.58$  V versus SHE) [43], and self-combination of azide radicals becomes significant in high concentrations ( $k(\text{N}_3^\bullet + \text{N}_3^\bullet) = 4.4 \times 10^9$   $\text{L mol}^{-1} \text{s}^{-1}$ ) [45].  $\text{Br}_3^-$  is clearly not sufficiently strong oxidant to induce ECL of RhB, which is indicated by a rapid quenching of ECL when concentration is over  $10^{-2}$  M.

A previous study using pulse radiolysis technique revealed that both of  $\text{N}_3^\bullet$  and  $\text{Br}_2^{\bullet-}$  reacted rapidly with RhB in aqueous solution. Amount of radical formed at the end of the  $\text{N}_3^\bullet$  reaction was demonstrated by intense spectra [50]. The  $k(\text{N}_3^\bullet + \text{RhB})$  is about  $5.0 \times 10^9$   $\text{L mol}^{-1} \text{s}^{-1}$  in aqueous solution [50]. However,  $k(\text{Br}_2^{\bullet-} + \text{RhB})$  is unknown. We

assume that  $k(\text{Br}_2^{\bullet-} + \text{RhB})$  is quite similar to  $k(\text{N}_3^\bullet + \text{RhB})$ . Thus, slight enhancement of present ECL by both of  $\text{N}_3^\bullet$  and  $\text{Br}^\bullet$  or  $\text{Br}_2^{\bullet-}$  means that they are better suitable for the present ECL system than the hydroxyl radical itself.

$E^\circ(\text{I}^\bullet/\text{I}^-)$  is ca. 1.3 V [43], which seems to be making  $\text{I}^\bullet$  strong enough an oxidant to produce ECL, but  $\text{I}_2^{\bullet-}$  (prevailing at high iodide ion concentrations), seems to be too weak an oxidant for the system ( $E^\circ(\text{I}_2/\text{I}_2^{\bullet-}) = 0.21$  V [43]). Thus, a clear quenching occurs when concentration of iodide ion increase. Similar phenomena were observed e.g. in luminol system under similar conditions [21].

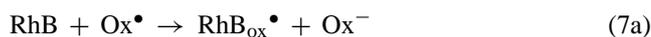
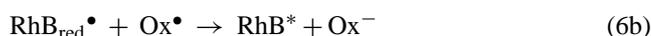
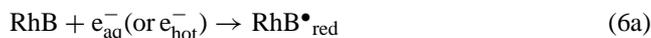
Ethanol has the strongest quenching effect on ECL of the system by reaction with hydroxyl radicals and produces strongly reducing secondary radicals ( $\text{C}_2\text{H}_4\text{O}^{\bullet-}$ ) by hydrogen abstraction as main secondary radicals (84.3%) [33,51]. Obviously, the reducing secondary radicals cannot act as an oxidant in the present system and thus quench the ECL. In fact, ethanol has been an efficient quencher of ECL in a number of aromatic luminophores studied by using our pulse polarization method [21,24,52].

Generally, the quenching effects seemed to be similar during cathodic pulse and after the pulse, so no additional information seems to be extractable from the TR-ECL signals of scavenging experiments. All in all, the effects of hydrated electron and hydroxyl radical scavengers on the ECL of RhB system support hydrated electron and hydroxyl radical playing an important role in the excitation ECL pathways when oxide film is thinner than few nanometers.

### 3.5. Mechanism of ECL

The formal reduction potential of RhB has been reported to be  $-0.52$  V versus SHE at pH 7 in aqueous solution [42] and  $-0.75$  V in alkaline aqueous solution [53]. RhB is oxidized at 1.24 V versus SHE in acidic conditions on  $\text{SnO}_2$  glass electrode and at about 1.21 V versus SHE on Pt-electrode in acetonitrile solution [53]. However, because RhB is rapidly oxidized by azide radical having reduction potential 1.3 V versus SHE [43], the oxidation potential of solvated RhB is somewhat lower, probably about 1.0 V.

In principle, the ECL excitation route of RhB can be reduction-initiated oxidative excitation (red–ox) pathway (reactions (6a) and (6b)) or the oxidation-initiated reductive excitation (ox–red) pathway (reactions (7a) and (7b)). The mechanisms are as follows:



In the equations  $\text{Ox}^\bullet$ , a one-electron oxidant, could be a hydroxyl radical, or a better suitable one-electron oxidant

such as an azide radical produced by scavenging the hydroxyl radical. The first pathway involves a one-electron reduction of rhodamine B to a radical intermediate followed by one-electron oxidation to the original oxidation state. The second pathway goes in reverse. Finally, light is emitted by radiative relaxation of excited  $\text{RhB}^*$  molecules. The initial step (6a) is known to proceed at almost diffusion controlled rate with the second-order rate constant  $k(\text{RhB} + e_{\text{aq}}^-) = 3.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$  [54] in an aqueous solution, but the rate constant of the second step (6b) is unknown.

On the other hand, the ox-red pathway can be rapidly initiated by a hydroxyl radical (7a) ( $k(\text{OH}^\bullet + \text{RhB}) = 9 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$ ) [54]. In addition, some secondary radicals produced from coreactants such as from azide ion ( $k(\text{N}_3^\bullet + \text{RhB}) = 5.0 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$ ) [49] are also capable of this. Azide radical is beneficial because it is known to almost plainly react as a one-electron oxidant unlike the hydroxyl radical [45].

The enthalpies of the excitation steps of each pathway can be evaluated on the basis of basic thermodynamics:

$$-\Delta H^\circ = -\Delta G^\circ - T \Delta S^\circ = (E_{\text{ox}}^\circ) - (E_{\text{red}}^\circ) - T \Delta S^\circ \quad (9)$$

where  $E_{\text{ox}}^\circ$  and  $E_{\text{red}}^\circ$  are the standard electrode potentials of oxidant and reductant, and the other symbols have their usual meanings (it is assumed that the entropy term is about 0.16 eV) [55]. Taking into the consideration the above-mentioned redox potentials of RhB and the fact that the 0–0 transition of RhB is at 567 nm (Fig. 1), i.e., at about 2.18 eV this excited state can be reached via red-ox pathway by any oxidants having their formal reduction potential higher than about 1.84 V versus SHE at pH 7, and higher than about 1.59 V versus SHE in more basic solution. Thus, azide radical is not sufficiently strong oxidant for this, although hydroxyl radical is [46]. Therefore, it is concluded that in the presence of a relatively high concentration of azide ions only the ox-red excitation pathway is possible. On the other hand, any reductants having their formal reduction potential clearly below ca.  $-1.33 \text{ V}$  versus SHE should be able to carry out the final excitation step in ox-red pathway on thermodynamic basis. Because hydrated electron is known not to follow Marcus electron transfer theory (it reduces very rapidly also extremely strongly oxidizing species) [33], it is believed that the ECL in the present systems is mainly generated by ox-red pathway. On the thermodynamic basis the red-ox pathway is also possible in the absence of added azide ions.

However, in the case of oxide films thicker than about 10 nm and for then existing weak ECL, we propose that a modification of red-ox pathway is valid in which hydrated electrons are replaced by heterogeneously transferred electrons from the bottom of the conduction band of aluminum oxide and somewhat above it at the oxide/solution interface as discussed above in Section 3.3.

### 3.6. Analytical applicability of ECL

We studied the analytical applicability of the ECL of RhB using peroxydisulfate ions and azide ions as coreactants. However, due to the increase of blank emission induced by peroxydisulfate ions, a better analytical performance was observed in azide-containing buffer solution. Luminescence lifetime of RhB ECL is ca.  $12 \mu\text{s}$  in the presence of peroxydisulfate ions, and  $19 \mu\text{s}$  in the presence of azide ions. Both lifetimes are longer than that of the background luminescence and thus facilitate the discrimination of this signal from the background. RCL lifetime of RhB was reported previously to be about  $15 \mu\text{s}$  on the basis of pulse radiolysis experiments [31].

Fig. 6 displays calibration plot for RhB in the presence of  $0.01 \text{ M NaN}_3$  as well as the ECL decay curve of  $1 \times 10^{-6} \text{ M}$  RhB under the same conditions in the inset. The time-resolved signal of RhB in the presence of azide ion was recorded for  $200 \mu\text{s}$  directly after each excitation pulse, and cathodic ECL was recorded during the excitation pulses. The ECL response is linear from  $10^{-10}$  to  $10^{-5} \text{ M}$ . The detection limit is not better by time-resolved detection, but still the ECL lifetime is much longer than that of e.g. coumarines we have studied [56]. Thus, RhB-based labels combined with other electrochemiluminescent labels with different lifetimes can provide multiparameter assays by application of time discrimination. For example, RhB-derivatives could be used as labels holding moderate lifetime of ECL, combining with another label with very short ECL lifetime, e.g. above-mentioned coumarines, which display no ECL after the excitation pulse. On the other hand, RhB has relatively strong cathodic ECL intensity, and relatively short luminescence lifetime of ECL compared with Tb(III) chelates (lifetime of Tb(III) chelate ECL is ca. 2–2.5 ms). Therefore, combining cathodic ECL signal of RhB with TR-ECL signal of Tb chelates provides another option

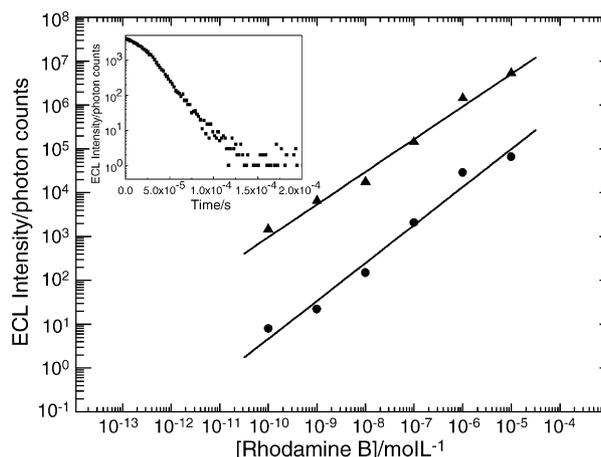


Fig. 6. Calibration plot of RhB using disposable aluminum electrodes: (▲) cathodic ECL during pulse; (●) time-resolved ECL. Conditions: all measurements were made in  $0.05 \text{ M Na}_2\text{B}_4\text{O}_7$  at pH 9.2 buffer solution containing  $0.01 \text{ mol/L NaN}_3$ . Otherwise the same as in Fig. 2. Inset: decay curve of RhB after the cathodic pulse.

for applications. Even more interestingly, homogeneous ECL energy transfer assays which are often carried out with photoexcitation of Tb(III) chelate donors and using Rhodamine B acceptors [57] seem to be possible.

#### 4. Conclusions

A radiochemiluminescent compound, Rhodamine B, was observed to show strong ECL during cathodic pulse polarization of oxide-coated aluminum electrodes in a fully aqueous solution and, therefore, the derivatives of RhB can be suggested to be usable as electrochemiluminescent labels in bioaffinity assays. Support was obtained for the same primary radicals (hydrated electrons and hydroxyl radicals) being responsible of the excitation reactions in the present type of cathodic ECL as in RCL of RhB in aqueous solutions.

The ECL response allowed the detection of RhB below nanomolar concentration level and the calibration plot was linear over several decades of concentration. The presence of azide ion enhances the ECL intensity of RhB by generation of azide radicals in reaction with hydroxyl radicals, because azide radical is a better oxidant for the system than hydroxyl radical. The ECL intensity has a wide plateau within a relatively wide pH range and the maximum intensity is observed in slightly basic solutions usable for bioaffinity assays. The relatively long lifetime of the ECL of RhB allows to suggest that the derivatives of RhB can be used in multilabel assays based on time-discrimination together with some shorter-lived ECL displaying labels such as coumarins [56]. It is also considered possible that other rhodamine dyes in the xanthene dye family may possess better ECL properties for more sensitive time-resolved detection.

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