Cathodic Electrochemiluminescence of Lucigenin at Disposable Oxide-coated Aluminum Electrodes

Qinghong Jiang[1], Markus Håkansson[1], Johanna Suomi[1], Timo Ala-Kleme[2], Sakari Kulmala[1*]

[1]Laboratory of Analytical and Inorganic Chemistry, Helsinki University of Technology,P.O. Box 6100, FIN-02015 HUT, Finland

[2]Laboratory of Analytical Chemistry, Departrment of Chemistry, University of Turku, FIN-20014, Finland.

Abstract

Electrogenerated chemiluminescence (ECL) of lucigenin is induced at oxide-coated aluminum electrode in aqueous solution by cathodic pulse polarization. This ECL can be enhanced by the presence of coreactants such as peroxodisulfalte. The present method is based on the injection of hot electrons into the aqueous electrolyte solution, which probably results in the generation of hydrated electrons as reducing mediators. The successive one-electron redox reactions result in the excited states of lucigenin or its fragmentation products. The method can detect lucigenin over several orders of magnitude of concentration with detection limit below nanomolar concentration level. In addition, the relatively long lifetime of the ECL of lucigenin makes time-resolved detection possible. This study suggests that the derivatives of lucigenin can be utilized as electrochemiluminescent labels in aqueous solution in bioaffinity assays at thin insulating film-coated cathodes. The cathodic ECL reaction mechanisms are discussed.

Key words: *Electrogenerated chemiluminescence, ECL, Lucigenin, Hot electron, Time-resolved Detection, Oxide-coated Aluminum Electrode*

1. Introduction

The chemiluminesence (CL) of lucigenin (Luc^{2+}, N,N'-dimethyl-9-9'-biacridinium dinitrate) has been known since 1935 [1]. It is known to produce strong chemiluminescence (CL) in the presence of hydrogen peroxide and reducing agents in alkaline solution. The mechanism of chemiluminescence under such conditions has been examined in detail [2-6]. Although the reactions undergone by lucigenin are quite complex, generally the CL mechanism seem to involve 3 reaction steps: (1) One-electron transfer to Luc^{2+} producing radical $Luc^{\bullet+}$; (2) $Luc^{\bullet+}$ then reacts with molecular oxygen or a superoxide radical ($O_2^{\bullet-}$) and yields an extremely unstable dioxetane-type intermediate; (3) The decomposition of this intermediate provides an excited state of N-methyl-acridone (NMA^*), which is the primary emitter, emitting at ca. 452nm.

In addition, it has been proposed that either Luc^{2+} or dimethylbiacridene (DBA), a product of lucigenin two-electron reduction, would be secondary emitters [3,4,6]. Both DBA and Luc^{2+} could act as energy acceptors from excited NMA^* by singlet-singlet energy transfer, finally emitting at 505 nm or 510nm, respectively. It has been generally suggested that the observed CL is mainly due to both the emission of excited NMA^* and secondary emitters (i.e. DBA^* and Luc^{2+*}) simultaneously giving a mixed broad band light emission [3,4,6].

The electrochemiluminescence of lucigenin in non-aqueous [2] and in aqueous solutions have been investigated as well [2,5-7]. The light emission was observed to occur at the cathode and the proposed mechanism for electrochemiluminescence of lucigenin by means of electrolysis was suggested to be analogous to that of chemiluminesecence of lucigenin [2-4].

Papadopoulos et al. observed that when irradiated oxygen saturated solutions of amines or amides were added to aqueous solutions of lucigenin [8], intense CL was induced with N,N-dimethylbiacridylidene (DBA) as the major emitting species. Emission maximum of DBA was reported to be at 505 nm.

The luminesence of lucigenin is of interest, e.g. because of its applications in determinations of trace metals. In addition, it has been extensively applied to the detection of substances generated from biological tissues, for example, superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide and epinephrine [9-13] and enzymatic systems (e.g. xanthine-xanthine oxidase system) in neutral or relatively weakly alkaline solutions (pH 7-10) [14-16]. Allen and Faulkner et al. have proposed that the chemiluminescence of lucigenin in such biological systems requires the generation of the one-electron reduced lucigenin ($Luc^{\bullet +}$) as an essential step of light generating pathways [17, 18]. ECL of lucigenin has been used to detect riboflavin [19] human chorinic gonadotropin [20] and hemin [21].

In previous studies [22-26], we have demonstrated that many kinds of luminophores can be excited by cathodic pulse-polarization at conductor/insulator/electrolyte (C/I/E) tunnel junction electrodes e.g. oxide-coated aluminum, silicon and magnesium electrodes. These thin insulating film-coated electrodes are known to act as pulsed cold-cathodes and tunnelemit hot electrons (e_{hot}^-) into aqueous solutions in an analogous manner to that occurring in solid-state devices [27-30].

If the energy of tunnelemitted hot electrons is above the conduction band edge of water, they may enter the conduction band of water and are likely to become hydrated (e_{aq}^-) after thermalization and solvation [31,32]. In practice, cathodic reductions may be produced simultaneously by heterogeneously transferred electrons from the bottom of the conduction band of the insulating film, via the surface states of the insulating film and by presolvated hot and hydrated electrons [25]. Meanwhile, strongly oxidizing species such as sulfate and hydroxyl radicals can be cathodically generated from purposely-added coreactants or form dissolved oxygen [22,23,25,33,34]. In addition, F^+-centers existing in oxide film of C/I/E electrodes may act as oxidants [2,23,33]. Hence, highly reducing and oxidizing conditions are simultaneously achieved in the vicinity of the electrode surface.

General ECL excitation routes for luminophores based on our cathodic pulse polarization method 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 by the cathodically generated species at the oxide film/electrolyte interface by successive one-electron transfer steps normally resulting in the luminophore in its original oxidation state but now in its singlet or triplet excited state [22-26]. However, sometimes the ECL mechanism is based on the decomposition of the luminophore [22].

In the present work, we studied the possibility of the generation of cathodic ECL of lucigenin at thin oxide film-coated aluminum electrodes and the ECL mechanisms involved.

2. Experimental

The measurements were carried out using a coulostatic pulse generator [35] with 120 μC, 20 Hz, -50 V cathodic pulses. The ECL intensities of lucigenin were detected through an interference filter with a center wavelength at 500 nm and transmission band half-width of 10 nm, using single photon counting and apparatus described previously [36,37]. In a two-electrode cell, the disposable planar aluminum electrode (effective area 63.6 mm^2) was used as a cathode and a small-diameter Pt-wire electrode (diameter 0,9 mm, effective area 2,6 mm^2) was used as an anode. Aluminum electrodes used here were made from nominally 99.9 % pure aluminum 0.3 mm thick band (Merk Art. 1057, batch 720 K22720857). The band was normally used as covered with its 2-3 nm thick natural oxide film and cut in 15 mm×15 mm slides.

The ECL spectra were measured in a 1-cm polystyrene cuvette, in which an Al strip working electrode and a Pt wire counter electrode were set between two PTFE support pieces. Photoluminescence measurements were made in quartz cuvettes using Perkin-Elmer LS-5 luminescence spectrometer. ECL spectrum was measured using Ocean Optics USBFL-2000 spectrometer.

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 mA cm^{-2} and then potentiostatically until the

current density decayed below 10 μA cm^{-2}. The thickness was calculated by using the anodization ratio value 1.4 nm V^{-1} [38,39].

Most of the measurements were made in 0.05 mol/L sodium tetraborate buffer adjusted to pH 7.8 with sulphuric acid unless otherwise mentioned. This buffer is known to be quite unreactive with hydrated electrons and hydroxyl radicals, as well as with sulfate radicals [40]. NaI, NaBr, $NaB_4O_7.10H_2O$, NaN_3, $K_2S_2O_8$, $NaNO_3$, $NaNO_2$, H_2O_2 were pro analysi grade reagents from Merck. Ethanol was pro analysi grade products from Primalco. Lucigenin was pro analysi grade reagent from Sigma, and no purification of lucigenin was made before use.

3. Results and discussion

3.1 Spectra of Lucigenin

Cathodic ECL spectrum of lucigenin produced at an oxide-coated aluminum electrode and fluorescence emission spectrum are presented in Fig.1. The molecular structure of lucigenin is presented in the inset of Fig.1. The fluorescence emission spectrum has two very closely located peaks at 495 nm and 510 nm, respectively. The ECL spectrum has a broad band with a maximum at 510 nm. Based on the information got from spectra, we cannot attribute the current ECL emission to NMA*, which emits at shorter wavelengths at about 450 nm.[5] In fact, the ECL spectrum is asymmetrical and, on the contrary, seems to be composed also of a weaker emission at longer wavelengths, peaking at about 560 nm (Fig. 1). Thus, it is assumed that the main emitter could be excited Luc^{2+*} or excited DBA^*, or mixture of both, because both of them have emission maxima around 510 nm [2-7]. Possibly, the weak emission at longer wavelengths is originated from the excited triplet state $^3Luc^{2+*}$.

3.2 Effect of pH on ECL

The dependence of the ECL of lucigenin on pH in air-saturated tetraborate buffer solution is presented in Fig. 2. The ECL intensity is relatively constant within pH range from 4 to 9, which is close to the pH range of stability of Al_2O_3 film [34].

When pH was below 4, the ECL decreased considerably with decreasing pH. In the present system, we believe that the ECL decreases in acidic solutions mainly due to the dissolution of the oxide film. Another reason for this is that hydrated electron is fast converted to its conjugated acid, a hydrogen atom, in highly acidic solutions ($k(e_{aq}^- + H^+) = 2.3 \times 10^{10}$ L $mol^{-1}s^{-1}$) [40]. Based on our previous studies [22-26], hydrated electrons are assumed to be the primary reducing species to trigger most of the ECL reaction pathways studied by us so far. In alkaline conditions, ECL intensity started to slightly decrease when pH exceeded 9 due to the thinning of oxide film. However, ECL intensity started to slightly increase when pH was higher than 11. Alkaline solutions above pH 12 were not used, because it is sure that the insulating film is then dissolved. We suggest that the slight increase in ECL when pH exceeds 11 is due to commencing of chemiluminescence in which at least aluminum metal, low-valent aluminum ions and hydrogen atoms can act as reducing species [41]. Under highly alkaline conditions Al_2O_3 film dissolves rapidly and metallic aluminum metal gets in direct contact with strongly alkaline aqueous solution. Then the relevant primary reaction mechanism is quite probably analogous to that previously studied by us in case of chemiluminescence of luminol induced by dissolution of aluminum metal in alkaline aqueous solution [42]. In addition, weak CL might be generated solely on the basis of reactions between lucigenin and hydroxide ions having very high concentration. Further studies on this CL of lucigenin are in progress in our laboratory.

3.3 Effect of oxide film thickness on ECL

The native oxide film on aluminum can be grown thicker by anodic oxidation [38]. Anodic oxide films have been proposed to be a mixture of amorphous and γ-Al_2O_3 [38], and the proportion of crystalline composition, i.e. γ-Al_2O_3, in anodic oxide film

increase with increase in film thickness [38]. The effect of oxide film thickness on the ECL intensity is presented in Fig.3. The results are analogous to those previously observed with metal chelates and organic luminophores [25,33,43].

As previously, the total cathodic pulse current was not dependent on oxide-film thickness [25]. Therefore, it is again proposed that only the tunneling mechanism changes as the oxide film thickness is increased. In case of ultra thin insulating films (thickness < ca. 4 nm) direct field-assited tunneling is predominating, i.e., electrons do not enter the conduction band of Al_2O_3 but tunnel from an energy level through the barrier to an equal energy level. In this case, no considerable loss of electron energy occurs [44]. Therefore, electrons reaching aqueous solution with energy above the conduction band edge of water can become hydrated electrons after thermalization and solvation, and ECL pathways can be initiated by hydrated electrons. Fowler-Nordheim (F-N) tunneling is predominant electron transport mechanism in the case of thicker oxide films (> 4 nm). Here, electrons are first tunneled to the conduction band of the oxide and then transported by the field in the conduction band. During transport the electrons partially lose their energy in inelastic scattering and partially gain energy in the electric field. After tunneling through thicker oxide films in F-N tunneling regime, the electrons are transferred into the electrolyte either from the bottom of the oxide conduction band or somewhere above it at the oxide/electrolyte interface. Due to the band bending under strong cathodic polarization, the formation of hydrated electrons is no longer possible and thus ECL intensity is exponentially decreased as the insulating oxide becomes thicker. Depending on the properties of the oxide film conduction band a distribution of electron energy is produced which can have the mean value above the energy of the conduction band edge of the oxide at the surface of the electrode in the oxide/electrolyte interface [44].

It is interesting to note that ECL intensity started to slightly increase when oxide film thickness exceeded 12 nm, but then again followed by continuous decrease in intensity with further increase in the oxide film thickness. The slight enhancement in ECL at about thickness of 12 nm is also displayed by the intrinsic electroluminescence (IEL) [45] of the oxide film (Fig. 3) that was measured in the absence of lucigenin. IEL of oxide-coated aluminum electrode has a very broad

emission band between 400 nm and 600 nm [45]. However, this cannot be the source of the ECL enhancement in the presence of lucigenin, because the changes in the IEL intensity would not be observable in the present experiment (the y-axis of Fig. 3 is logarithmic). Therefore, we assume that lucigenin, which is quite easily reduced ($E°(Luc^{2+}/Luc^{+\bullet})$ = –0.13 V *vs*. SHE) [6] when compared with the other luminophores we have studied [24,32,46] is producing considerable ECL also under F-N tunneling regime.

3.4 Effect of free radical scavengers on the ECL

If the common excitation routes for luminophores based on our cathodic pulse polarization method were valid in the present study, free radicals would be the primary species inducing the present ECL. Therefore, free radical scavengers would affect significantly on the present ECL. First, the effects of hydrated electron scavengers on ECL were investigated (Fig.4). $Co(NH_3)_6^{3+}$ is the strongest hydrated electron quencher amongst the tested electron scavengers. When the concentration of $Co(NH_3)_6^{3+}$ exceeds 1×10^{-5} M, ECL intensity decreases abruptly. The order of the quenching capability of the tested scavengers is $Co(NH_3)_6^{3+}$, nitrite ion and nitrate ion. The order of quenching capability is related to second-order reaction rate constants of these scavengers reacting with hydrated electrons ($k(e_{aq}^- + Co(NH_3)_6^{3+})=8.7\times10^{10}$ L mol^{-1}s^{-1}, $k(e_{aq}^- + NO_3^-) = 9.7\times10^9$ L mol^{-1}s^{-1} and $k(e_{aq}^- + NO_2^-) = 4.1\times10^9$ L mol^{-1}s^{-1}) [40].

The rate constant $k(e_{aq}^- + Co(NH_3)_6^{3+})$ is about 20 times higher than $k(e_{aq}^- + NO_2^-)$, thus $Co(NH_3)_6^{3+}$ strongly quenched the present ECL already in its lower concentrations. Nitrite ions quench the present ECL more strongly than nitrate ions. The reason for that is that nitrite also reacts rapidly with hydroxyl radical [$k(\cdot OH + NO_2^-)=1.0\times10^{10}$ L mol^{-1}s^{-}1] [40] and hence it consumes hydrated electrons and hydroxyl radicals from the excitation pathways. Analogous phenomena were observed also in our previous studies [23,45,47]. It is worth noting that both $Co(NH_3)_6^{3+}$ and nitrate ion are unreactive towards hydrogen atom ($k(H^\bullet + Co(NH_3)_6^{3+})<9\times10^4$ L mol^{-1}s^{-1}, $k(H^\bullet + NO_3^-)=1.4\times10^6$ L mol^{-1}s^{-1}) [40]. Thus, hydrogen atom cannot be the reducing mediator in the present system. The pronounced

quenching of the ECL by $Co(NH_3)_6^{3+}$ and nitrate ion support the assumption that hydrated electron is an essential species for the ECL excitation routes also in the present ECL system.

Peroxodisulfate ions enhanced the ECL signal of lucigenin from 10^{-6} M, the most at about 10^{-3} M, after which the increase in concentration of peroxodisulfate resulted in quenching of the ECL. Peroxodisulfate ions react with the hydrated electrons, resulting in strongly oxidizing sulfate radical as follow:

$$e_{aq}^- + S_2O_8^{2-} \rightarrow SO_4^{\bullet -} + SO_4^{2-} \quad (1)$$

The rate constant $k(e_{aq}^- + S_2O_8^{2-})$ is 1.2×10^{10} L $mol^{-1}s^{-1}$ [40]. Sulfate radical is a strong one-electron oxidant (E° = 3.4V vs. SHE) [48] capable of oxidizing a number of aromatic compounds very difficult to oxidize, and luckily, it reacts very sluggishly with water [40]. Thus $SO_4^{\bullet -}$ can probably oxidize also Luc^{2+} but surely at least $Luc^{\bullet +}$ in highly exothermic reaction. However, the ECL intensity decreases when the concentration of $S_2O_8^{2-}$ ions exceeds 10^{-3} M, because hydrated electrons are too efficiently consumed from light generating pathways by reacting with excess $K_2S_2O_8$, and, in addition, part of the $SO_4^{\bullet -}$ radicals are also lost by recombination reaction ($k(SO_4^{\bullet -} + SO_4^{\bullet -}) = 5.1\times10^8$ L $mol^{-1}s^{-1}$) [40].

Hydrogen peroxide slightly enhanced the present ECL at 3×10^{-3} M, after which an increase in concentration of scavenger resulted in quenching of ECL signal by rapid reaction with hydrated electrons ($k(e_{aq}^- + H_2O_2) = 1.2\times10^{10}$ $Lmol^{-1}s^{-1}$) [40]. First, we should take into the consideration that in all the present cases the solutions were air-equilibrated which means that the oxygen concentration was always close to $2x10^{-4}$ M [23,49]. Thus, also superoxide radical, hydrogen peroxide and hydroxyl radicals were rapidly generated:

$$O_2 + e^-_{aq} \rightarrow O_2^{\bullet -} \quad (2a)$$

$$O_2^{\bullet -} + 2H^+ + e^-_{aq} \rightarrow H_2O_2 \quad (2b)$$

$$H_2O_2 + e^-_{aq} \rightarrow {}^{\bullet}OH + OH^- \quad (2c)$$

with the second-order rate constants k_{2a} = 1.9x10^{10} L mol^{-1}s^{-1}, k_{2b} = 1.3x10^{10} L mol^{-1}s^{-1} and k_{2c} = 1.2x10^{10} L mol^{-1}s^{-1} , respectively [21].

Secondly, hydroxyl radical has a strong tendency also to other reaction modes in addition to that of one-electron oxidation [40]. Therefore, sulfate radical that has less tendency to these side reactions and also is considerably stronger oxidant than hydroxyl radical [48], is clearly better oxidant for the present ECL system than hydroxyl radical.

In addition, F^+-center (one electron trapped in an anion vacancy in aluminum oxide) existing in oxide film can act as strongly oxidizing species capable of oxidizing hydroxyl ion (or surface hydroxyl groups) to hydroxyl radicals in aqueous solution by following reaction [26]:

$$F^+\text{-center} + OH^- \rightarrow \text{F-center} + OH^\bullet \qquad (3)$$

The F^+-centers are regenerated by an anodic current peak after each cathodic pulse. If this anodic current peak is cut off by a series diode with the cell, the ECL intensity is strongly quenched.

If hydroxyl radicals would be generated as above shown and if they would be of vital importance in the ECL pathways, hydroxyl radical scavengers should give significant effects on the present ECL. The effects of different hydroxyl radical scavengers on the ECL are presented in Fig.5. It reveals that all the tested hydroxyl radical scavengers quench the present ECL. Amongst the scavengers, iodide ions quenched the present ECL the most strongly when its concentration exceeded 10^{-4} M, and ethanol was a very efficient quencher, which started to quench the present ECL at the concentration of 10^{-5}M. The order of quenching capability of tested scavengers is iodide ions > thiocyanate ions > ethanol > azide ions > bromide ions. In addition, chloride doesn't quench ECL significantly.

The explanation for that is related to that these halide or pseudohalide ions produce a series of oxidizing secondary radicals initiated by one-electron oxidation reactions as follows:

$$OH^{\bullet} + X^{-} \rightarrow OH^{-} + X^{\bullet} \quad (4)$$

$$X^{\bullet} + X^{-} \rightarrow X_2^{\bullet -} \quad (5)$$

$$X^{\bullet} + X^{\bullet} \rightarrow X_2 \quad (6)$$

The reaction rate constants for hydroxyl radical in reaction with chloride, bromide, iodide, azide, thiocyanate and ethanol are: 4.3×10^{9} (pH 2, unreactive at pH 11), 2.2×10^{8} (at pH 7), 1.1×10^{10}, 1.2×10^{10}, 1.1×10^{10} and 1.9×10^{9} L $mol^{-1}s^{-1}$, respectively [40].

The quenching effects of different scavengers on ECL are related to the redox potential of the oxidizing secondary radicals and their corresponding rate constants. In general, the lower redox potential and the higher reaction rate constant of the scavenger, the stronger quenching effect on ECL intensity is observed. In the tested conditions, chloride did not react with hydroxyl radicals ($E°(OH^{\bullet}/OH^{-})$=2.3V vs. SHE) because its formal reduction potential is smaller than that of chlorine atom ($E°(Cl^{\bullet}/Cl^{-})$ = 2.41V vs. SHE) [49,50]. Thus, no significant effect of chloride on lucigenin-ECL is observed.

In addition, the reaction of iodide with hydroxyl radical produces weak oxidizing agents, i.e. $I^{\bullet}$ ($E°(I^{\bullet}/I^{-})$ = 1.33V *vs.* SHE) and especially $I_2^{\bullet -}$ ($E°(I_2/I_2^{\bullet -})$ = 0.21V *vs.* SHE) [50], prevailing at high iodide ion concentrations. Thus, strong quenching of the lucigenin ECL is observed with the increase in the concentration of iodide. A similar ECL quenching effect by thiocyanate ions is obtained as well because the $(SCN)_2^{\bullet -}$ is also a weak oxidizing agent ($E°((SCN)_2^{2-}/(SCN)_2^{\bullet -})$ = 0.21V *vs.* SHE). In addition, for thiocyanate ion, the rate constants of reaction (5) and (6) are very high [51]. Therefore, thiocyanate ions have strong quenching effect on lucigenin ECL when its concentration exceeds 10^{-4} M.

Bromide ($E°(Br^{\bullet}/Br^{-})$ = 1.92V *vs.* SHE) slightly enhances lucigenin ECL at 10^{-3} M. Similarly, a weak increase in ECL is observed in the presence of azide ($E°(N_3^{\bullet}/N_3^{-})$ = 1.33V *vs.* SHE) at 10^{-3} M. It indicates that $Br^{\bullet-}$ and $N_3^{\bullet}$ are capable of participating in light generating pathways more efficiently than hydroxyl radicals. Further increase in concentration of bromide results in formation of Br_3^{-}($E°(Br_2/Br_2^{\bullet-})$ = 0.58V vs. SHE) [50], $Br_2^{\bullet-}$ is clearly not a sufficiently strong oxidant for the present ECL system. When concentration of bromide is over 10^{-2} M, $Br_2^{\bullet-}$ prevails in solution, therefore, quenching ECL is observed. Similarly, the amount of azide radicals would be decreased by production of molecular nitrogen through self-combination of azide radicals ($k(N_3^{\bullet}+ N_3^{\bullet})$ = 4.4×10^9 L $mol^{-1}s^{-1}$) at high concentration of azide [51]. Therefore, above 10^{-3} M, azide ions quench the present ECL. Analogous phenomena were observed in luminol system under similar conditions [52].

Ethanol significantly quenches present ECL by reacting with hydroxyl radicals and producing strongly reducing secondary radicals $C_2H_4O^{\bullet-}$($E°(C_2H_5OH/C_2H_4O^{\bullet-})$ = -1.2 V *vs.* SHE) [53] by hydrogen abstraction as the main secondary radicals (84.3%) [40,54]. Obviously, the reducing secondary radicals cannot excite ECL in the present system. Thus, ethanol quenches ECL at relatively low concentration of 10^{-5} M. However, ethanol is not the strongest quencher amongst the tested hydroxyl radical scavengers. The explanation is related to rate constant of ethanol reacting with hydroxyl radicals, which is about 10-fold smaller than that of iodide. In addition, the fluorescence quantum efficiency (Φ_{FL}) of DBA^* in ethanol is much higher than that in aqueous solution [2]. Thus, it is quite possible that the presence of high concentration of ethanol is beneficial for DBA^* emission, which might be an emitter in the tested system.

In short, the effects of hydroxyl radical and hydrated electron scavengers on ECL of lucigenin support the assumption that the hydrated electrons and the oxidizing species with properties similar to those of the hydroxyl radical are generated during cathodic pulse polarization and play important roles in excitation ECL pathway when the ultra thin oxide film coated electrodes are applied as working electrodes.

3.5 Mechanism of ECL

As mentioned above, two half-wave potentials for lucigenin reduction in 0.10 M KCl aqueous solutions have been reported to be 0.02 V and –0.13 V (*vs.* SHE), independent of pH in the range pH 0.7 - 10.8 [3]. In addition, Okajima et al. have reported that the reduction potential $E°(Luc^{2+}/Luc^{+\bullet})$ is –0.13 V (*vs*. SHE) [6], and the two-electron reduction product of lucigenin can be reoxidized at 0.7 V vs. SHE [6] at a gold electrode. Hydrated electrons (E° = –2.9V vs. SHE) [40] generated by cathodic pulse polarization of thin oxide-coated aluminum electrode can easily reduce Luc^{2+} to $Luc^{\bullet-}$, and even to $lucigenin^0$, but unfortunately, the rate constants have not ever been measured nor those of in reaction with hydroxyl radicals.

Taking into consideration the redox potential values of lucigenin, the ECL excitation route of lucigenin could be the reduction-initiated oxidative excitation (red-ox) pathway (6-8). The mechanism is as follows:

$$Luc^{2+} + e_{aq}^{-}(\text{or } e_{hot}^{-}) \rightarrow Luc^{\bullet+} \quad (7a)$$

$$Luc^{\bullet+} + Ox^{\bullet} \rightarrow Luc^{2+*} \quad (7b)$$

$$Luc^{2+*} \rightarrow Luc^{2+} + h\nu \quad (\lambda = 510 \text{ nm}) \quad (7c)$$

Where, $Ox^{\bullet}$ is a one-electron oxidant, such as $OH^{\bullet}$, $SO_4^{\bullet-}$ or other suitable one-electron oxidant generated by scavenging the hydroxyl radical. Based on the basic thermodynamics, the standard potentials of the oxidant and the reductant needed in the excitation step can be evaluated using the equation:

$$-\Delta H° = nF[(E_{0x}°) - (E_{Red}°)] - T\Delta S° \quad (8)$$

where $E_{0x}°$ and $E_{Red}°$ are the standard reduction 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]. In the present case, the enthalpy of the excitation step equals to the energy of emission light at wavelength 510 nm, i.e, 2.43 eV. This excited state can be reached by oxidants having their redox potential higher than about 2.46 V vs. SHE. This can be demonstrated by high enhancement of ECL by peroxodisulfate ions

in Fig.4. Therefore, the ECL in the present system is generated by red-ox pathway if sulfate radical is present (or hydroxyl radical at sufficiently low pH). However, bromine atom or azide radicals are not sufficiently strong oxidants for this red-ox pathway.

Thus, during part of the hydroxyl radical scavenging experiments the ox-red or some other excitation pathway must have been predominant. It is not considered impossible that lucigenin could be oxidized by azide radical ($E°(N_3^{\bullet}/N_3^-)$=1.33V *vs*. SHE) [40]. This would then mean that Luc^{3+} would have formal reduction potential e.g. around 1 V vs. SHE and the whole excitation sequence would be:

$$Luc^{2+} + Ox^{\bullet} \rightarrow Luc^{\bullet 3+} \quad (9a)$$

$$Luc^{\bullet 3+} + e_{aq}^-(\text{or } e_{hot}^-) \rightarrow Luc^{2+*} \quad (9b)$$

$$Luc^{2+*} \rightarrow Luc^{2+} + h\nu \quad (\lambda = 510 \text{ nm}) \quad (7c)$$

Thus, due to the highly negative reduction potential of hydrated electron (-2.9 V vs. SHE) [40] the ox-red excitation pathway is easily possible on thermodynamic grounds. In addition, hydrated electron is not following Marcus theory and is reducing extremely rapidly with highly oxidizing species near diffusion controlled rate [40]. Thus, it is suggested that both ox-red and red-ox excitation pathways are important in the present case.

Because CL of lucigenin is a very complex process with several reactions proceeding in parallel, it is quite possible that also dioxetane type of intermediate is produced [5]. In aerated solution, a parallel reductive excitation pathway involving an energy transfer step from NMA* to lucigenin is plausible:

$$Luc^{2+} + e_{aq}^-(\text{or } e_{hot}^-) \rightarrow Luc^{\bullet +} \quad (10)$$

$$O_2 + e_{aq}^-(\text{or } e_{hot}^-) \rightarrow O_2^{\bullet -} \quad (2a)$$

$$Luc^{\bullet +} + O_2^{\bullet -} \rightarrow \text{dioxetane} \rightarrow NMA + NMA^* \quad (11)$$

$$NMA^* + Luc^{2+} \rightarrow NMA + Luc^{2+*} \quad (12)$$

$$Luc^{2+*} \rightarrow Luc^{2+} + h\nu \quad (\lambda=500 \text{ nm}) \quad (13)$$

In principle, all the CL pathways involving formation of dioxetane type of intermediates by reaction with superoxide radical or hydrogen peroxide are quite possible (due to the fast reactions 2a and 2b). However, more studies are required before the emission mechanism/mechanisms can be confirmed.

4. Analytical applicability of ECL

Intense ECL from lucigenin was obtained in the presence of peroxodisulfate ions with an optimal concentration of 10^{-3} M. The luminescence lifetime of lucigenin ECL at oxide-covered aluminum electrode is 18.4 μs (Fig. 6). This lifetime is longer than that of the IEL, facilitating the time-discrimination of the ECL from the background.

Calibration plots of lucigenin ECL in the presence or absence of 10^{-3} M $K_2S_2O_8$ are shown in Fig.6. The inset in Fig.6 displays the ECL decay curve of 1×10^{-6} M lucigenin in the presence of 10^{-3} M $K_2S_2O_8$. The time-resolved signals of lucigenin in presence of 10^{-3} M $K_2S_2O_8$ were recorded with 1.0 ms gate time and 5 μs delay time. The calibration plot of lucigenin in the absence of 10^{-3} M $K_2S_2O_8$ is linear from 10^{-9} to 10^{-5} M, while in the presence of 10^{-3} M $K_2S_2O_8$ a wider linear calibration range is obtained from 10^{-10} to 10^{-6} M. Thus, in this case it is useful to use peroxodisulfate ions as coreactants.

According to the present results it can be suggested the suitable derivatives of lucigenin could be used as labels in bioaffinity assays such as immunoassays or DNA-probe assays. Sometimes, the time-resolved measurement might be useful, although the luminescence lifetime in the present case is far from those of Tb(III) chelates [46,56,57] .

Lucigenin itself contains no functional groups that allow conjugation to biological molecules of interest. However, two lucigenin derivatives containing conjugation groups, 9,9'-bisacridinium-N,N'-diaceticacid ethyl ester (BADE) and N, N'-di-(3-sulfopropyl)-9,9'-basacridinium (SPBA) have been synthesized [58,59]. We assume the present ECL technique could have a good prospect by using these derivatives as labels. Unfortunately, the present ECL is not applicable for the detection of

superoxide radical ($O_2^{\bullet -}$), because superperoxide radical is also generated from dissolved oxygen in the present system.

4. Conclusions

Intense ECL of lucigenin was produced by cathodic pulse polarization at thin insulating film-coated electrodes in fully aqueous solution. The results obtained suggest that the hydrated electrons, superoxide and hydroxyl radicals are primary species for the generation of ECL of lucigenin in aerated solutions by the present cathodic pulse polarization method. It seems that there is no single excitation pathway that would be valid only.

The ECL response allowed the detection of lucigenin below nanomolar concentration level and over several orders of magnitude of concentration. The presence of peroxodisulfate ion strongly enhances the ECL of lucigenin and the optimal concentration of peroxodisulfate ion is ca. 1 mM under present experimental conditions. The existence of lucigenin derivatives (i.e, BADE and SPBA) having conjugation groups to link them to biological molecules of interest indicates that they can be used as a labels in bioaffinity assays based also on our cathodic pulse polarization method.

We assume that e.g. oxide coated magnesium and silicon electrodes can be also applied as a working electrodes for the cathodic excitation of lucigenin which will be studied in the near future in our laboratory.

References:

1. K.Gleu , W. Petsch, Angew. Chem., 48 (1935) 57.
2. K.D. Legg, D.M Hercules, J. Am. Chem. Soc., 91 (1969) 1902.
3. R. Maskiewicz, D. Sogah, T.C, Bruice, J. Am. Chem. Soc., 101 (1979) 5355.
4. R. Maskiewicz, D. Sogah, T.C, Bruice, J. Am. Chem. Soc., 101 **(**1979) 5347.
5. Y.G. Sun, H. Cui, X.Q. Lin, J. Lumin., 92 (2001) 205.
6. T. Okajima, T. Ohsaka, J. Electroanal. Chem., 534 (2002) 181.
7. K.E Haapakka, J.J Kankare, Anal. Chim. Acta., 130 (1981) 415.
8. K. Papadopoulos, T. Triantis, D. Dimotikali, J. Nikokavouras, J. Photochem. Photobiol. A: Chem., 124 (1999) 85.
9. I. Minkenberg, E. Ferber, J. Immunol. Methods, 71 (1984) 61.
10. H. Gyllenhammar, J. Immunol. Methods**.,** 97 (1989) 209.
11. H. Gyllenhammar , J. Clin. Lab. Immunol. 28 (1989) 97.
12. H. Sasamoto, M. Maeda, A. Tsuji , Anal. Chimi. Acta, 310 (1995) 347.
13. Y.Y. Su, J. Wang, G. Che, Talanta, 65 (2005) 513.
14. Z. Bai, L. M Harvey, B. McNeil, Biotechnol. Bioeng. 75 (2001) 204.
15. I.B. Afanas'ev, E.A. Ostrakhovitch, E.V. Mikhal'chik, L.G. Korkina, Luminescence, 16 (2001) 305.
16. L. Greenlee, I. Fridovich, P. Handler, Biochemi., 1 (1962) 779.
17. R. C. Allen, Methods Enzymol., 133 (1986) 449.
18. K. Faulkner, I. Fridovich, Arch. Biochme. Biophys., 337 (1997) 115.
19. C. Zhang, H. Qi, Anal. Sci., 18 (2002) 819.
20. M. Yamada, Microchem. J. 58 (1998) 105.
21. G.N. Chen, L. Zhang, R.E. Lin, Z.C. Yang, J.P. Duan, H.Q. Chen, D.B. Hibbert, Talanta, 50 (2000) 1275.
22. S. Kulmala, T. Ala-Kleme, A. Kulmala, D. Papkovsky, K. Loikas, Anal. Chem., 70 (1998) 1112.
23. S. Kulmala, A. Kulmala, M. Helin, I. Hyppänen, Anal. Chim. Acta, 359 (1998) 71.
24. S. Kulmala, M. Helin, T. Ala-Kleme, L. Väre, Anal. Chim. Acta, 386 (1999) 1.
25. S. Kulmala, T. Ala-Kleme, L. Heikkila. L. Väre, J. Chem. Soc. Faraday Trans., 93 (1997) 3107.
26. T. Ala-Kleme, S. Kulmala, M. Latva, Acta. Chem. Sand., 51 (1997) 541.

27. N. Koshida, T. Ozaki, X. Sheng, H. Koyama, Jpn. J. Appl. Phys., 34 (1995) 705.
28. G. van Gorkum, A. Hoeberechts, Philips Tech. Rev., 43 (1987) 49.
29. S. Borson, D. DiMaria, D. Fischetti, F. Pesavento, P. Solomon, D. Dong, J. Appl. Phys., 57 (1985) 1302.
30. E. Savoye, D. Anderson, J. Appl. Phys., 38 (1967) 3245.
31. Y. Pleskov, Z. Rotenberg, in: H. Gerischer, C. Tobias (Eds.), Advances in Electrochemistry and Electrochamical Engeering, p. 3-155. Wiley, New York, (1978).
32. E. Hart, M. Anbar, The Hydrated Electron, Wiley, New York, (1970).
33. S. Kulmala, K. Hapakka, J. Alloys Compd., 225 (1995) 502.
34. T. Ala-Kleme, S. Kulmala, L. Vare, P. Juhala, M. Helin, Anal. Chem., 71 (1999) 5538.
35. S. Kulmala, M. Hakansson, A.-M. Spehar, A. Nyman, J. Kankare, K. Loikas, T. Ala-Kleme, J. Eskola, Anal. Chim. Acta, 458 (2002) 271.
36. J. Kankare, K. Fälden, S. Kulmala, K. Haapakka, Anal. Chim. Acta, 256 (1992) 17.
37. T. Ala-Kleme, S. kulmala, M. Latva, Acta Chem. Scand., 51 (1997) 541.
38. J. Diggle, T. Downie, C. Goulding, Chem. Rev., 69 (1969) 365.
39. A. Despic, Modern Aspects of Electrochemistry. 20, p. 400-503 plenum Press, New York, (1989) (Chapter 6) and references therein.
40. G. Buxton, J. Phys. Chem. Ref. Data, 17 (1988) 513 and references cited therein.
41. Q. Jiang, M. Kotiranta, K. Langel, J. Suomi, M. Håkansson, A.-M. Spehar, T. Ala-Kleme, J. Eskola, S. Kulmala, Anal. Chim. Acta, 541 (2005) 177.
42. S. Kulmala, C. Matachescu, A. Kulmala, D. PapKovsky, M. Hakansson, H. Ketamo, P. Canty, Anal. Chim. Acta., 453 (2002) 253.
43. S. Kulmala, T. Ala-Kleme, H. Joels, A. Kulmala, J. Radioanal. Nucl. Chem., 232 (1998) 91.
44. D.J. DiMaria, E. Cartier. J. Appl. Phys. 78 (1995) 3883.
45. S. Kulmala, T. Ala-Kleme, A. Hakanen, K. Haapakka, J. Chem. Soc. Faraday Trans., 93 (1997) 165.
46. S. Kulmala, A. Kulmala. T. Ala-Kleme, J. Pihlaja, Anal. Chim. Acta, 367 (1998) 17.
47. P. Canty, L.Väre, M. Håkansson, A.-M. Spehar, D. Pakovsky. T. Ala-Kleme, J. Kankare, S. Kulmala, Anal. Chim. Acta, 453 (2002) 269.

48. R. Memming, J. Electrochem. Soc., 116 (1969) 785.

49. W. Koppenol, J. Butler, Adv. Free Rad. Biol. Med., 1 (1985) 91.

50. D. Standury, Adv. Inorg. Chem. Rad. Chem., 33 (1989) 69.

51. P. Neta, R. Huie, A. Ross, J. Phys. Chem. Ref. Data, 17 (1988) 1027 and the references cited therein.

52. C. Matachedscu, S. Kulmala, E. Laine, P. Raerinne, Anal. Chim. Acta, 349 (1997) 1.

53. R.W. Alder, M. Bonifacic, K.D. Asmus, Chem. Soc. Perkin Trans., II, (1986) 277. P. Wardmam, J. Phys. Chem. Ref. Data, 18 (1989) 1637.

54. C. Matachescu, S. Kulmala, T. Ala-Kleme, H. Joela, Anal.Chem., 69 (1997) 3385.

55. L. Faulkner, H. Tachikawa , A. J. Bard. J. Am. Chem. Soc., 94 (1972) 691.

56. S. Kulmala, T. Ala-Kleme, M. Latva, K. Loikas, H. Takalo, J. Fluor., 8 (1998) 59.

57. S. Kulmala, A. Kulmala, M. Helin, I. Hyppanen, Anal. Chim. Acta, 359 (1998) 71.

58. T. Werner, K. Fahnrich, C. Huber, O.S. Wolfeis, J. Photochem. Photoobiol., 70 (1999) 585.

59. C. Huber, K. Fahnrich, O.S. Wolfbeis, J. Photochem. Photoobiol. A, 128 (1999) 111.

Figures and Figure Captions

Fig.1

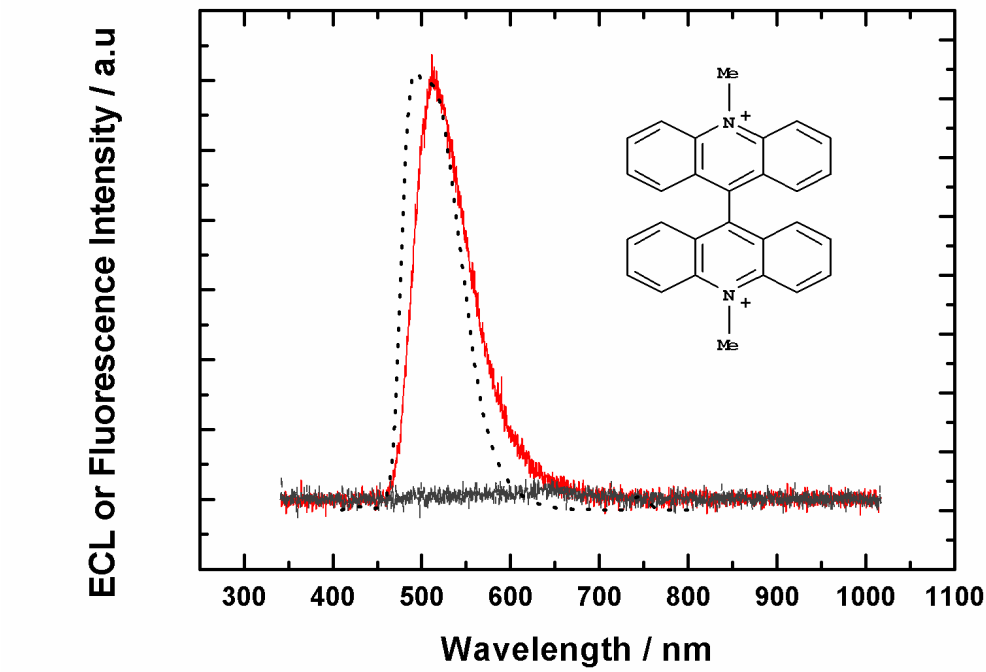

Fig.1. ECL spectrum and fluorescence emission spectrum of lucigenin. Conditions: fluorescence emission spectrum (dot line) was measured by Perkin-Elmer LS-50B spectrometer with scan speed 240 nm min^{-1}, excitation wavelength (λ_{ex}) 369 nm; ECL spectrum (solid line) was measured by Ocean Optics USBFL-2000 spectrometer integrated for 1000 ms; Solution for ECL spectrum was 1×10^{-3} M lucigenin in 3×10^{-3} M $K_2S_2O_8$, 0.05M $Na_2B_4O_7$ buffer at pH 7.8; Solution for fluorescence spectrum is 1×10^{-5} M lucigenin, 0.05M $Na_2B_4O_7$ buffer at pH 7.8. Coulostatic pulse generator: pulse voltage -45 V, pulse frequency 20 Hz, pulse charge 120 μC, aluminum strip cathode, platinum wire anode. Molecular structure of lucigenin is given in the inset.

Fig.2

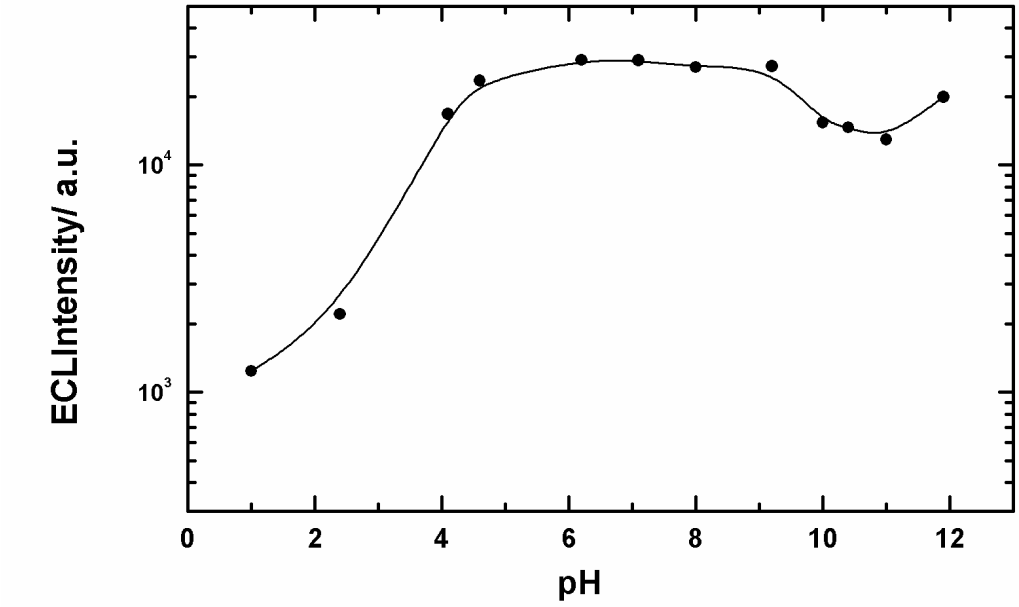

Fig.2. Effect of pH on the lucigenin ECL. Conditions: 1.0×10^{-5} M lucigenin in 0.1 M Na_2SO_4 supporting electrolyte solutions. Solutions were adjusted to the desired pH with sulfuric acid or sodium hydroxide; pulse voltage –50V, pulse frequency 80 Hz, pulse charge 100 μC. ECL was measured by Perkin-Elmer LS-5 spectrometer in Phosphorescence mode with excitation light path closed, scan speed 240 nm min^{-1}, delay time 0.1 ms, gate time 13 ms.

Fig.3

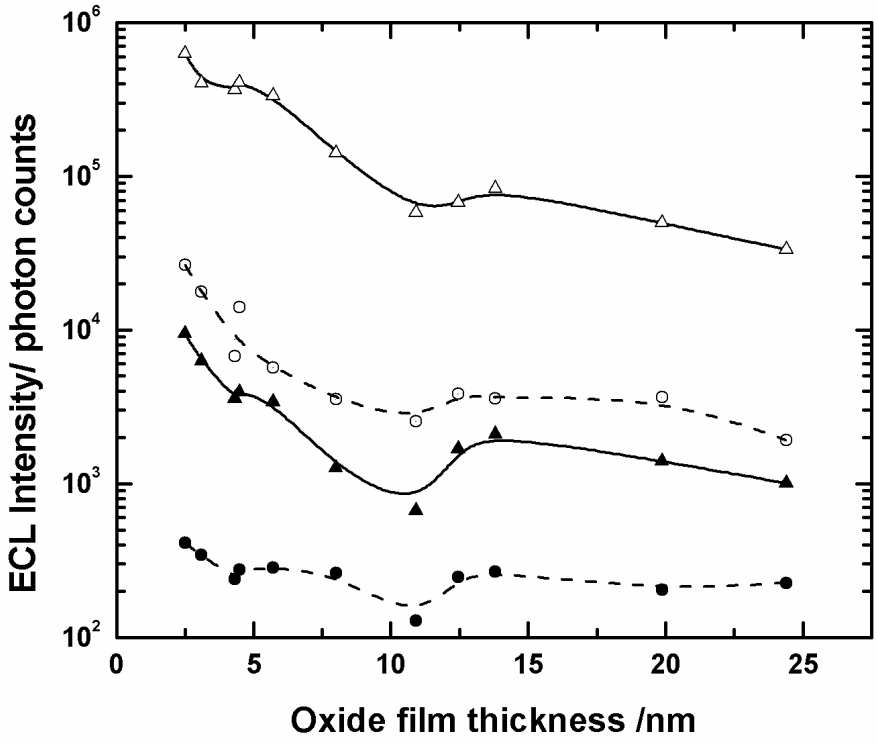

Fig.3. Effect of oxide film thickness on the ECL intensity of lucigenin (solid lines) and blank (dashed lines). (○, △) Cathodic ECL; (●, ▲) TR-ECL. Conditions: 1.0×10^{-6} M lucigenin in 0.05 M $Na_2B_4O_7$ buffer solution at pH 7.8. Aluminum electrodes with varied oxide thickness were used as disposable working electrodes. TR-ECL: delay time 5 μs, gate time 1.00 ms. ECL and TR-ECL intensities were integrated over 1000 excitation cycles. All signals were measured through a 500-nm interference filter with half-width of the transmission band ca. 10 nm. Pulse voltage – 45 V, pulse frequency 20 Hz, pulse charge 120 μC.

Fig.4

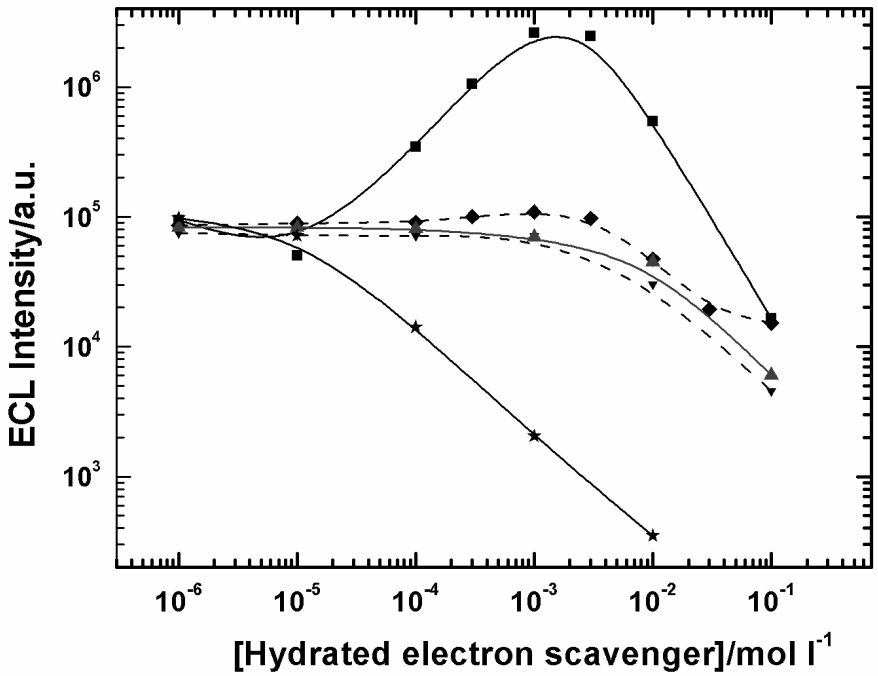

Fig.4. Effect of several hydrated electron scavengers on the ECL intensity of lucigenin (1×10^{-5} M). (▪) Cathodic ECL, $K_2S_2O_8$; (♦)Cathodic ECL, H_2O_2; (▼) Cathodic ECL, $NaNO_2$; (▲) Cathodic ECL, $NaNO_3$; (✯) Cathodic ECL,$Co(NH_3)_6^{3+}$ Conditions: measurements were made in 0.05 M $Na_2B_4O_7$ buffer solution at pH 7.8, otherwise the same as in Fig. 2.

Fig.5

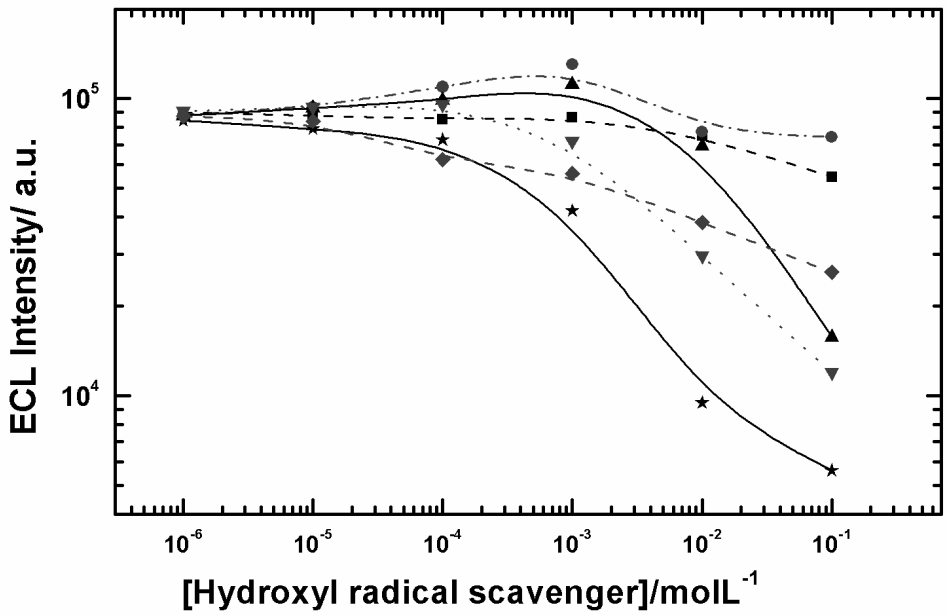

Fig.5. Effect of different hydroxyl radical scavengers on the ECL intensity of lucigenin (1×10^{-5}M). (●) NaBr, (▲) NaN_3, (▪) NaCl, (♦) ethanol, (▼) NaSCN, (✯)

NaI; Conditions: measurements were made in 0.05 M $Na_2B_4O_7$ solution at pH 7.8, otherwise the same as in Fig. 2.

Fig.6

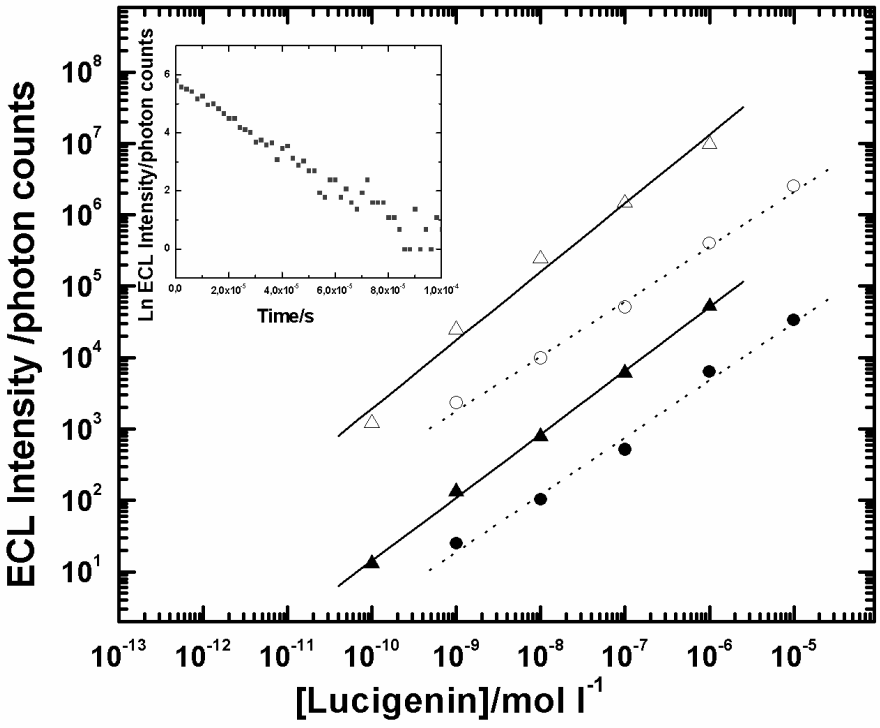

Fig.6. Calibration plot of lucigenin using disposable oxide-coverd aluminum electrodes. (△) ECL in the presence of 1 mM $K_2S_2O_8$, (▲) TR-ECL in the presence of 1 mM $K_2S_2O_8$ (○) ECL in the absence of $K_2S_2O_8$, (●) TR-ECL in the absence of $K_2S_2O_8$. Conditions: all measurements were made in 0.05M $Na_2B_4O_7$ buffer solutions at pH 9.2. Otherwise the same as in Fig. 3. ECL decay curve of lucigenin is shown in the inset.