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Electrochemiluminescent behavior of melatonin and its important derivatives in the presence of $Ru(bpy)_3^{2+}$

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Abstract

Melatonin and some of its important derivatives were found to be able to enhance the ECL of $Ru(bpy)_3^{2+}$ in an alkaline Britton–Robinson buffer solution. The optimum conditions for the enhanced ECL, such as the selection of applied potential mode, type of buffer solution, pH effect and effect of $Ru(bpy)_3^{2+}$ concentration have been investigated in detail in this paper. Under the optimum conditions, the enhanced ECL is linear with the concentration of melatonin and its derivatives over the wide range, and the detection limit for these compounds was found to be in the range of 5.0×10^{-8} to 1.0×10^{-10} mol L^{-1}. The proposed procedure was applied for the determination of drug in tablets with recoveries of 85–93%. A possible mechanism for the enhanced ECL of $Ru(bpy)_3^{2+}$ by melatonin and its derivatives was proposed, and the relationship between molecular structure of melatonin and its derivatives and the enhanced ECL behavior was also discussed.

Keywords: Melatonin and its derivatives; Electrochemiluminescence; $Ru(bpy)_3^{2+}$

1. Introduction

Melatonin (MT) and its important derivatives (MTS), such as 5-methoxyindole-3-acetic acid (MIAA), *N*-acetyl-5-hydroxytryptamine (NAHT), 5-methoxytryptamine (5-MT) and *N*-acetyl-5-methoxy-6-hydroxytryptamine (HMT) (their structures are shown in Table 1) are strongly bioactive, they have regulation action for the genital [1], endocrine [2] and immune [3] system of human. They have recently increased interest as a treatment of sleep disorder and prevention of aging [4]. The contents of these indole-hormones in human body have been used as the important index for clinical examination and diagnosis. Therefore, the development of a method for determination of melatonin and its metabolites or derivatives in biological samples is especially important.

Melatonin in biological samples can be detected by several methods, such as HPLC [5–8], UV [9], fluorimetry [10,11] and RIA [12]. Some chemiluminescent methods have been used for determination of melatonin and its derivatives [13–15]. It has been noted that melatonin and its derivatives belong in the indole aromatic compound. They all have the aromatized conjugation system and can produce the free radical ion under electrolysis [20,22], which is very favorable to give ECL. However, no attention has been paid to use the ECL method for determination of melatonin and its derivatives, even the investigation for ECL of indole compounds has been rarely reported [16–19]. It has been found that melatonin and its derivatives would enhance the ECL of $Ru(bpy)_3^{2+}$ in an alkaline Britton–Robinson (B–R) buffer solution. The aim of the present study is to investigate the behavior of this ECL system and to develop a sensitive ECL method for determination of melatonin and its derivatives. The mechanism of this ECL system has been also investigated

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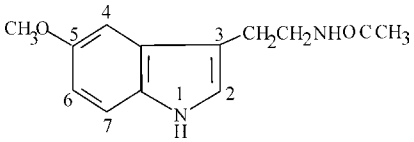
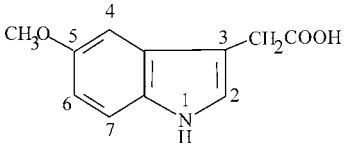
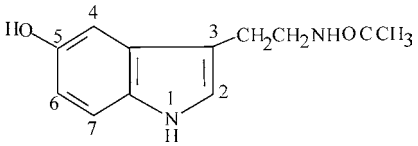
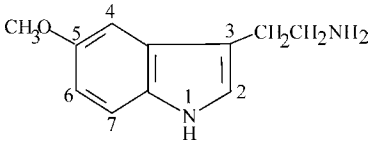
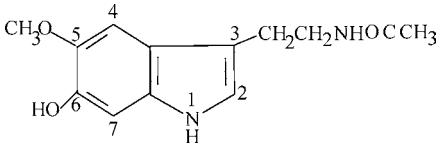
Table 1
Structure of MT and relative derivatives

Compounds	Structure	Abbreviation
Melatonin (*N*-acetyl-5-methoxytryptamine)		MT
5-Methoxyindole-3-acetic acid		MIAA
N-Acetyl-5-hydroxytryptamine		NAHT
5-Methoxytryptamine		5-MT
N-Acetyl-5-methoxy-6-hydroxytryptamine		HMT

and proposed using electrochemical and spectroscopic method.

2. Experimental

2.1. Chemicals and solution

Melatonin (MT), 5-methoxyindole-3-acetic acid (MIAA), *N*-acetyl-5-hydroxy-tryptamine (NAHT), 5-methoxytryptamine (5-MT), *N*-acetyl-5-methoxy-6-hydroxy-tryptamine (HMT) and $Ru(bpy)_3^{2+}$ were purchased from Sigma. Pyridoxine was purchased from Shanghai Chemical Company. Other chemicals were analytical grade or better and double distilled water was used throughout.

The 1.0×10^{-2} mol L^{-1} stock solutions of MTS were prepared by dissolving the required amount of sample in alcohol–water solution (10 + 90, v/v), respectively. 1.0×10^{-2} mol L^{-1} stock solution of $Ru(bpy)_3^{2+}$ was prepared by dissolving the required amount of sample in water. All these stock solutions were stored under refrigeration. Testing solution was prepared by diluting the stock solutions with appropriate buffer solutions before used. The buffer system was a Britton–Robinson (B–R) buffer prepared by titrating a stock solution containing 0.04 mol L^{-1} acetic acid, 0.04 mol L^{-1} phosphoric acid and 0.04 mol L^{-1} boric acid with 0.2 mol L^{-1} sodium hydroxide to the desired pH value.

2.2. Apparatus

2.2.1. ECL detection system

ECL measurements were performed using a system make in our laboratory, consisting of a BPCL Ultra-Weak Chemiluminescence Analyzer (Institute of Biophysics, Academia Sinica, Beijing, China), a potentiostat, an electrochemical cell and a computer control system. A block diagram of the system is shown in Fig. 1. The potentiostat mainly included a waveform generator, which could perform linear-, triangular- and square-wave voltage-sweeps. A conventional three-electrode system was used, and a glassy carbon electrode (GCE) was used as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl electrode (sat. KCl) as the reference electrode. A commercial 5 ml cylindroid glass cell was used as ECL cell, and it was put directly in front of the photomultiplier tube.

2.2.2. Other instruments

A BAS 100A electrochemical analyzer (Bioanalytical Systems, Purdue, IN) was used for all electrochemical measurements.

2.3. Procedure for ECL measurement

One milliliter of sample solution and 1 ml of 1.0 mmol L^{-1} $Ru(bpy)_3^{2+}$ were added to a 10 ml volumetric flask, and

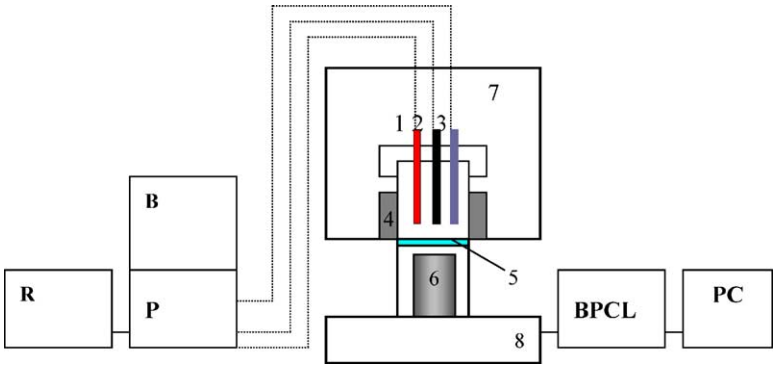


Fig. 1. Experimental setup for the ECL. (1) Reference electrode; (2) working electrode; (3) counter electrode; (4) jacket for localization; (5) quartz window; (6) photomultiplier tube; (7) detector chamber; (8) base frame; B: negative high voltage power supply; P: potentiostat; R: recorder; BPCL: CL detector; PC: computer controller and data processing system.

diluted with buffer solution to the required volume; 2.5 ml of this mixture solution was transferred to the ECL cell. A triangular voltage was scanned in the range of 0–1600 mV with the scan rate of 50 mV s^{-1}, and the ECL signal was then recorded.

The working electrode was pretreated prior to use by polishing their surfaces with aqueous slurries of alumina powders (average particle diameters: 1.0 μm and 0.3 μm α-Al_2O_3) on a polishing microcloth and rinsed with water and then sonicated in acetone.

3. Results and discussion

3.1. ECL behavior of MT and its derivatives in the presence of Ru(bpy)$_3^{2+}$

The primary experiments showed that the MT and its derivatives did not give ECL by themselves at GCE in the absence of Ru(bpy)$_3^{2+}$, however, they enhanced the ECL of Ru(bpy)$_3^{2+}$ at GCE, and the enhanced ECL intensity (ΔI) was dependent on the chemical and electrochemical factors, such as the mode of applied potential, the type of buffer solution, pH of the media solution and concentration of Ru(bpy)$_3^{2+}$.

3.1.1. Selection of the mode of applied potential

Square, triangular and symmetric double step pulse voltage scanning were selected to examine the ECL behavior of Ru(bpy)$_3^{2+}$/MT system at GCE over the potential range of 0–1600 mV. The results showed that only under the mode of triangular and square scanning, MT would enhance the ECL of Ru(bpy)$_3^{2+}$ obviously and stably, and the higher signal-noise-ratio could be obtained under the triangular scanning mode. The situations of other derivatives mentioned above were found to be similar to that of MT.

Under the mode of triangular scanning, the effect of scanning rate on the enhancement of the ECL (ΔI) of Ru(bpy)$_3^{2+}$ has been examined and the results is shown in Fig. 2. It can be known from Fig. 2 that ΔI is increased with the scanning rate below 40 mV s^{-1}, beyond that, ΔI reaches the maximum and constant, therefore, 50 mV s^{-1} was selected as the scanning rate for subsequent studies.

3.1.2. Effect of buffer solution

The ECL behavior of Ru(bpy)$_3^{2+}$/MT system in different buffer media (pH 11), such as B–R buffer solution, Na_2HPO_4–NaH_2PO_4 (PBS) buffer solution, $NaHCO_3$–Na_2CO_3 buffer solution (CS) and H_3BO_3–NaOH buffer solution (BS) were investigated in detail. The experiments showed that the maximum enhanced ECL intensity could be obtained in B–R buffer solution.

3.1.3. Effect of pH

In B–R buffer solution, the effects of MT, HMT, NAHT, 5-MT and MIAA (concentrations were 1.0×10^{-8} mol L^{-1}) on the enhancement for ECL of Ru(bpy)$_3^{2+}$ have been examined under different pH value. The results showed that for all of the five compounds mentioned above, the enhanced ECL intensity (ΔI) was increased quickly with pH below pH 11, in the range of pH 11–12, ΔI reached the maximum and constant, beyond pH 12, ΔI was decreased slightly with the increasing of pH. Therefore, pH 11.6 was selected as the optimum pH for subsequent experiments.

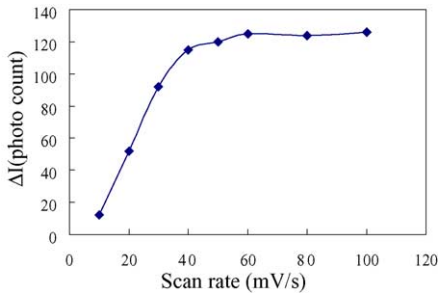


Fig. 2. Effect of sweep rate on the enhanced ECL intensity (ΔI) B–R buffer solution (pH 11.6), [Ru(bpy)$_3^{2+}$] = 1.0×10^{-6} mol L^{-1}, [MT] = 1.0×10^{-8} mol L^{-1}.

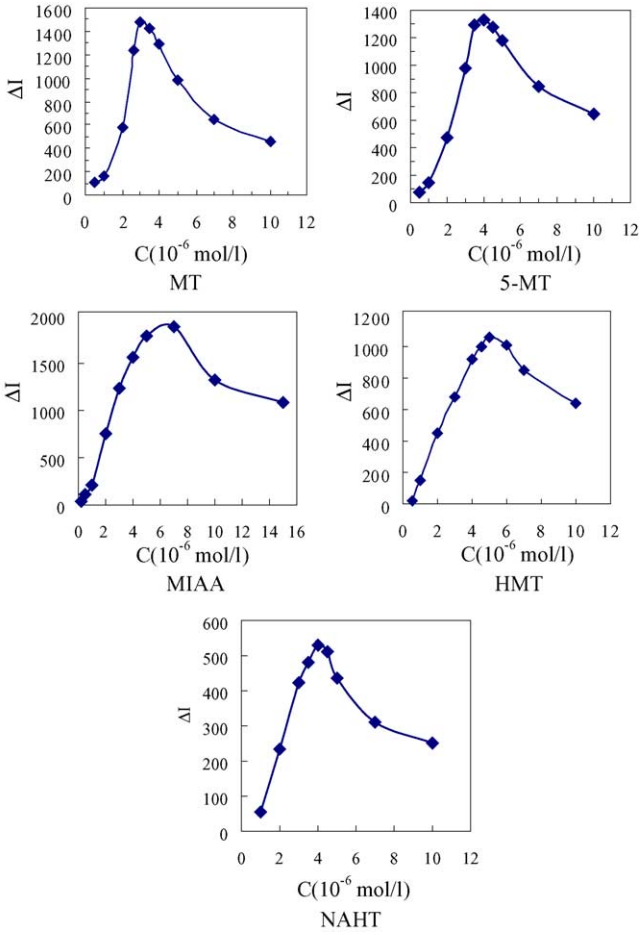


Fig. 3. Effect of $Ru(bpy)_3^{2+}$ concentration on the enhanced ECL intensity (ΔI) of MTS. B–R buffer solution (pH 11.6).

3.1.4. Effect of $Ru(bpy)_3^{2+}$ concentration

In pH 11.6 B–R buffer solution, $Ru(bpy)_3^{2+}$ itself would give ECL at the potential of 1300 mV, and the ECL intensity was enhanced with increased concentration of $Ru(bpy)_3^{2+}$. The effect of $Ru(bpy)_3^{2+}$ concentration on the enhanced ECL has been investigated when MT and its derivatives was 1.0×10^{-6} mol L^{-1}, and the results are shown in Fig. 3. Fig. 3 indicates that ΔI is increased with the concentration of $Ru(bpy)_3^{2+}$ in the lower concentration range of $Ru(bpy)_3^{2+}$, when the concentration of $Ru(bpy)_3^{2+}$ is higher than $(3.0–5.0) \times 10^{-6}$ mol L^{-1}, ΔI is decreased with the increase of $Ru(bpy)_3^{2+}$ concentration. For MT, NAHT, MIAA, 5-MT and HMT, the optimum concentrations of $Ru(bpy)_3^{2+}$ for obtaining the best ECL response were 3.0×10^{-6} mol L^{-1}, 4.0×10^{-6} mol L^{-1}, 7.0×10^{-6} mol L^{-1}, 5.0×10^{-6} mol L^{-1} and 4.0×10^{-6} mol L^{-1}, respectively.

3.1.5. Linear response range and detection limit

Under the above optimum conditions, the linear response range and the detection limits for the MT and its derivatives were measured. The results are listed in Table 2, in which the logarithm of ECL intensity is well linear with the logarithm of the concentration for all of abovementioned compounds. The detection limit was defined as three times the concentration corresponding to the standard deviation of the blank. As shown in Table 2, the detection limit for MT and it derivatives is in the range of 5.0×10^{-8} to 1.0×10^{-10} mol L^{-1}.

3.1.6. Analytical application

The simultaneous determination of melatonin in the presence of other indolic compounds in biological sample was also studied. The effect of some compounds such as serotonin, tryptophan, dopamine, uric acid, vitamin and glucose that may coexist in bio-sample was studied. The results indicated that there was no significant difference in determination of melatonin in presence of these compounds. However, direct measurement of melatonin in presence of NAHT, HMT, 5-MT, HIAA and MIAA would be very difficult due to the proximity of the ECL characteristic of these compounds, which indicate a need for the association of separation technique, i.e. HPLC or FIA, in clinic examination.

The optimized procedure was applied for the assay of melatonin in tablets sold as a pharmaceutical preparation. The grinded powder of tablets (Nurture Diagnostics Company, USA) was completely mixed with alcohol–water (10 + 90, v/v) by stirring and shaking. After standing for 5 min, the mixture was then filtered and the precipitate was washed several times with solvent. The filtrate and the washing were collected quantitatively in 50 ml flask and diluted appropriately with water before measurement. Aliquots of these solutions were analyzed by the same method described for the calibration curve. The results of five determinations gave a mean melatonin content of 3.01 mg per capsule with R.S.D. 3.8%. The percentage recovery of melatonin, based on the average of five replicate measurements was found to be 85–93% (see Table 3). The results were statistically compared with those obtained by the reported method [23].

Table 2
The linear response range and the detection limits

Compound	Linear range (mol L^{-1})	Regression equation	Correlation coefficiency	Detection limit (S/N = 2) (nmol L^{-1})
MT	5.0×10^{-9} to 1.0×10^{-7}	$\log(I) = 0.13 \log(C) + 4.38$	0.9948	1.0
MIAA	3.2×10^{-9} to 3.2×10^{-7}	$\log(I) = 0.97 \log(C) + 11.04$	0.9911	0.1
5-MT	3.2×10^{-9} to 1.0×10^{-6}	$\log(I) = 0.48 \log(C) + 6.16$	0.9885	1.0
NAHT	1.0×10^{-7} to 1.0×10^{-5}	$\log(I) = 0.35 \log(C) + 5.31$	0.9629	50.0
HMT	3.2×10^{-8} to 1.0×10^{-6}	$\log(I) = 0.20 \log(C) + 4.74$	0.9941	10.0

Table 3
Analysis of pharmaceutical formulations by the proposed and reported methods

Pharmaceutical formulations	Labeled amount (mg g^{-1})	Amount found[a] (mg g^{-1})		Added amount (mg g^{-1})	Founded amount (mg g^{-1})	Recovery (%)
		D ($\pm\sigma$)	R			
Melatonin tablets 1	3	3.06 ($\pm$ 0.13)	3.14	1.50	1.32	88.1
Melatonin tablets 2	3	2.95 ($\pm$ 0.12)	3.08	2.32	2.15	92.7
Melatonin tablets 3	3	3.02 ($\pm$ 0.09)	3.01	1.00	0.85	85.0

[a] Average of five determinations. D, proposed ECL method; R, reported method [23].

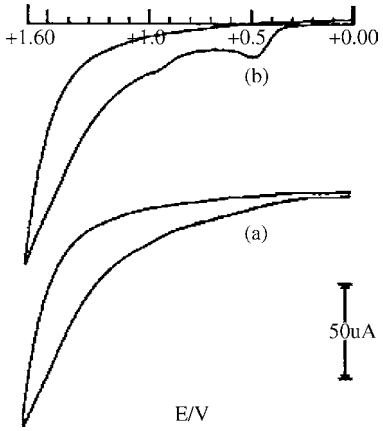


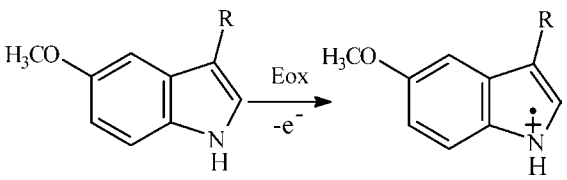
Fig. 4. Cyclic voltammogram of MT solution at GCE: (a) control experiment without MT, (b) [MT] = 1.0×10^{-4} mol L^{-1}. Scan rate: 100mV s^{-1}, B–R buffer solution, pH 11.6.

3.2. Mechanism for the ECL of MT and its derivatives in the presence of $Ru(bpy)_3^{2+}$

3.2.1. Cyclic voltammetry of MT-$Ru(bpy)_3^{2+}$ system

The cyclic voltammogram of MT in B–R buffer solution (pH 11.6) at a GC electrode was recorded. As shown in Fig. 4(b), MT gives an irreversible oxidation wave at 0.5 V, which indicates that MT is oxidized at GC electrode to give a radical cation $MT^{\bullet+}$ [20] (see Scheme 1). In Fig. 4(b), a small irreversible oxidation peak in the range of 0.9–1.0 V is also observed, which is probably due to the re-oxidation of the MT oxidation product [20].

The previous literature reported that the oxidation-reduction reaction of $Ru(bpy)_3^{2+}$ in the range of 1.1–1.35 V in the alkaline solution was an irreversible process. The cyclic voltammetric behavior of $Ru(bpy)_3^{2+}$/MT system in pH 11.6 B–R buffer solution is shown in Fig. 5, and the result shows that MT still give an irreversible oxidation wave at 0.5 V, which indicates that MT free radical cation is produced in this system. Fig. 5 also shows that $Ru(bpy)_3^{2+}$ is oxidized at 1.2 V to give a quasi-irreversible redox process. It was found that when the applied potential was lower than 1.0 V, the ECL was not observed in this system. The ECL was appeared when the applied potential was higher than 1.0 V and reached the maximum at 1.3 V, beyond which the ECL was decreased, which indicated that the ECL process apparently occurred with the electrogeneration of $Ru(bpy)_3^{3+}$.



Scheme 1. R=$CH_2CH_2NHCOCH_3$.

3.2.2. The effect of surfactant on the ECL behavior of $Ru(bpy)_3^{2+}$/MT

In general, below the CMC of surfactant, the single surfactant molecule is easily adsorbed on the surface of electrode, and the association or exclusion is occurred between the surfactant molecule and the excited state ion of analyte to form a "active layer" or "inactive layer" on the surface of electrode. Therefore, addition of a suitable surfactant is favorable to investigate the form of the substance adsorbed on the electrode surface. The cationic surfactant cetyltrimethylammonium bromide (CTMAB), anionic surfactant sodium dodecylsulphate (SDS) and non-ionic surfactant Triton X-100 were added to the $Ru(bpy)_3^{2+}$ (3.0×10^{-6} mol L^{-1})/MT (1.0×10^{-8} mol L^{-1}) system respectively to examine the effects of surfactant on this ECL system. The experimental results showed that increases in ECL efficiency have been observed in 1.3×10^{-6} mol L^{-1} CTMAB media ($\geq$ 4-fold) and in 0.004% Triton X-100 media ($\geq$ 8-fold), respectively, while a decrease in ECL efficiency has been observed in 1.5×10^{-6} mol L^{-1} SDS media ($\geq$ 8-fold). Because the proton on N1 position of indole ring (see the structure in Table 1) is easily dissociated in alkaline solution, MT is partly in the form of anion. An increase of the applied voltage (over the range of 0.2–0.5 V) will facilitate the electrochemical

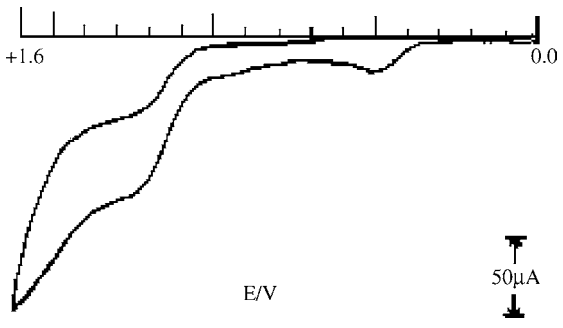


Fig. 5. Cyclic voltammogram of $Ru(bpy)_3^{2+}$/MT system at GCE [MT] = 1.0×10^{-4} mol L^{-1}, [$Ru(bpy)_3^{2+}$] = 1.0×10^{-3} mol L^{-1}. Scan rate: 100 mV s^{-1}, B–R buffer solution, pH 11.6.

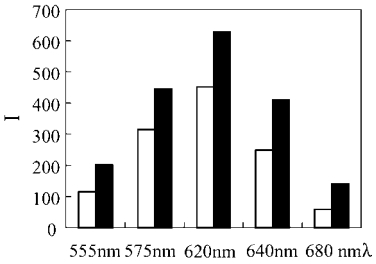


Fig. 6. ECL spectrum of $Ru(bpy)_3^{2+}$/MT system in B–R buffer solution (pH 11.6). $[Ru(bpy)_3^{2+}] = 3.0 \times 10^{-6}$ mol L^{-1}, $[MT] = 1.0 \times 10^{-8}$ mol L^{-1}, $[Ru(bpy)_3^{2+}] = 3.0 \times 10^{-6}$ mol L^{-1}.

generation of the reductive free radical intermediate of MT, serotonin and other indoles, which mechanism is proposed in the previous research [20,22]. The cationic and neutral surfactant is easier to attract the MT free radical and anion on the surface of electrode to form the "active layer", which contribute to the rapidly increase of the ECL intensity of system. While the anionic surfactant is easier to associate with the excited $Ru(bpy)_3^{2+}$ to form the "inactive layer" on the surface of electrode, which will increase the non-radiative transition and further decrease the ECL efficiency.

3.2.3. The ECL spectrum

The ECL emission spectrum for $Ru(bpy)_3^{2+}$ (3.0×10^{-6} mol L^{-1}) in pH11.6 B–R buffer solution from 535 nm to 680 nm was obtained by using a series of filters. The result showed that the maximum emission wavelength was 620 nm, after addition of 1.0×10^{-8} mol L^{-1} of MT, the ECL was enhanced obviously (see Fig. 6). However, the maximum emission wavelength was still at 620 nm, which indicated that the luminophore of $Ru(bpy)_3^{2+}$/MT system was still $[Ru(bpy)_3^{2+}]^*$.

3.2.4. Comparison of the ECL intensity of MT with that of its derivatives

The ECL intensity of MT and its derivatives (all in 1.0×10^{-7} mol L^{-1}) has been examined under the same conditions (pH 11.6 B–R buffer solution, 50 mV s^{-1} of scan rate, $Ru(bpy)_3^{2+}$ 3.0×10^{-6} mol L^{-1}), and the results are shown in Fig. 7. The order of the enhanced ECL for the system of MT and its derivatives is, thus, given by MIAA > MT > 5-MT > HMT > NAHT, which indicates that the indole ring structure of MTS plays an important role for enhancement of ECL, and the type of substituted group and its position on the indole ring would affect the ECL intensity. Therefore, the different substituting group introduced into the indole ring maybe led to different behavior for the enhancement of ECL.

3.2.5. ECL mechanism for MT and its derivatives

MT and its derivatives (I) are weakly acidic, the proton on N1 position of the indole ring is easily dissociated to produce the anion (II) in alkaline solution. The anion can be electrolyzed under a certain applied potential (E_1) to form the active free radical intermediate (IV). Meanwhile, the compound (I) is oxidized in the lower potential range of 0.2–0.5 V to form the free radical cation (III), this free radical cation is also possible to lose a proton to form the active neutral free radical intermediate (IV). However, as the neutral free radical intermediate (IV) is very unstable [22], if there is no active substance in the system, this acid-base equilibrium tends toward formation of free radical cation (III), which is further oxidized in the potential range of 0.9–1.0 V to form a cation (V), while this process is not followed by ECL. In the presence of $Ru(bpy)_3^{2+}$, the reductive neutral free radical intermediate (IV) is easier to react with the electrogenerated $Ru(bpy)_3^{3+}$ to produce the excited $Ru(bpy)_3^{2+*}$ to give ECL. Therefore, the ECL reaction mechanism of MT and its derivatives in this system is proposed in Fig. 8.

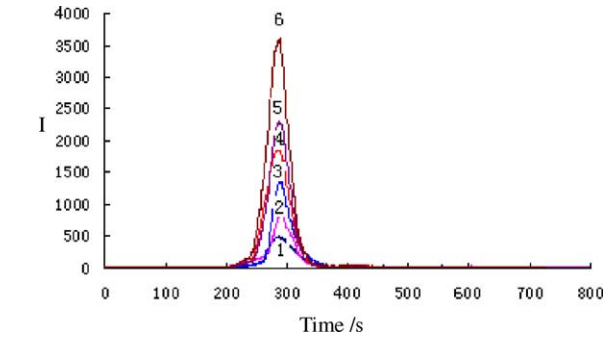
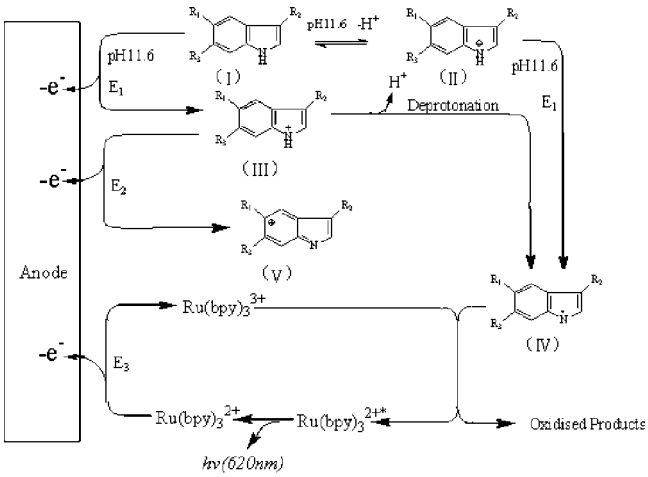


Fig. 7. Comparison of the ECL intensity for MT and related derivatives (1) $Ru(bpy)_3^{2+}$ blank solution; (2) NAHT; (3) HMT; (4) 5-MT; (5) MT; (6) MIAA.

The experiments showed that the indole ring structure of the MTS play the most important role for enhancement of



R_1=OCH_3 or OH

R_2=$CH_2CH_2NHCOCH_3$ or $CH_2CH_2NH_2$ or CH_2CH_2COOH

R_3=OCH_3 or H

E_1=0.2~0.5 V; E_2=0.9~1.0 V; E_3=1.3 V

Fig. 8. The proposed mechanism for the ECL reaction of $Ru(bpy)_3^{2+}$ with a MTS.

the ECL of $Ru(bpy)_3^{2+}$, however, the enhanced ECL was also closely related to the functional group substituted onto the indole ring of MTS. In our experiments it is evident that the enhanced ECL intensity of MTS with 5-methoxy group, such as MIAA, MT and 5-MT, is stronger than that of MTS with phenolic hydroxyl group, such as HMT and NAHT, this is because of the introduction of methoxy group on the indole ring increase the electronic delocalization of aromatic nucleus, which is favorable to form a neutral free radical intermediate to react with $Ru(bpy)_3^{2+}$. The previous research has indicated that the compound containing phenolic hydroxyl group would inhibit the ECL of $Ru(bpy)_3^{2+}$ in neutral and alkaline solution, while introduction of carboxyl group would enhance the ECL of $Ru(bpy)_3^{2+}$ [21]. MIAA contains not only a methoxy group at C5 of the indole ring, but also a carboxyl group on the side chain, so it has the strongest enhancement ability for ECL. As NAHT has a phenolic hydroxyl group at C5, it is the weakest one for enhancement of ECL. HMT has both methoxy and phenolic hydroxyl group at C5 and C6, respectively, this is the reason that its enhancement ability for ECL is lower than that of MIAA, MT and 5-MT, but stronger than that of NAHT.

4. Conclusion

Melatonin and some of its derivatives have been found to be able to enhance the ECL of $Ru(bpy)_3^{2+}$, and their enhancement ability for ECL was not only dependent on the structure of indole ring but also on the substituted group introduced onto the indole ring. Based on which a possible mechanism for the enhancement ECL of $Ru(bpy)_3^{2+}$ by melatonin and its derivatives has been proposed, and the relationship between structure of MTS molecule and the enhanced ECL behavior also has been explained. The enhanced ECL intensity is linear with the concentration of MTS over a wide range. Under the optimum conditions, the detection limit for these compound was found to be in the range of 5.0×10^{-8} to 1.0×10^{-10} mol L^{-1}, based on which a sensitive method is possible to be developed for determination of melatonin and its derivatives.

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