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A novel electrochemiluminescence tetracyclines sensor based on a $Ru(bpy)_3^{2+}$-doped silica nanoparticles/Nafion film modified electrode

Xiaomei Chen [a], Limin Zhao [a], Xiaotian Tian [a], Sai Lian [a], Zhiyong Huang [a,c,*], Xi Chen [b]

[a] College of Food and Biological Engineering, Jimei University, Xiamen 361021, China
[b] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China
[c] Fujian Provincial Key Laboratory of Food Microbiology and Enzyme Engineering, Xiamen 361021, China

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ABSTRACT

A novel method for the determination of tetracyclines (TCs) using electrochemiluminescence (ECL) based on a $Ru(bpy)_3^{2+}$-doped silica nanoparticles (RuSiNPs)/Nafion film modified electrode is presented in this paper. The RuSiNPs were prepared by a water-in-oil microemulsion method. The characterization results indicated that the thus-prepared RuSiNPs presented a uniform size of 45 nm and retained the original electrochemical properties of $Ru(bpy)_3^{2+}$. Importantly, the ECL response on RuSiNPs/Nafion film modified electrode was greatly enhanced by TCs. Under the optimum conditions, the ECL intensity versus the concentration of TCs was found to be linear over the range of 1–100 μmol L^{-1} for tetracycline (TC), 0.1–100 μmol L^{-1} for oxytetracycline (OTC) and 1–100 μmol L^{-1} for chlortetracycline (CTC). The detection limits ($S/N=3$) were 0.23 μmol L^{-1} for TC, 0.10 μmol L^{-1} for OTC and 0.16 μmol L^{-1} for CTC. Moreover, due to the electrostatic interaction between $Ru(bpy)_3^{2+}$ and silica matrix, the leaching of $Ru(bpy)_3^{2+}$ was greatly reduced, therefore, the ECL sensor exhibited excellent repeatability and stability in the detection of TCs. Based on these investigations, the proposed ECL approach was successfully used to analyze the TCs content in drugs.

1. Introduction

Tetracyclines (TCs) such as tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) (Scheme 1) are a class of broad-spectrum antibiotics. As they have a good bactericidal effect, they are widely used in aquaculture and are also used in nutrition and feed additives throughout the agricultural sector. However, the improper or excessive using TCs can lead to antibiotic residues, which pose a serious threat to human health. Therefore, it is of great importance to develop an accurate and simple method for the determination of TCs in the pharmaceutical analysis and food security. Many methods for the determination of TCs have been developed, such as microbiological methods [1]; thin-layer chromatography (TLC) [2]; high performance liquid chromatography (HPLC) [3–5]; enzyme-linked immunosorbent assay (ELISA) [6]; liquid chromatography–mass spectrum (LC–MS) [7]; capillary electrophoresis (CE) [8,9]; capillary electrophoresis–mass spectrometry (CE–MS) [10]; fluorescence [11] and chemiluminescence (CL) [12,13]. Generally, the above approaches are performed in the centralized laboratories, requiring extensive labor and analytical resources, and often resulted in a lengthy turnaround time.

Electrochemiluminescence (ECL) is a well-known high sensitivity detection method depending on the generation of an optical signal triggered by an electrochemical reaction. In recent years, $Ru(bpy)_3^{2+}$-based ECL has become a powerful analysis tool because of its high ECL efficiency, good electrochemical stability and wide linear range [14,15]. There are several reports using $Ru(bpy)_3^{2+}$-based ECL in the detection of TCs [16–18]. Pang et al. demonstrated that TCs could inhibit the ECL signal of $Ru(bpy)_3^{2+}$/tripropylamine (TPA) system [17]. Lately, Guo et al. found that the $Ru(bpy)_3^{2+}$/2-(dibutylamino) ethanol (DBAE) ECL system could also be inhibited by TCs [18]. Based on these ECL signal changes, they established a simple detection method for TCs. However, in these liquid phase ECL applications, as an expensive ECL reagent, $Ru(bpy)_3^{2+}$ has been consumed greatly, resulting in environmental pollution and costly analysis. Moreover, these studies are based on the inhibition ECL systems, which may easily influence by the experimental conditions, resulting in a false signal. Therefore, considerable efforts are continuing towards the development of $Ru(bpy)_3^{2+}$-based ECL methods for the determination of TCs.

Recently, the studies on the immobilization of $Ru(bpy)_3^{2+}$ and its derivations to form a solid state ECL sensor have becoming an attractive research field. Various approaches, including sol–gel technology [19,20], Langmuir–Blodgett technique [21], ion-exchangeable polymer film [22] and layer-by-layer self-assembly [23], have been adopted for immobilizing $Ru(bpy)_3^{2+}$. However, these ECL approaches have several drawbacks, such as lacking long-term stability

* Corresponding author. Tel.: +86 592 6181487; fax: +86 592 6180470.
E-mail address: zhyhuang@jmu.edu.cn (Z. Huang).

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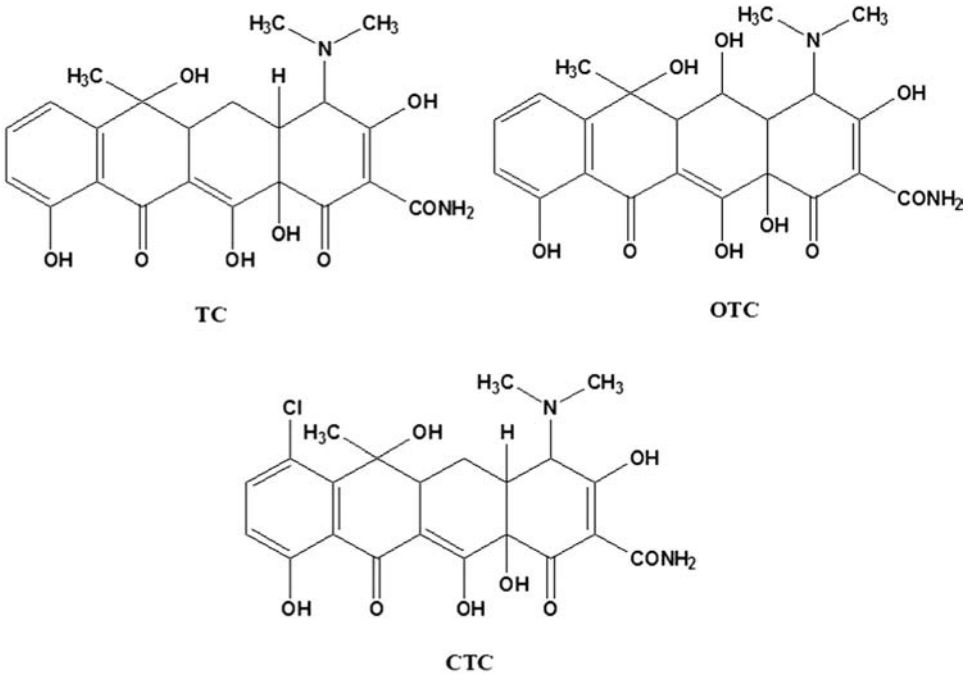

Scheme 1. Molecular structures of TC, OTC and CTC.

due to the leakage of $Ru(bpy)_3^{2+}$, and low sensitivity due to the limited amount of $Ru(bpy)_3^{2+}$ immobilized on the electrode. Several years ago, $Ru(bpy)_3^{2+}$-doped silica nanoparticles (RuSiNPs) with a core–shell structure incorporated $Ru(bpy)_3^{2+}$ molecules in the silica matrix have been applied in ECL [23–25]. Because of the strong electrostatic interaction between $Ru(bpy)_3^{2+}$ and silica matrix, the leaching of $Ru(bpy)_3^{2+}$ is negligible. Furthermore, as a large amount of $Ru(bpy)_3^{2+}$ are three-dimensionally encapsulated inside each silica nanoparticles, the original electrochemical properties of $Ru(bpy)_3^{2+}$ can be retained in these RuSiNPs. Nowadays, the RuSiNPs-based ECL sensors have been used in the determinations of DNA [26], enzyme [27], amines [28] and proteins [29], however, to the best of our knowledge, there are no report concerning the detection of TCs using RuSiNPs-based ECL sensors.

In this work, we prepared the RuSiNPs through a water-in-oil microemulsion method and immobilized them on the glassy carbon electrode (GCE) with the help of Nafion. Different from the previous inhibition ECL systems, we found that in the presence of TCs, the ECL signal can be greatly enhanced on RuSiNPs/Nafion modified GCE. Based on this observation, a simple and sensitive method for the determination of TC, OTC and CTC was developed. Our results showed that the as-proposed ECL sensor for the TCs detection displayed high sensitivity and good stability. Moreover, this method has been applied for the determination of TCs content in drugs with satisfactory results.

2. Experimental

2.1. Reagents and materials

Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$) were purchased from J&K. Triton X-100 (TX-100), tetraethyl orthosilicate (TEOS) and Nafion were obtained from Sigma. 1-Hexanol, cyclohexane, ethanol, acetone and ammonium hydroxide ($NH_3 \cdot H_2O$, 25 wt%) were purchased from Xilong Chemical Co., Ltd. TCs were purchased from Aladdin. A series of TCs standard solutions were prepared by diluting 10 mg mL^{-1} TCs with 0.1 mol L^{-1} phosphate buffer solutions (PBS, pH 8.5) and stored in the refrigerator (4 °C). Three different kinds of TC Tablets, OTC Tablets and CTC Hydrochloride Eye Ointment were purchased randomly form a drugstore in Xiamen, China. All the other chemicals were of analytical grade, and the water used throughout the experiment was purified using a water purification system (TKA, Germany).

2.2. Apparatus

The morphology of the RuSiNPs was examined by LEO1530 scanning electron microscope (SEM) and TECNAI F-30 transmission electron microscopy (TEM). The fluorescence spectroscopy was obtained from a fluorescence spectrophotometer (Varian, USA) and the pH values were measured using a pH510 meter (Eutech, Singapore). The ECL experiments were performed on a Model MPI-A ECL detection system with the voltage of the photomultiplier tube (PMT) setting at −800 V (Xi'an Remax Electronic Science-Tech Co. Ltd., Xi'an, China). All experiments were carried out with a three-electrode system including a GCE coated with RuSiNPs/Nafion film as the working electrode, a Pt wire as the counter electrode and an Ag wire as the reference electrode. All the potentials reported in this paper are referred to the Ag wire.

2.3. Preparation of RuSiNPs

RuSiNPs were prepared with a water-in-oil microemulsion method according to the literatures with a little modification [25,30]. In brief, 1.77 mL of TX-100 was mixed with 7.5 mL of cyclohexane, 1.8 mL of 1-hexanol, and 400 μL of 20 mmol L^{-1} $Ru(bpy)_3^{2+}$ aqueous solution. In the presence of 100 μL of TEOS, a polymerization reaction was initiated by adding 60 μL of $NH_3 \cdot H_2O$. The reaction was allowed to be stirred for 24 h to complete the reaction. After that, the product was precipitated by acetone, followed by centrifugation and washing with ethanol and water several times to remove any surfactant molecules. Finally, the orange RuSiNPs were obtained after drying.

2.4. Sample preparation

For the TC tablets, before grinding, 10 pieces of TC tablets were weighted, and then the average amount of one tablet was calculated. After grinding to fine powder, the amount equal to one tablet was weighted accurately, and then transferred into a 50 mL volumetric flask. After that, 0.1 mol L^{-1} HCl was added into the flask to solute the powder. The solution was diluted 30 times with 0.1 mol L^{-1} PBS (pH 8.5) when in use. Additionally, OTC tablets were deal with by a similar approach with TC.

For the CTC, 0.4 g aliquot of sample was weighed accurately, and then transferred into a separating funnel. After that, 5 mL ether was added to dissolve the sample. Then, the mixture was extracted by 2 mL of 0.1 mol L^{-1} HCl for three times. Finally, all the extracted liquid was transferred into a 10 mL volumetric flask and diluted with water to the scale.

2.5. Fabrication of the modified electrode

Before modification, each GCE was polished with 0.3 and 0.05 μm α-Al_2O_3, and then dried at room temperature. 8 mg RuSiNPs were dispersed in 1 mL ethanol by ultrasonication and then the suspension was mixed with Nafion (0.4 wt%) in the volume ratio of 1:1, so that the concentration of RuSiNPs in the resulting suspension was 4 mg mL^{-1}. After that, 4 μL RuSiNPs/Nafion mixture was placed on the surface of GCE and dried at room temperature. When not in use, the modified electrodes were kept in dry state at room temperature.

3. Results and discussion

3.1. Characterization of RuSiNPs

As the morphology of NPs influences the spectral and electrochemical properties, NPs with a uniform size is one of the key steps to improve the ECL sensitivity and stability. Fig. 1A shows the SEM and TEM images of the RuSiNPs. It is clear that the well dispersed RuSiNPs have a uniform size (Fig. 1A) and the diameter of the RuSiNPs was around 45 nm (the inset of Fig. 1A). Fig. 1B shows the fluorescence spectrum of $Ru(bpy)_3Cl_2$, RuSiNPs suspension and the RuSiNPs/Nafion composite. Comparing with $Ru(bpy)_3Cl_2$ solution, the maximum emission wavelength (λ_{em}) of RuSiNPs and the RuSiNPs/Nafion exhibited a slight red shift (~4 nm), which might because of the interaction between $Ru(bpy)_3^{2+}$ and Si–OH. Moreover, after mixing with Nafion, the RuSiNPs/Nafion composite presented the same λ_{em} with that of RuSiNPs, and they exhibited a same intensity in the same concentrations (1 mg mL^{-1}). This result confirmed that $Ru(bpy)_3^{2+}$ was successfully doped in SiO_2 nanoparticles and the RuSiNPs/Nafion maintained the photochemical characteristic well in the Nafion film.

3.2. Electrochemistry and ECL of RuSiNPs/Nafion film modified GCE

The electrochemical and ECL behavior of the RuSiNPs/Nafion film modified GCE has been investigated in PBS solution with and without 20 μmol L^{-1} TC (Fig. 2). As shown in the cyclic voltammograms (CVs), in the potential scan region from 0.2 to 1.1 V, the novel electrode showed a reversible redox wave with an oxidation peak at 0.89 V and a corresponding reduction peak at 0.77 V due to the electrochemical transition between Ru(III) and Ru(II), which was in accordance with the previous report [25]. It should be noted that comparing with the blank solution, only a slight increase was obtained in the oxidation peak at 0.89 V after the addition of TC, however, in the I_{ECL}/E curves, the ECL signal increased considerably in the presence of 20 μmol L^{-1} TC. As shown in Fig. 2, the onset luminescence occurred at 0.61 V, and then rose steeply until it reached a maximum at +0.97 V. This result indicated that RuSiNPs have been effectively modified onto

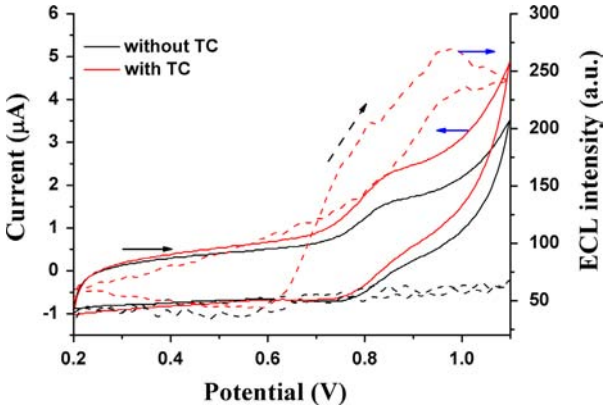

Fig. 2. CVs and I_{ECL}/E curves of the RuSiNPs/Nafion film modified GCE in 0.1 mol L^{-1} PBS (pH 8.5) without (black line) and with 20 μmol L^{-1} TC (red line). Scan rate: 80 mV s^{-1}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

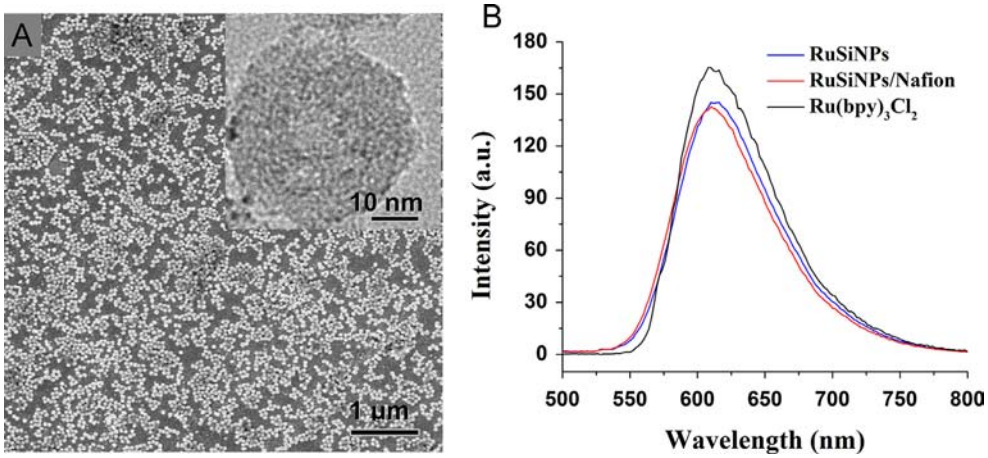

Fig. 1. (A) SEM image of RuSiNPs. The inset shows TEM image of RuSiNPs. (B) The fluorescence spectrum of 5 mM $Ru(bpy)_3Cl_2$ (black line), 1 mg mL^{-1} RuSiNPs suspension (blue line), and 1 mg mL^{-1} RuSiNPs/Nafion composite (red line). $\lambda_{ex\text{-}Ru(bpy)3Cl2}$: 570 nm; $\lambda_{ex\text{-}RuSiNPs}$: 460 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

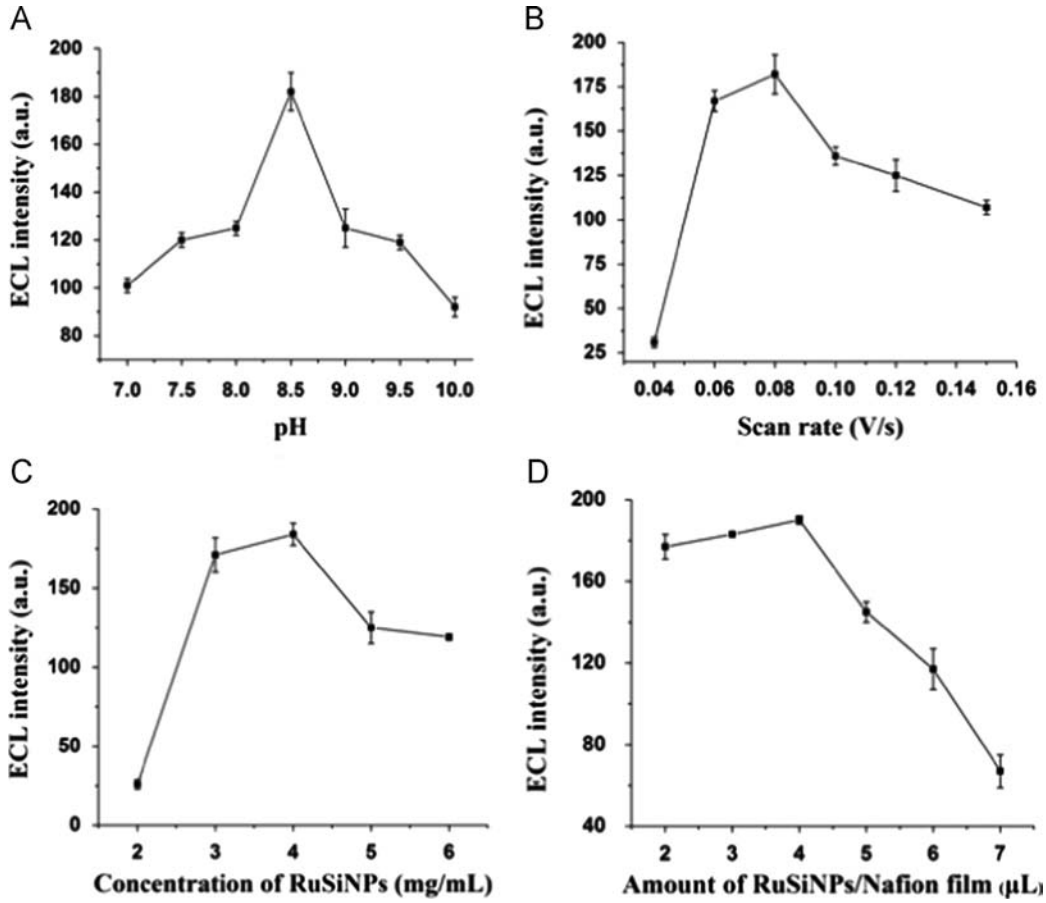

Fig. 3. Effect of (A) pH, (B) scan rate, (C) RuSiNPs concentration and (D) the amount of the RuSiNPs/Nafion film on the ECL intensity of 20 μmol L^{-1} TC.

the electrode surface and the enhancement of ECL intensity by TC at 0.97 V is applicable for the determination of TC.

3.3. Optimization of the experimental conditions

Since the ECL of $Ru(bpy)_3^{2+}$–TC is a pH-dependent reaction [16], we studied the effect of pH on the ECL response (Fig. 3A). It is seen that the ECL intensity increased with the increasing of pH value and reached the maximum value at a pH of 8.5. Then, the ECL intensity decreased as pH was further increased. This phenomena is consistent with the electrocatalytic reaction of $Ru(bpy)_3^{2+}$–TPA system. The ECL signal increased with the pH value from 6.5 to 8.5, implying that the deprotonation of TC is required during the ECL process. However, at high pH values, the redundant OH^- ions undergo a competing reaction with $Ru(bpy)_3^{2+}$ [31], which resulted in the decrease of ECL intensity. Therefore, a pH of 8.5 was selected for subsequent experiments.

The effect of scan rate on the ECL intensity was also studied (Fig. 3B). With the increase of scan rates, the ECL intensity increased and achieved the maximum value when the scan rate was at 80 mV s^{-1}. It has been reported that the ECL intensity was influenced by the formation and diffusion of the intermediate on the electrode [32]. At a lower scan rate, the formation of the intermediate is very slow, which cause a lower ECL intensity. However, at higher scan rate, shorter reaction time led immediately to lower concentration and smaller transmission of the reaction, resulting in a lower ECL intensity. Therefore, 80 mV s^{-1} was chosen as the scan rate in the further experiment.

Furthermore, the concentration of RuSiNPs in the composite film on the ECL intensity was investigated (Fig. 3C). ECL intensity increased with the increasing of RuSiNPs concentration, which attributed to the fact that more $Ru(bpy)_3^{2+}$ immobilized on the electrode with more RuSiNPs. However, if the concentration was higher than 4 mg mL^{-1}, the Nafion film cannot immobilize so many RuSiNPs, and moreover, the composite film cracked easily from the electrode, leading to the decrease of ECL intensity. Additionally, we studied the effect of the amount of the RuSiNPs/Nafion film on ECL response (Fig. 3D). The ECL intensity increased with the increasing of the RuSiNPs/Nafion film in a lower amount, however, when the amount was larger than 4 μL, ECL intensity began to reduce. This was because that thick film might absorb and scatter the ECL emission within the films and prevented TC from reacting on the electrode surface [30].

3.4. Method evaluation

Under the selected conditions, a calibration curve was plotted for the ECL intensity and TC concentration using the RuSiNPs/Nafion film modified GCE (Fig. 4). The ECL intensities presented a good linearity with the concentration of TC ranging from 1 to 100 μmol L^{-1} with a low detection limit ($S/N=3$) of 0.23 μmol L^{-1}, which was comparable with some other reported TC sensors (Table 1) [17,18,33–36]. The regression equation of ECL peak intensity versus the TC concentration (μmol L^{-1}) was $I_{ECL}=17.975+7.9036C$ with a correlation coefficient of 0.9962. Moreover, with nine repeated injections of TC (20 μmol L^{-1}), the relative standard deviation (RSD) of peak heights was 1.6%, showing the good repeatability of the proposed method.

The stability of the RuSiNPs/Nafion film modified GCE under the present conditions was studied. As shown in the inset of Fig. 4, the RSD of the ECL emission upon 10 continuous potential cycles in 0.1 mol L^{-1} PBS (pH 8.5) containing 20 μmol L^{-1} TC was 4.4%, suggesting the good stability of the ECL sensor. The long-term

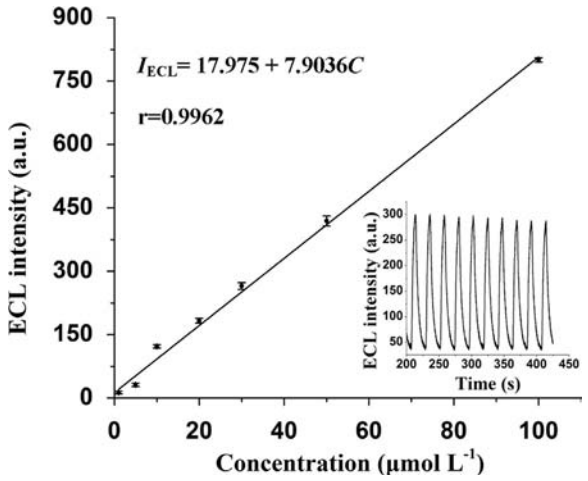

Fig. 4. Calibration curve of TC concentration and ECL intensity. The inset shows ECL emission of 20 μmol L^{-1} TC on a RuSiNPs/Nafion film modified GCE under continuous CV circles. Scan rate: 80 mV s^{-1}; pH 8.5, PBS; the concentration of RuSiNPs: 4 mg mL^{-1}; the amount of RuSiNPs/Nafion film: 4 μL.

Table 1
Comparisons of the linear range and detection limit of different electrodes in the detection of TC.

Electrode	Linear range (μmol L^{-1})	Detection limit (μmol L^{-1})	References
Pt foil	0.045–22.5	0.009	[17]
Pt disk	9×10^{-5}–9×10^{-3}	4.5×10^{-6}	[18]
GME[a]	2.25–22.5, 22.5–225	0.23	[33]
MWCNT-GCE[b]	2.5–100	0.12	[34]
mvRuO/RuCN[c]-GCE	–	0.23	[35]
Ni-GCCME[d]	5.6–180	0.06	[36]
RuSiNPs/Nafion-GCE	1–100	0.23	This work

[a] GME: gold microelectrode.
[b] MWCNT-GCE: multi-walled carbon nanotubes-glassy carbon electrode.
[c] mvRuO/RuCN: mixed-valence ruthenium oxide/ruthenium cyanide.
[d] Ni-GCCME: nickel-glassy carbon chemically modified electrode.

storage stability of the present proposed TC sensor was studied over a week by monitoring its ECL response with a continuous usage (every day) and stored in the air at room temperature when not in use. It was found that the response of the ECL sensor gradually decreased to approximately 85% of its initial value within a week, suggesting that the RuSiNPs/Nafion film modified GCE exhibits a long service life in the sensing of TC. This can be attributed to three factors: (i) $Ru(bpy)_3^{2+}$ is a stable luminous reagent, its photochemical characteristics ensure the chemiluminescence stable for a long time; (ii) the strong electrostatic interaction between $Ru(bpy)_3^{2+}$ and silica matrix makes silica matrix a suitable environment for the enrichment of $Ru(bpy)_3^{2+}$, and prevent the leaching of $Ru(bpy)_3^{2+}$ from the electrode; (iii) as an ion-exchange polymer, Nafion is very resistant to chemical attack, thus can firmly fix RuSiNPs in the film.

Additionally, the interference of co-existing substances was studied in the determination of 20 μmol L^{-1} TC. The tolerant maximum concentration of the co-existing substance was determined, in which the substance caused an ECL intensity change of approximately ±5%. Based on the experimental results, the tolerable concentration ratios for interference at the 5% level were over 1000 folds for Na^+, K^+ and Cl^-, 100 folds for Mg^{2+}, Fe^{3+}, Fe^{2+}, NH_4^+, Cu^{2+}, Zn^{2+}, SO_4^{2-}, CO_3^{2-} and NO_3^-.

To further verify the extensive application and reliability of the ECL sensor, it was applied in the determinations of OTC and CTC.

Table 2
ECL determination results of TC tablets using a RuSiNPs/Nafion film modified GCE (n=6).

Sample	Labeled (g)	Amount (g)	Added (g)	Found (g)	Recovery (%)	R.S.D. (%)
1	0.250	0.278	0.140	0.414 ± 0.02	97.1 ± 6.7	4.8
			0.280	0.537 ± 0.01	92.5 ± 4.4	2.3
			0.560	0.892 ± 0.03	109 ± 5.0	3.6
2	0.250	0.215	0.110	0.319 ± 0.02	94.5 ± 7.2	6.1
			0.220	0.445 ± 0.02	105 ± 7.6	4.4
			0.440	0.613 ± 0.03	90.5 ± 3.8	4.3
3	0.250	0.241	0.140	0.378 ± 0.01	97.9 ± 4.2	3.3
			0.280	0.488 ± 0.02	88.2 ± 2.2	3.4
			0.560	0.776 ± 0.03	95.5 ± 5.5	4.0

Table 3
ECL determination results of OTC tablets using a RuSiNPs/Nafion film modified GCE (n=6).

Sample	Labeled (g)	Amount (g)	Added (g)	Found (g)	Recovery (%)	R.S.D. (%)
1	0.250	0.242	0.120	0.358 ± 0.01	96.7 ± 3.3	4.0
			0.240	0.469 ± 0.02	94.5 ± 4.3	4.0
			0.480	0.767 ± 0.02	109 ± 4.5	3.0
2	0.250	0.263	0.130	0.393 ± 0.02	100 ± 8.0	4.5
			0.260	0.514 ± 0.02	96.5 ± 7.2	3.6
			0.520	0.836 ± 0.03	110 ± 5.0	3.7
3	0.250	0.254	0.130	0.390 ± 0.01	105 ± 5.5	1.8
			0.260	0.505 ± 0.01	96.5 ± 5.2	2.7
			0.520	0.767 ± 0.02	98.7 ± 4.4	3.0

The ECL intensities for the detection of OTC and CTC as a function of concentrations were found to be linear from 0.1 to 100 μmol L^{-1} for OTC and 1–100 μmol L^{-1} for CTC with the detection limit (S/N=3) of 0.10 μmol L^{-1} for OTC and 0.16 μmol L^{-1} for CTC, respectively. The regression equations of ECL peak intensity versus the concentration (μmol L^{-1}) are I_{ECL}= 6.2387+5.7936C with a correlation coefficient of 0.9943 for OTC, and I_{ECL}=17.282+7.287C with a correlation coefficient of 0.9916 for CTC.

3.5. Detection of real sample

The proposed method has been applied to evaluate TCs content in the drug samples. Before determination, all the TCs samples were diluted by 0.1 mol L^{-1} PBS (pH 8.5) to an appropriate concentration. Then, the ECL behaviors of these samples were tested under their optimum conditions. It should be mentioned that in order to facilitate the comparison of the labeled values on the medicine bottles, all the tested results were converted into "g" as the unit. As shown in Tables 2–4, the values obtained on the ECL sensor are consistent with the labeled values and the recoveries ranged from 88.2% to 109% for TC tablets, 94.5% to 110% for OTC tablets and 95% to 107% for CTC hydrochloride eye ointment, further verifying the practicability of the TCs ECL sensor based on RuSiNPs/Nafion film modified GCE.

4. Conclusions

In summary, this study proposed a novel ECL sensor based on a RuSiNPs/Nafion film modified GCE for the determination of TCs. Based on the results, the $Ru(bpy)_3^{2+}$ encapsulation interior of the silica matrix maintains its electrochemical activities and also reduces $Ru(bpy)_3^{2+}$ leaching from the electrode, which make the proposed sensor present good stability for TCs determination. This approach provides a new facile route to construct effective TCs sensors, further experiments, such as using the RuSiNPs/Nafion

Table 4
ECL determination results of CTC hydrochloride eye ointment using a RuSiNPs/Nafion film modified GCE ($n=6$).

Sample	Labeled (g)	Amount (g)	Added (g)	Found (g)	Recovery (%)	R.S.D. (%)
1	0.0020	0.0015	0.00072	0.0022 ± 0.0001	97.2 ± 4.6	2.7
			0.0014	0.0030 ± 0.0001	107 ± 4.2	2.8
			0.0028	0.0042 ± 0.0001	96.4 ± 2.0	1.3
2	0.0020	0.0022	0.0010	0.0032 ± 0.0001	100 ± 3.9	1.9
			0.0020	0.0042 ± 0.0001	100 ± 6.4	3.0
			0.0040	0.0060 ± 0.0002	95.0 ± 4.3	3.1
3	0.0020	0.0016	0.00082	0.0024 ± 0.0001	97.6 ± 7.2	2.5
			0.0016	0.0033 ± 0.0001	106 ± 3.5	1.7
			0.0032	0.0049 ± 0.0001	103 ± 2.4	1.6

film modified electrode in capillary electrophoresis coupled with electrochemiluminescence (CE–ECL) system and practical application of this ECL sensor in the determination of TCs in food, are underway.

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