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Original Article

The analytical determination and electrochemiluminescence behavior of amoxicillin



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ABSTRACT

A novel electrochemiluminescence (ECL) luminophor of amoxicillin was studied and found to generate ECL following the oxidation or reduction of amoxicillin. The amoxicillin oxidation state was also found to eliminate the reduction state, generating ECL. When solutions of amoxicillin were scanned between +1.5 V and –1.0 V with a graphite electrode in the presence of cetyltrimethyl ammonium bromide using KCl as the supporting electrolyte, ECL emissions were observed at potentials of –0.7 V and +0.5 V. The ECL intensity at –0.7 V was enhanced by H₂O₂. Based on these findings, an ECL method for the determination of the amoxicillin concentration is proposed. The ECL intensities were linear with amoxicillin concentrations in the range of 1.8×10^{-8} g/mL to 2.5×10^{-7} g/mL, and the limit of detection (signal/noise = 3) was 5×10^{-9} g/mL. The fluorescence of amoxicillin had the greatest emission intensity in a neutral medium, with the emission wavelength dependent on the excitation wavelength. The experiments on the ECL mechanism for amoxicillin found that the electrochemical oxidation products of dissolved oxygen and active oxygen species contributed to the ECL process. The data also suggest that the hydroxyl group of amoxicillin contributed to its ECL emission.

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1. Introduction

Amoxicillin (AM), a β -lactam antibiotic, is used to treat infections caused by certain bacteria, such as pneumonia, bronchitis, venereal disease, and ear, lung, nose, urinary tract, and skin infections. It may also be used before surgery or

dental work to prevent infection [1,2]. The structure of AM is shown in Figure 1. The analytical methods used for the detection of AM in biological fluids are mainly based on high-performance liquid chromatography [3,4], spectrophotometry [5,6], capillary electrophoresis [7], fluorometry [8], and chemiluminescence [9–14].

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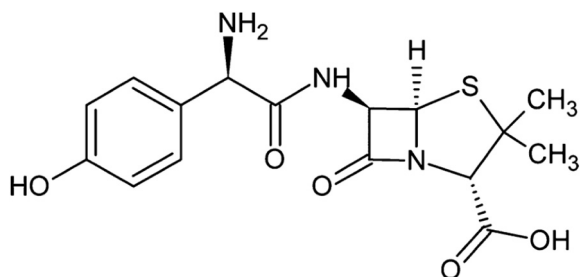


Fig. 1 – Molecular structure of amoxicillin.

Electrochemiluminescence (ECL) has emerged as a useful analytical technique in which luminescence is produced when an analyte is in the vicinity of an electrode surface with an applied potential [15]. Two commonly used ECL systems are the Ru(2,2-bipyridine) $_3^{2+}$ system and the luminol system. Both of these systems are usually applied in the positive potential region. However, the luminol system is known to suffer from interference by metal ions. The Ru(2,2-bipyridine) $_3^{2+}$ system is commonly operated at high potential (at or near +1.3 V), thus limiting its applicability. The ECL determination of β -lactamases employs the Ru(2,2-bipyridine) $_3^{2+}$ system [16], a system requiring the relatively expensive reagent Ru(2,2-bipyridine) $_3^{2+}$. Therefore, a new ECL system that does not employ this expensive reagent ECL would be of significant value. However, to date, only indole, tryptophan, and quercetin, which exhibit a different ECL behavior from luminol and Ru(2,2-bipyridine) $_3^{2+}$, have been reported [17,18]. A new ECL luminophor activated at a negative potential would represent a novel approach. To our knowledge, some quantum dots [19,20] and the Ru(2,2-bipyridine) $_3^{2+}$ system used in the presence of peroxysulfate can produce ECL emissions at negative potential [21]. This paper aims to use a fluorescent material that can be either oxidized or reduced electrochemically to generate ECL. Based on the ECL phenomena of the fluorescent material, we hope to develop a new ECL analytical method that widens the range of applications of ECL. The work reported in this paper demonstrates that electrochemical oxidation and reduction of amoxicillin generated ECL at a graphite electrode using a triangular pulse with KCl as the supporting electrolyte. The intensity of the ECL was enhanced by both H $_2$ O $_2$ and the surfactant hexadecyl trimethyl ammonium bromide. In addition, the oxidation state of amoxicillin eliminated its reduction state and generated ECL at a negative potential.

2. Methods

2.1. Reagents

All reagents were of analytical grade. Double-distilled water was used throughout the experiments. Amoxicillin was biochemical-reagent grade and was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (88%, Beijing, China). A stock solution of amoxicillin (1.00 \times 10 $^{-3}$ g/mL) was prepared by dissolving 0.1136 g of amoxicillin in 10 mL of 0.10M hydrochloric acid and

then diluting the resulting solution with water to a final volume of 100 mL. After the stock solution of amoxicillin had been stored for 1 week, the pH was adjusted to 7.0 using 0.1M NaOH. Standard working solutions were prepared daily by appropriate dilution of the stock solution with water. Additionally, 0.10M H $_2$ O $_2$ and 1.25mM hexadecyl trimethyl ammonium bromide (CTAB) solutions were prepared as required. Amoxicillin capsules (Harbin General Pharmaceutical Factory, China) were purchased from a local hospital. Urine samples were collected from three healthy individuals from the Hospital of Shaanxi Normal University in Xi'an.

2.2. Apparatus

Cyclic voltammograms and the ECL signals produced in the electrolytic cell were detected and recorded using an MPI-Electrogenerated Chemiluminescence Analyzer (Xi'an Remax Analyse Instrument Co. Ltd., Xi'an China). The ECL measurements were performed using a three-electrode ECL system that included a graphite electrode as the working electrode (ϕ = 4.0 mm), a platinum wire as the counter electrode, and an Ag/AgCl (Sat. KCl) electrode as the reference electrode. The ECL cell was placed directly in front of a photomultiplier tube (PMT) window and was enclosed in a light-tight box. The PMT was operated at -900 V, and the window was only opened towards the working electrode to eliminate background ECL from the counter-electrode. The working electrode was fixed on a Teflon cover to ensure reproducibility of the electrode position. All of the potentials were measured and reported relative to the reference electrode. UV-visible absorption spectra were measured with a TU-1901 Spectrophotometer (Beijing Currency Instrumental Co. Ltd., Beijing, China). The fluorescence spectra were measured with a Cary Eclipse Fluorescence Spectrometer (Varian, Palo Alto, CA, USA). The ECL spectra were measured using a Cary Eclipse Fluorescence Spectrometer by shielding the excitation optical window during constant potential scanning of the 2.5 \times 10 $^{-4}$ g/mL AM/0.25M KCl/1.25mM CTAB/0.25M H $_2$ O $_2$ at -0.7 V at the graphite electrode.

2.3. Procedure for ECL profiles and cyclic voltammograms

Each electrolytic cell contained a mixture of 1.0 mL of 1.0M KCl, 1.0 mL of 5.0 \times 10 $^{-3}$ M CTAB, 1.0 mL of different concentrations of AM solution, and 1.0 mL of 1.0M H $_2$ O $_2$. The ECL cell was placed in front of the PMT. Both ECL profiles versus potentials and the corresponding cyclic voltammograms were recorded simultaneously as the potential of the working electrode was scanned between +1.5 V and -1.0 V at a scan rate of 100 mV/s. When a potential of -0.7 V was applied at the graphite electrode, the ECL kinetic profiles were recorded. The ECL spectrum was measured using a Cary Eclipse fluorescence spectrometer while shielding the excitation optical window during the constant potential scanning of the 2.5 \times 10 $^{-4}$ g/mL AM/0.25M KCl/1.25mM CTAB/0.25M H $_2$ O $_2$ at -0.7 V at the graphite electrode.

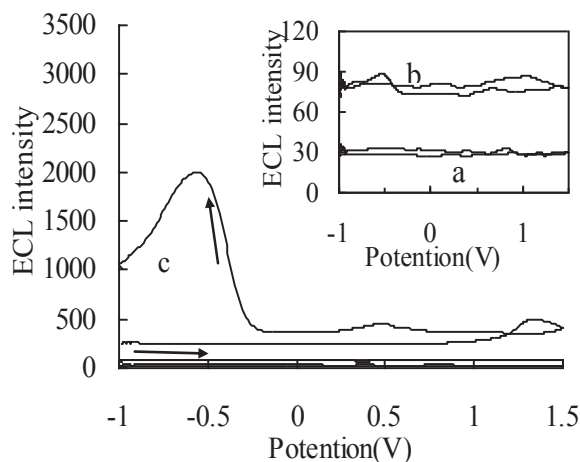


Fig. 2 – Electrochemiluminescence potential curves of (a) 0.25M KCl/1.25mM cetyltrimethyl ammonium bromide solution; (b) 0.25M KCl/1.25mM cetyltrimethyl ammonium bromide solution + 0.25M H₂O₂; (c) 0.25M KCl/1.25mM cetyltrimethyl ammonium bromide solution + 0.25M H₂O₂ + 1.0×10^{-5} g/mL amoxicillin

3. Results and discussion

3.1. ECL characteristics of AM

When the potential was scanned between +1.5 V and –1.0 V at the graphite electrode in a solution of 0.25M KCl/1.25mM CTAB, no ECL signal was detected, as shown in Figure 2 curve (a). In addition, no ECL signal was detected upon addition of 0.25M H₂O₂ to the solution of 0.25M KCl/1.25mM CTAB, as shown in Figure 2 curve (b). In contrast, when amoxicillin was added, a weak ECL signal and a strong ECL signal were detected at approximately +0.5 V and –0.7 V, respectively, as shown in Figure 2 curve (c).

3.2. ECL mechanism of AM

3.2.1. The electrochemical behavior of AM

The ECL behavior of AM at the electrode was associated with the electrochemical oxidation-reduction process. Therefore, the electrochemical behavior of different concentrations of AM in a 0.25M KCl/1.25mM CTAB solution was investigated. The potential scans from 0 V → –1.0 V → +1.0 V → 0 V were found to contain a cathode current peak at approximately –0.7 V and a weak anode current peak at +0.5 V, as shown in Figure 3A. Moreover, the greater the concentration of AM is, the higher is intensity of the peak current. Based on these results, we conclude that AM is reduced at –0.7 V and is oxidized at +0.5 V. The ECL-potentials of 2.5×10^{-4} g/mL AM/0.25M KCl/1.25mM CTAB solutions were also recorded between +1.0 V and –1.0 V. The scans contained two ECL peaks at –0.7 V and +0.5 V, as shown in Figure 3(B). The ECL peaks of AM at –0.7 V and +0.5 V coincided with the electrochemical peaks, suggesting that the ECL peaks at –0.7 V and +0.5 V resulted from the electrochemical reduction of AM at –0.7 V and the electrochemical oxidation of AM at +0.5 V.

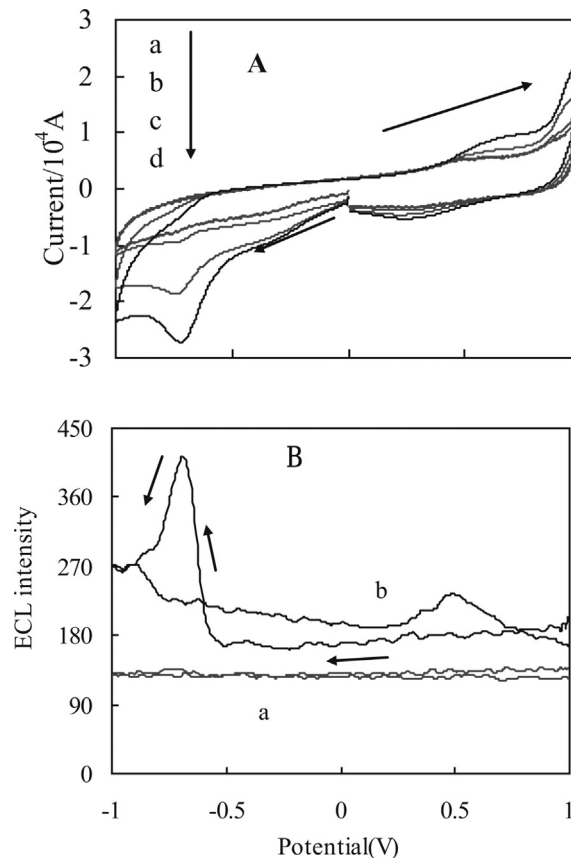


Fig. 3 – (A) Cyclic voltammograms (scan rate:100 mV/s) of different concentrations of amoxicillin in a 0.25M KCl/1.25mM cetyltrimethyl ammonium bromide solution (a: 1.25×10^{-4} g/mL; b: 2.5×10^{-4} g/mL; c: 7.5×10^{-4} g/mL; d: 2.0×10^{-3} g/mL); (B) electrochemiluminescence profiles in 2.5×10^{-4} g/mL amoxicillin/0.25M KCl/1.25mM cetyltrimethyl ammonium bromide solutions in the absence of H₂O₂ either deoxidized by nitrogen for 10 min (a) or not deoxidized by nitrogen (b) during potential scanning at a scan rate of 100 mV/s.

The only difference between AM and ampicillin is the presence of an OH group on the benzene ring in the former. However, when ampicillin solutions were scanned between +1.5 V and –1.5 V at the graphite electrode using KCl as the supporting electrolyte, ECL signals were not observed, even when H₂O₂ was added. These observations suggest that the OH group of AM plays an important role in the ECL mechanism following oxidation at +0.5 V during the anodic potential scan.

Additional phenolic antibiotics, namely, tetracycline and adriamycin, were also investigated. The results show that these two antibiotics do not generate ECL emissions at a graphite electrode between +1.5 V and –1.5 V potentials using KCl as the supporting electrolyte. These results demonstrate that not all phenolic antibiotics with hydroxyl groups generate ECL following electrochemical reduction and oxidation. They also indicate that the hydroxyl group was not the only group involved in the ECL process and that the hydrolyzed structure of AM, especially of an -S- group, may have also played an important role in the observed ECL.

3.2.2. Reactive oxygen free radical participation in the ECL of AM

After 2.5×10^{-4} g/mL AM/0.25M KCl/1.25mM CTAB solution was purged with nitrogen for 10 minutes, no ECL signal was detected when the potential was scanned between +1.0 V and -1.0 V, as shown by curve (a) in Figure 3B. This finding suggests that dissolved oxygen participates in the ECL process of AM; dissolved oxygen can be oxidized or reduced to produce reactive oxygen free radicals. To determine which oxygen free radicals were involved in ECL process, the following investigations were performed. Sulfoarea and hydroquinone, two scavengers of hydroxyl radicals ($\cdot\text{OH}$), were added into the solutions of 2.5×10^{-4} g/mL AM/0.25M KCl/1.25mM CTAB (the final concentration of sulfur urine or hydroquinone was 5.0×10^{-4} g/mL). Following the addition of the scavengers, the ECL occurring at -0.7 V during the potential scan from +1.5 V to -1.0 V decreased dramatically. Addition of ascorbic acid, a scavenger of superoxide anion radicals (O_2^-), also decreased the ECL intensity at -0.7 V. These two findings demonstrated that both hydroxyl radicals ($\cdot\text{OH}$) and superoxide anion radicals (O_2^-) participated in the ECL of AM at -0.7 V.

3.3. ECL character of AM at different potential scanning mode

3.3.1. The 0 V \rightarrow +2.0 V potential scanning process

The potential of a solution of 6.25×10^{-4} g/mL AM/0.25M KCl/1.25mM CTAB/0.25M H_2O_2 was scanned from 0 V to +2.0 V at a rate of 100 mV/s with a concomitant recording of the ECL signal. The results are shown in Figure 4A. No ECL signal was detected within the 20 seconds interval during the potential scan from 0 V to +2.0 V in Figure 4A. When the scan finished, we found that the ECL intensity slowly increased to approximately 1500 after 50 seconds. From these results, it can be deduced that AM can be oxidized and the resulting oxidized AM may emit luminescence by an ECL mechanism consistent with a slow luminescence process.

3.3.2. The 0 V \rightarrow -2.0 V potential scanning process

The potential of a solution of 6.25×10^{-4} g/mL AM/0.25M KCl/1.25mM CTAB/0.25M H_2O_2 was scanned from 0 V to -2.0 V at a

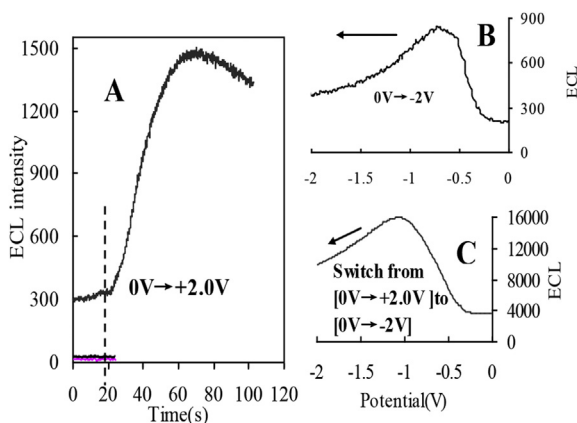


Fig. 4 – Electrochemiluminescence of the solution of 6.25×10^{-4} g/mL amoxicillin/0.25M KCl/1.25mM cetyltrimethyl ammonium bromide/0.25M H_2O_2 during different potential scan at a scan rate of 100 mV/s.

rate of 100 mV/s with a concomitant recording of the ECL signal. The results are shown in Figure 4B, where an ECL signal appeared at -0.5 V and reached a maximum intensity of approximately 800 at -0.7 V. From these results, it can be deduced that AM can be reduced and will produce an ECL emission as a result of the reduction.

3.3.3. Switch from [0 V \rightarrow +2.0 V] potential scanning process to [0 V \rightarrow -2.0 V] potential scanning process

The potential of a solution of 6.25×10^{-4} g/mL AM/0.25M KCl/1.25mM CTAB/0.25M H_2O_2 was scanned from 0 V to +2.0 V at a rate of 100 mV/s. The potential was then scanned from 0 V to -2.0 V. The ECL signal was recorded during the potential scan from 0 V to -2.0 V, and the result is shown in Figure 4C. It can be seen that ECL signals appeared at -0.5 V and a tremendous ECL peak appeared at -1.0 V, with the intensity of the peak approaching 16,000. When one compares the ECL-time curves covering the potential scan from 0 V to -2.0 V in Figure 4B and Figure 4C, it appears that different patterns strongly influenced the ECL of AM, with the intensity of the ECL in Figure 4C > 20 times higher than the ECL intensity in Figure 4B. Moreover, after AM was oxidized in the anode potential range (0 V \rightarrow +2.0 V), the potential peak of AM was shifted to -1.0 V during the potential scan from 0 V to -2.0 V.

In conclusion, AM was oxidized during the potential scan in the positive regions (0 V \rightarrow +2.0 V) while slowly emitting ECL. AM underwent reduction during the potential scan in the negative regions (0 V \rightarrow -2.0 V) and produced an ECL emission at -0.7 V. The oxidation state of AM can eliminate the reduction state of AM, generating immense ECL emission at -1.0 V.

3.3.4. The ECL spectrum of AM

First, the fluorescence emission spectra of AM in different media were investigated, as shown in Figure 5. As seen in Figure 5, the fluorescence (FL) intensities were the highest in the neutral medium and higher in the acidic medium than in the basic medium. These findings were consistent with the ECL data, which showed the highest intensities in the neutral medium and higher intensities in the acidic medium than in the basic medium. In the acidic medium, when the excitation wavelength was < 400 nm, the emission peaks at 486 nm or at 531 nm. When the excitation wavelength was > 400 nm, the emission peaks were shifted to between 494 nm and 501 nm. In the basic medium, when the excitation wavelength was at 360 nm, the emission peak was at 489 nm. When the excitation wavelength was in the range of 370 nm to 430 nm, the emission peak appeared at either 489 or 531 nm. When the excitation wavelength was > 430 nm, the emission peak was red shifted along with the excitation wavelength. The maximum emission wavelength at 570 nm when the excitation wavelength was either at 470 nm or at 480 nm.

In the neutral medium, the emission peak had a tendency to red shift when the excitation wavelength was red shifted. When the excitation wavelength was 360 nm, 370 nm, 380 nm, 390 nm, 400 nm, 410 nm, 420 nm, 430 nm, 440 nm, 450 nm, 460 nm, 470 nm, and 480 nm, the maximum emission peaks were found to be 478 nm, 478 nm, 478 nm, 481 nm, 478 nm, 478 nm, 488 nm, 504 nm, 507 nm, 513 nm, 521 nm, 525 nm, and

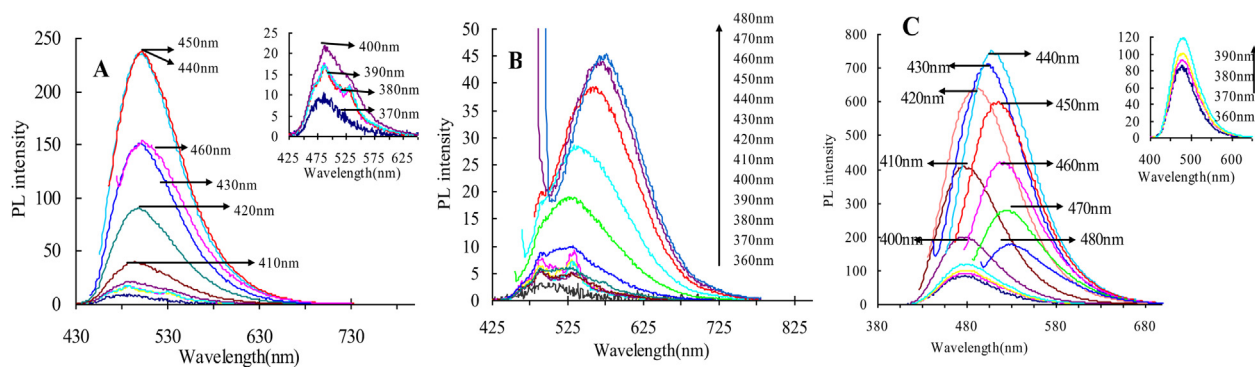


Fig. 5 – The fluorescence of amoxicillin placed for at least 1 week in: (A) an acid medium; (B) alkaline medium; and (C) neutral medium at different excitation wavelength.

528 nm, respectively. These results demonstrate that the FL of AM is complicated and significantly impacted by the medium in which it is measured. As a result of these complications, the only solid conclusion that can be drawn is that the FL intensity of AM is highest in the neutral medium.

The maximum emission peak of the FL of AM was at 303 nm when the AM was prepared freshly in the acidic medium, as shown in Figure 6A. When Figure 6A and Figure 5 are compared, it can be seen that the AM has hydrolyzed and that the hydrolysis product has a different FL emission profile. This phenomenon has been previously reported [16]. The ECL spectrum of the 6.25×10^{-4} g/mL AM/0.25M KCl/1.25mM CTAB/0.25M H₂O₂ system at -0.7 V was also investigated, as shown in Figure 6B. It can be seen that the maximum ECL peak was at 534 nm, with a second lower-intensity peak at approximately at 630 nm. Based on the emission spectral band of an excited state singlet oxygen [19,20], the emission peaks were found at approximately 460 nm, 520 nm, 575 nm, 630 nm, and 700 nm. Therefore, the emission peak at 630 nm in Figure 6B was attributed to the excited state singlet oxygen. The 534 nm emission band may result from the hydrolysis product of AM because the emission peak was near 528 nm peak observed in Figure 5C.

Based on the above discussion, we propose two types of excited state luminophors in the AM-CTAB/H₂O₂ ECL system. One is the excited state singlet oxygen, and the other is the hydrolysis product of AM. Moreover, according to references

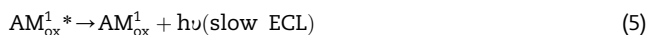
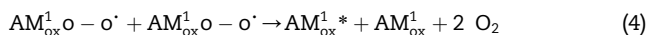
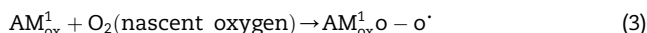
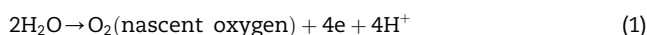
[19,21] and as outlined below, O₂ (dissolved oxygen) can accept different electrons, forming different radicals.

- O₂ + e → O₂^{-•};
- O₂ + 2e + 2H⁺ → H₂O₂ or O₂^{-•} + e + → 2H⁺ → H₂O₂;
- O₂ + 3e + 3H⁺ → H₂O + •OH or H₂O₂ + e + H⁺ → H₂O + •OH

Therefore, we propose the ECL mechanism of AM-CTAB/H₂O₂ system as follows:

AM → AM¹ hydrolysis

The 0 V → +2.0 V potential scanning process



The 0 V → -2.0 V potential scanning process

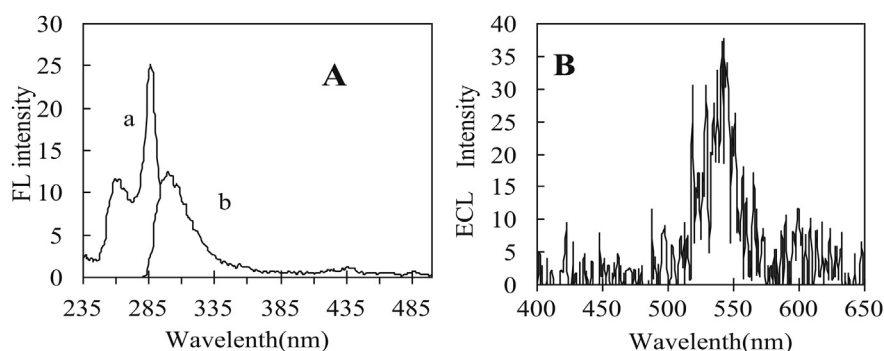
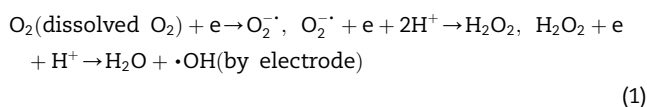
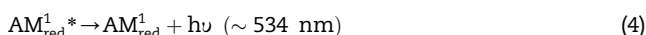
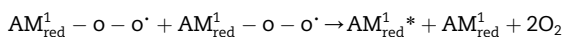
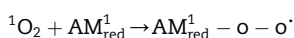
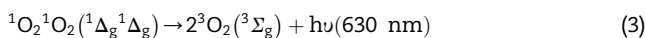
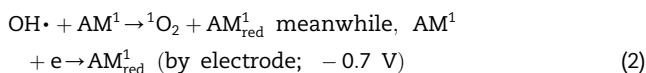


Fig. 6 – (A) The fluorescence freshly prepared amoxicillin and (B) electrochemiluminescence spectra of 6.25×10^{-4} g/mL amoxicillin/0.25M KCl/1.25mM cetyltrimethyl ammonium bromide/ 0.25M H₂O₂ system at -0.7 V.

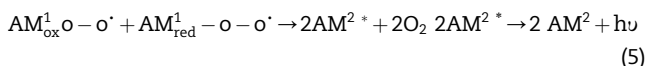
Table 1 – Analytical results of sample.

Samples	Claimed	Proposed method ^a	UV method
1	250	246	251
2	250	252	250
3	250	250	249

^a Average of five measurements.



After 0 V → +2.0 V potential scanning process following
0 V → -2.0 V potential scanning process



The proposed mechanism provides a pathway for the enhancing effect of H₂O₂ on ECL. In the absence of H₂O₂, the concentration of ·OH generated from dissolved oxygen was low, whereas in the presence of H₂O₂, the concentration of ·OH was greatly increased and significantly increased the ECL intensity of AM.

3.4. Optimization of the experimental conditions

3.4.1. Effect of medium

The effect of three different media on the ECL intensity in the presence of 2.0 × 10⁻⁶ g/mL AM/0.25M KCl/1.25mM CTAB was investigated. These experiments demonstrated that the ECL intensity was highest in the neutral medium and that the ECL intensity in the acidic medium was higher than the intensity in the basic medium. The reason for the different ECL intensities in the three different media is not clear. However, a similar pattern of FL intensity was observed, indicating that the ECL luminophores correlated with the FL luminophores. Based on these findings, the neutral medium was used in further experiments.

3.4.2. Effect of hydrogen peroxide concentration

The effect of hydrogen peroxide concentrations in the range of 0.04–0.36M on the ECL intensities in the presence of 2.0 × 10⁻⁶ g/mL AM/0.25M KCl/1.25mM CTAB was investigated at the graphite electrode when the potential was scanned from +1.5 V to -1.0 V. The ECL intensities increased as the concentrations of hydrogen peroxide increased, reaching a maximum value when 0.25M hydrogen peroxide was used. Higher concentrations of hydrogen peroxide decreased the ECL intensities.

3.4.3. Effect of CTAB concentration

The effect of CTAB concentrations in the range of 0.5–5.0mM on the ECL intensities in the presence of 2.0 × 10⁻⁶ g/mL AM/0.25M KCl/0.25M H₂O₂ was investigated at the graphite electrode when the potential was scanned from +1.5 V to -1.0 V. The ECL intensities increased with increasing concentrations of CTAB up to 1.25mM; above this concentration, the ECL intensities decreased.

3.5. Analytical performance

Under the selected experimental conditions, the ECL intensities were linear with amoxicillin concentrations in the range of 1.8 × 10⁻⁸ to 2.5 × 10⁻⁷ g/mL. The linear regression equation was $I_{\text{CL}} = 183.5 + 18.3 C$ (×10⁻⁸ g/mL) with a correlation coefficient of R² = 0.9943. The detection limit was 5 × 10⁻⁹ g/mL (signal/noise = 3). The relative standard deviation (n = 11) for 1.0 × 10⁻⁷ g/mL amoxicillin was 2.4%.

3.6. Selectivity

The effect of some commonly used co-existing compounds in pharmaceutical preparations and urine, such as excipients and additives, was studied by analyzing a standard solution of 1.0 × 10⁻⁷ g/mL amoxicillin. The tolerated limit for a foreign species was taken as the largest amount yielding a relative error < 5%. No interference was observed when the solutions of amoxicillin contained 500-fold magnesium; 100-fold glucose, sucrose, stearate, maltose and fructose; and equal concentrations of ascorbic acid and uric acid. Equal concentrations of co-existing compounds and ampicillin interfered with ECL detection.

3.7. Application

The proposed method was applied to the determination of amoxicillin in capsules. An accurately weighed amount of the powder was dissolved using a small quantity of HCl before

Table 2 – Determination of amoxicillin in urine samples.

Samples	Found ^a × 10 ⁻⁸ g/mL	Added × 10 ⁻⁸ g/mL	Recovered ^a × 10 ⁻⁸ g/mL	Recovery %	RSD
1	0.35	20.0	20.4	102	2.7%
2	0.77	40.0	41.2	101	3.1%
3	0.65	60.0	58.2	96	2.1%

RSD = relative standard deviation.

^a Average of five measurements.

being diluted further in water. After filtering, aliquots of the filtrate were further diluted with water until the final amoxicillin concentrations were within the working range. The result of the concentration determination of amoxicillin in capsules is shown in Table 1 and agreed well with the concentrations obtained using a UV method [22].

Blank urine samples (10.0 mL) were collected from three healthy volunteers. Each of the volunteers then took two amoxicillin capsules, and further urine samples (10.0 mL) were collected after 2 hours. After centrifuging the samples at 3000 rpm for 20 minutes, the supernatants were transferred into test tubes and analyzed by the proposed method. A known amount of AM standard solution was added to each 0.1 mL of urine sample, and the mixture was diluted to 100 mL with water prior to analysis. Each recovery was calculated by comparing the results obtained before and after the addition of AM standard, and the recoveries were found to be between 96% and 101% ($n = 5$), as shown in Table 2.

4. Conclusions

A novel ECL system of AM/H₂O₂/CTAB that exhibited enhanced ECL emissions at negative potential region of -0.7 V and a weak ECL emission at $+0.5$ V has been described. A mechanism involving singlet oxygen in the excited state and the hydrolysis product of AM as the ECL luminophor has been proposed. The unique ECL behavior in the negative potential region of -0.7 V has potential application in the ECL analysis of AM.

Conflicts of interest

All authors have no conflicts of interest to declare.

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