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Deciphering electrochemiluminescence generation from luminol and hydrogen peroxide by imaging light emitting layer



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1. Introduction

Electrochemiluminescence (ECL) refers to the light emission from excited luminophores generated by electrochemical reactions at the electrode surface [1,2]. Benefiting from its unique advantages including excellent spatiotemporal controllability, low background, and high sensitivity, ECL has emerged as one of leading signal transduction techniques in chemical analysis, biosensing, and clinical diagnostics [3-6]. 5-Amino-2,3-dihydro-1,4-phthalazinedione, namely luminol, is one of water-soluble luminophores in ECL and chemiluminescence which have been extensively used in immunoassays [7–10]. In general, luminol generates ECL at a relatively low potential in alkaline solutions in the presence of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), superoxide radical anion (O₂^{•-}), and hydroxyl radical (OH[•]) [11–13]. When generating the anodic ECL, luminol is first oxidized electrochemically and subsequently reacts with ROS to form excited state species, namely 3-aminophthalate, which eventually emits light at 425 nm [14]. While the generation of cathodic ECL from luminol strongly depends on dissolved oxygen [15], which can be significantly promoted by the electrode modification [10,16].

Although the research on ECL generation from luminol has been carried out for a long time since it was first reported in 1929 by Harvey [17], the reaction mechanism is a long-standing research topic and still

ABSTRACT

Electrochemiluminescence (ECL) of luminol is a luminescence process that proceeds in the presence of reactive oxygen species (e.g. hydrogen peroxide (H_2O_2)) at a suitable electrode potential, the reaction mechanism of which is complicated and remains ambiguous. In this work, we report a visualization approach for measuring the thickness of the ECL layer (TEL) of the luminol/H₂O₂ system to decipher the reaction process by combined use of the microtube electrode, ECL microscopy, and finite element simulations. With the increase of solution pH, the ECL image captured with the microtube electrode tends to vary from spot to ring, corresponding to the decrease of TEL from >9.1 μ m to ca. 4.3 μ m. We propose that different intermediates are involved in the course of ECL reaction. At a low pH (e.g. pH < 9), a relatively large TEL is most likely determined by the diffusion of oxidized and deprotonated luminol intermediate that is neutral and has a long lifetime. While at a high pH (e.g. pH in the range of 10 to 12), the ECL reaction is controlled by short-lived radical intermediates of both luminol and superoxide anion. The proposed mechanism is proved theoretically by finite element simulations and experimentally by the apparent effect of concentration ratio of luminol/H₂O₂.

remains ambiguous. For example, different mechanisms have been proposed for various electrode materials [18], including gold [19,20], platinum [21], indium tin oxide glass [22], and carbon-based electrodes [14,23-25]. Moreover, a multiplicity of mechanisms exist in terms of experimental conditions, such as the applied potential [19], and solution pH [26]. Current methodologies used for studying ECL mechanisms of luminol are usually based on the conventional cyclic voltammetry and ECL intensity-potential measurements [27–32]. The influence of various factors, including the potential scan direction and range, solution pH, concentration of luminol, electrocatalytic property and redox reactivity of nanoparticles, has been addressed [27]. For example, possible mechanisms at different potentials have been proposed using the boron-doped diamond electrode, at which H_2O_2 can be in situ produced from carbonate. In this case, ECL generation from luminol depends on the applied potential, carbonate concentration, and solution pH [24].

On the other hand, ECL microscopy (ECLM) has recently become a powerful tool with high throughput and high surface sensitivity for imaging analysis of latent fingerprints [33], micro-/nano-structures [34–38], and live cells [39–43]. Benefiting from its ability to visualize the electrode reaction, ECLM has also manifested itself in deciphering reaction mechanisms [44–46]. For instance, ECL reactivity at the single-particle level can be resolved by ECLM to clarify the socalled low oxidation potential mechanism in bead-based immunoassays

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Scheme 1. Schematic illustration of measuring the TEL of luminol/ H_2O_2 system by combined use of MTE and ECLM. L = deprotonated luminol. WE = working electrode, RE = reference electrode, CE = counter electrode. EMCCD = electron multiplying charge coupled device.

[45]. In terms of ECL images of microbeads functionalized by tris(2,2'bipyridyl)ruthenium (Ru(bpy)₃²⁺), it is clarified that the lifetime of coreactant (namely tri-*n*-propylamine, TPrA) radical cation determines essentially the size of microbeads used [45,47]. Recently, the oxidativereduction route and catalytic route of Ru(byy)₃²⁺/TPrA system have also been rationalized by the combined use of ECLM and microtube electrode ensemble (MTE) [46]. The ECL pattern captured for an MTE phenomenologically varies from ring- to spot-shape when increasing the concentration of Ru(bpy)₃²⁺, suggesting the remarkable increase of thickness of ECL layer (TEL) and the significant contribution of catalytic route. It also indicates that the variation of TEL revealed by ECLM can provide insightful information on the reaction mechanism.

In this work, we intend to clarify the ECL reaction mechanism of luminol/H₂O₂ system using ECLM and MTE. As shown in Scheme 1, an MTE consisting of gold microtubes with uniform size is placed under an upright microscope, allowing the acquisition of ECL image from the top side. Note that the ECL reaction mainly occurs at the inner surface of a microtube electrode. Spot- and ring-shaped ECL patterns were observed at pH < 9 and 10-12, respectively, corresponding to a TEL of >9.1 μ m and ~4.3 μ m. We propose that the reaction is likely controlled by oxidized and deprotonated luminol with a long lifetime and by short-lived radical intermediate species, respectively, at low and high pH. The mechanistic hypothesis is also corroborated theoretically by finite element simulations and experimentally by the effect of concentration ratio of luminol/H₂O₂.

2. Material and methods

2.1. Chemicals and materials

All chemicals and reagents were of analytical grade and used as received without further purification. Tin(II) chloride (SnCl₂, 97.5%) was bought from J&K Scientific Ltd. Formaldehyde (HCHO, 37–40%), sodium sulfite (Na₂SO₃), hydrochloric acid (HCl, 36–38%), sodium hydroxide (NaOH), dichloromethane (CH₂Cl₂), methanol and hydrogen peroxide (H₂O₂, 30 wt%) were purchased from Sinopharm Chemical Reagent. 8-amino-5-chloro-7-phenylpyrido [3,4-d]pyridazine-1,4 (2H, 3H) dione sodium salt (LO12) was obtained from Wako chemical (USA). The commercial gold electroless plating solution containing Na₃Au(SO₃)₂ (0.25 M) was bought from Changzhou Institute of Chemical Research. Silver nitrate (AgNO₃, 99.8%), trifluoroacetic acid (CF₃COOH, 99.5%) and luminol (C₈H₇N₃O₂) were obtained from Aladdin. The concentrated ammonium hydroxide solution (NH₃·H₂O, 25 wt%) was obtained from Sigma-Aldrich. Potassium ferricyanide (K₃[Fe(CN)₆], 99.0%) and potassium ferrocyanide (K₄[Fe(CN)₆], 99.0%) were received from Wenzhou Chemical Material Factory. The track-etched polycarbonate (PC) membrane with pores of 20.2 μ m (pore density 4 × 10⁴ cm⁻²) in diameter was purchased from Wuwei Kejin Xinfa Technology. All aqueous solutions were prepared with ultrapure water (18.2 MΩ·cm).

2.2. Preparation of MTE

The MTE was fabricated by electroless deposition of gold using the track-etched PC membrane as the template [48,49]. Scheme S1 briefly describes the preparation strategy of MTE. After wetting in methanol for 2 h, the PC membrane was sensitized with Sn^{2+} in a methanol solution containing 0.026 $\rm M~SnCl_2$ and 0.07 $\rm M$ trifluoroacetic acid for 45 min. Followed by rinsing with copious amounts of methanol, the membrane was immersed in 0.029 M [Ag(NH₃)₂]NO₃ for 10 min, which was freshly prepared by a dropwise addition of concentrated NH₃·H₂O into AgNO₃ aqueous solution (0.029 M). Subsequently, the membrane was immersed in the gold plating bath of ~5 mL containing 0.127 M Na₂SO₃, 0.0079 M Na₃Au(SO₃)₂ and ~0.25 M HCHO. After being stored at 4 °C for 24 h, a thin film of gold was deposited on both the outer surface and channel walls of the PC membrane. The prepared membrane was then rinsed thoroughly with ultrapure water and dried under N₂ stream. Prior to use, the gold layer coated on the top surface of the PC membrane was removed by adhesive tape. Finally, the resulting PC membrane was fixed onto a glass slide to obtain the MTE.

2.3. Instrumentation and Measurements

The morphology and structure of the PC template membrane and MTE were characterized by scanning electron microscopy (SEM, SU8010, Hitachi) at 3 kV. Energy dispersive X-ray spectrometry (EDS) characterization was conducted on an X-ray energy spectrometer (X-max 80, OXFORD Instruments).

Electrochemical characterizations of MTE were carried out on a CHI 920C electrochemical workstation (CH Instrument). A traditional threeelectrode configuration was used, in which the MTE, platinum wire, and Ag/AgCl electrode (saturated with KCl) served as the working, counter and reference electrodes, respectively.

ECL measurements were also performed using the classic threeelectrode system. ECL intensity was measured on the MPI-E ECL analytical system (Remex Analysis Instrument) with the photomultiplier tube (PMT) biased at 400 V. ECL imaging was carried out on an upright microscope (Nikon, ECLIPSE LV100ND) equipped with a water immersion objective (Nikon, CFI Apo 40 ×, N.A. 0.8) and an electron multiplying charge-coupled device (EMCCD) camera (Andor, iXon Ultra 897). The ECL reactions were triggered and controlled by a double-step potential (initial potential 0 V, pulse potential +0.6 V unless otherwise specified, pulse period 2 s, pulse time 1 s) implemented by a CHI440D electrochemical workstation. The exposure time of EMCCD camera was 4 s.

3. Results and discussion

The track-etched polycarbonate (PC) membrane consisting of 20.2 μ m-in-diameter pores was used as the template to prepare the MTE with uniform gold microtubes (more details in Supporting Information, Scheme S1 and Figs. S1, S2). Fig. 1a shows the top-view scanning electron microscopy (SEM) image of MTE after removing the gold layer from the top surface. We can see clearly that ensemble of well-separated gold microtubes are formed in the PC membrane. The inner and outer diameters are ca. 18.1 and 20.5 μ m, respectively. The inset of Fig. 1a displays the element distribution of gold (green) and carbon (red) measured by energy dispersive X-ray spectrometry, confirming that gold element indeed only exists on the channel walls of the template membrane (more characterizations in Supporting Information, Fig. S3). As shown in Fig. 1b, after dissolving the PC membrane in dichloromethane, discrete and uniform gold microtubes were obtained, with microtube lengths comparable to the thickness of the pristine PC membrane.



Fig. 1. Structure characterization of microtube electrode. (a) Top-view SEM image of MTE. The inset shows the element mapping of gold (green) and carbon (red) using energy dispersive X-ray spectrometry. (b) SEM image of gold microtubes obtained after dissolving polycarbonate template. The scale bars are $20 \ \mu$ m.



Fig. 2. Measurement of ECL intensity at low and high pH. ECL intensitypotential curves (red) overlaid with CVs (black) obtained with MTE in 0.2 M phosphate solutions containing 1 mM luminol and 10 mM H_2O_2 at (a) pH 9 and (b) pH 12. The PMT was biased at 400 V.

deposition of gold was also proved by electrochemistry measurements (Fig. S6). Indeed, cyclic voltammetry (CV) in 0.05 M H_2SO_4 solution displays fingerprint oxidation and reduction peaks of the gold surface (Fig. S6a). Furthermore, using Fe(CN)₆³⁻/Fe(CN)₆⁴⁻ as the redox probe, a current wave at ca. 0.3 V was also observed in CV (Fig. S6b).

The MTE was first used as the working electrode to determine the onset potential of ECL generation by luminol and H_2O_2 , as well as their respective oxidation potentials. Figs. 2 and S7 exhibit ECL intensity-potential curves overlaid with CVs in solutions of different pH containing 1 mM luminol and 10 mM H_2O_2 . Apparently, the onset potential of ECL generation shifts negatively with the increase of solution pH. Note that the light signal detected before the onset potential at a high pH can be ascribed to the chemiluminescence generated by luminol in the presence of H_2O_2 [50]. Moreover, as shown and compared in Fig. S8 and Table S1, the oxidation potential of H_2O_2 is lower than that of luminol and the onset potential of ECL generation is always higher than that of luminol at the electrode surface is necessary to produce ECL.

By combined use of MTE and ECLM, the TEL of the luminol/ H_2O_2 system can be measured, with which we intend to rationalize the reaction mechanism. Figs. S9, S10 display ECL images captured with MTE at pH of 9 and 12, respectively, with the applied potential varied from +0.4 V to +1.0 V. It can be seen that, if the solution pH is fixed (either 9 or 12), the pattern of ECL images at different potentials is almost the same (either spot or ring), although the brightness of ECL spot or ring (namely ECL intensity) is different. While increasing the solution pH, the pattern tends to change from spot to ring, indicative of the decrease of TEL at the microtube electrode surface. On the other hand, at these two pH, clear ECL images of MTE were only observed at a potential more positive than +0.5 V. Therefore, in the following studies, a double-step potential with a pulse potential of +0.6 V was applied to stimulate the ECL reaction (more experimental details in Supporting Information).

ECL images shown in Fig. 3a-f are captured with MTE in solutions at different pH between 8 to 12. With the increase of solution pH, the pattern of ECL images varies from spot to ring. The normalized ECL intensity profiles along the radial direction of MTE at different pH are exhibited in Fig. 3g,h for comparison. We can see that the ECL intensity reaches the maximum at the inner wall of MTE and then decreases grad-

ually away from the microtube electrode surface. Moreover, the ECL intensity at the microtube center in the solution with pH < 9 is apparently higher than that in the solution with pH at 10-12, indicating that a relatively thick ECL layer is formed in solutions with lower pH. If assuming the distance from the microtube electrode surface to the position where the light intensity is zero as the TEL, it is estimated to be about 4.3 μ m in solutions with pH between 10 to 12 in terms of the ECL intensity profile along the radial direction of MTE. While in solutions with pH < 9, the TEL is at least the half of diameter of MTE, namely >9.1 μ m. The significant difference in TEL indicates the existence of two distinct reaction pathways in solutions with low and high pH.

Based on ECLM results and literature reported previously [14,51], we propose that a large TEL at pH < 9 is controlled by the diffusion of long-lived species generated at the electrode surface. While at a high pH of 10-12, the reaction pathway involving short-lived radical intermediates is prevailing, leading to surface-confined ECL. As shown in Fig. 4, these two pathways are designated as Route I and Route II, respectively. Basically, when a potential of +0.6 V is applied to the electrode, both luminol and H_2O_2 can be oxidized to form luminol radical (LH[•]) and superoxide radical anion (O2'-). At a high pH, LH' is deprotonated to form luminol radical anion (L'-) that further reacts with O2'-, yielding the peroxide compound (LO $_2^{2-}$). Followed by the decomposition of LO_2^{2-} into the excited state, namely 3-aminophthalate dianion (AP^{2-*}), the light emission at a wavelength of 425 nm is obtained. Note that the radical intermediates including $\mathrm{O_2}^{{\boldsymbol{\cdot}}{\mathsf{-}}}$ and $\mathrm{L}^{{\boldsymbol{\cdot}}{\mathsf{-}}}$ are all short-lived (e.g. a half-life of ~40 ms (τ) for O₂^{•-}) [50], resulting in the confinement of ECL generation at the electrode surface by Route II and thus yielding a ringshaped ECL pattern. In this case, the diffusion distance of these radical intermediates is further estimated to be ca. 6 μm according to $(2D\tau)^{1/2}$ with a diffusion coefficient (D) of 5×10^{-6} cm²/s. In contrast, at a low pH, LH' formed at the electrode surface undergoes preferentially the disproportionation reaction to generate the neutral 3-aminophthalazo compound (L) ($k = 1.8 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) [51], which can subsequently react with hydrogen peroxide anion (HO_2^{-}) to yield LO_2^{2-} according to Route I. Because of the long lifetime of L, Route I is expected to extend remarkably the concentration profile of $\mathrm{LO}_2{}^{2-}$ at the electrode surface, so that the ECL pattern tends to appear as spot. The variation of the ECL pattern from spot to ring shown in Fig. 3 indeed confirms the transition of the reaction pathway, likely from Route I to Route II, with the increase of solution pH.

To further prove that the variation of TEL is associated with two different reaction routes exemplified in Fig. 4, finite element simulations using COMSOL were conducted. More calculation details can be found in Supporting Information (Figs. S12,S13, Table S2 and related description). Fig. 5a,b shows the top-view of calculated patterns for ECL generated at the surface of a single microtube electrode. The corresponding side-view distribution of ECL intensity is displayed in Fig. S14. Clearly, the ECL intensity reaches the maximum at the surface of the microtube electrode and gradually decreases towards the tube center. As seen from ECL profiles along the radial direction of MTE (Fig. 5c), ECL generation by Route II at a high pH (open circles) indeed makes the light-emitting layer more surface-confined than that at a low pH (solid dots). The TEL derived from the simulations are >9.1 μ m and ca. 4.1 μ m, respectively, for low and high pH, which are well consistent with experimental results (Fig. 3).

As mentioned above, a large TEL in solutions of a low pH can be attributed to the diffusion of long-lived species, L, generated at the electrode surface. As shown in Fig. 4, L can further react with HO₂⁻ to eventually generate the excited state species. In principle, at a high concentration of H₂O₂, L will be consumed considerably by HO₂⁻ at a high rate ($k = 5.0 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$) [51], resulting in the shrinkage of the concentration profile of L and thus a thin ECL layer at the electrode surface. Fig. 6a-c shows ECL images captured with MTE at pH 9 by adding different concentrations of H₂O₂, namely 0.1 mM, 1 mM and 10 mM, in solutions whereas keeping that of luminol the same. Although a blurry image with a weak ECL is observed at a very low concentration of H₂O₂ (0.1 mM), it is clear that the ECL layer extends when decreasing the



Fig. 3. Variation of TEL with the solution pH. (a-f) ECL images obtained with MTE in 0.2 M phosphate solution containing 1 mM luminol and 10 mM H_2O_2 at different pH. The scale bar is 20 μ m. A double-step potential (initial potential 0 V, pulse potential +0.6 V, pulse period 2 s, pulse time 1 s) was applied to stimulate the ECL reaction. The exposure time of EMCCD was 4 s. (g, h) ECL intensity profiles along the radial direction of two microtubes at low (g) and high (h) pH.



Fig. 4. Mechanistic pathways of ECL generation by luminol/H₂O₂ at low (Route I) and high (Route II) pH.

concentration of H_2O_2 from 10 mM to 1 mM (Fig. 6b,c). This trend can be identified more clearly in the ECL intensity profiles plotted in Fig. 6g. The results thus corroborate the assumption that a high concentration of H_2O_2 will result in the significant consumption of L and thus a small TEL. For comparison, similar experiments were also performed at pH 12. However, in this case, the ECL patterns appear ring-shaped (Fig. 6df) and all ECL intensity profiles almost overlap at different concentrations of H_2O_2 (Fig. 6h), indicating that the prevailing reaction pathway involves short-lived radicals (O_2 ⁻⁻ and L⁻⁻). It should be noted that finite element simulations (Fig. S15) also prove the above mechanistic hypotheses.

In order to examine whether the reaction mechanism shown in Fig. 4 is a general scheme for luminol analogues, a similar study was also performed with 8-amino-5-chloro-7-phenylpyrido [3,4-d]pyridazine-

1,4 (2H, 3H) dione. This compound is a luminol analogue usually called L012. L012 is known to emit much stronger luminescence than luminol in neutral solutions (results shown in Fig. S16), so that it is widely used in bioanalysis [39,41,52]. As shown in Fig. S17, ECL images captured for the L012/H₂O₂ system remain to be ring-shaped with the increase of solution pH. Note that a slightly changed TEL and more surface-confined ECL patterns were observed in comparison with luminol in the pH range of 7.5 to 13, which might be related to the difference in pK_a of luminol and L012 radicals. Furthermore, ECL images were also captured at pH 8 (Fig. S18a-c) and 12 (Fig. S18d-f) upon decreasing the concentration of H₂O₂. Similar to that observed for luminol, a low concentration of H₂O₂ results in a large TEL at pH 8 (Fig. S18g), whereas the TEL is almost the same at pH 12 and independent of the concentration of H₂O₂ (Fig. S18h).



Fig. 5. Simulated ECL intensity distribution at low and high pH. (a, b) Topview ECL patterns calculated for a single microtube electrode at low (a) and high (b) pH. (c) Calculated ECL intensity profiles along the radial direction of microtube at low (solid dots) and high (open circles) pH.

4. Conclusion

In summary, ECL imaging in conjunction with using MTE provides us a visualization method for measuring the TEL of luminol/ H_2O_2 , with which possible reaction mechanisms are proposed. As revealed by an upright ECLM, ECL generation at the microtube electrode surface gives rise to a spot pattern at low pH, which turns to a ring upon increasing the solution pH. The variation of ECL pattern figures out a remarkable decrease of TEL from >9.1 μ m to ca. 4.3 μ m. We propose that, at low pH,

the reaction pathway involving neutral intermediate species, L, with a long lifetime dominates. While at high pH, the prevailing route is most likely controlled by short-lived radical intermediates including O2 - and L⁻⁻. Finite element simulations well corroborate the above mechanistic hypotheses from the theoretical viewpoint. Experimentally, considering the limiting intermediate, L, can be considerably consumed at the microtube electrode surface via homogenous reaction with HO2⁻, the ECL layer at a low pH becomes more surface-confined when increasing the concentration of H₂O₂, thus proving the dominating role of L. Contrary to the case of high pH, the TEL is independent of the concentration of H_2O_2 and remains unchanged. With these results, we believe that the measurement of TEL by combined use of ECLM and MTE holds promise for deciphering ECL generation by different reaction systems. Last but not least, the measurement of TEL cannot only provide insightful mechanistic information but also offer a facile method of screening efficient bioanalytical and immunoassay systems [45,47].

Declaration of Competing Interest

The authors declare that they have no conflicts of interest in this work.

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Fig. 6. TEL affected by the concentration ratio of luminol/H_2O_2. (a-f) ECL images captured with MTE in 0.2 M phosphate solution containing 1 mM luminol and different concentrations of H_2O_2 (a, d: 0.1 mM; b, e: 1 mM; c, f: 10 mM) at pH 9 (a-c) and 12 (d-f). The scale bar is 20 μ m. A double-step potential (initial potential 0 V, pulse potential +0.6 V, pulse period 2 s, pulse time 1 s) was applied to initialize the ECL reaction. The exposure time of EMCCD was 4 s. (g, h) The variation of ECL intensity along the radial direction of MTE at pH 9 (g) and 12 (h) with different concentrations of H_2O_2 (blue: 0.1 mM; black: 1 mM; red: 10 mM).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fmre.2021.11.018.

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