An electrochemiluminescent biosensor for uric acid based on the electrochemiluminescence of bis-[3,4,6-trichloro-2-(pentyloxycarbonyl)-phenyl] oxalate on an ITO electrode modified by an electropolymerized nickel phthalocyanine film†

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Nickel(II) tetrasulfophthalo-cyanine (NiTSPc) could be electrodeposited onto an indium tin oxide (ITO) electrode to form an electropolymerization film of NiTSPc. The electrochemiluminescent (ECL) behavior of bis-[3,4,6-trichloro-2-(pentyloxy-carbonyl)-phenyl] oxalate (BTPPO) on this modified electrode was investigated in detail. The emission of ECL of BTPPO can be greatly enhanced by hydrogen peroxide at this modified electrode. Thus, a new ECL biosensor for uric acid was developed based on the enzymatic reaction of uric acid in the presence of uricase to produce H_2O_2. The developed sensor has been used to detect uric acid in real serum samples, and the results compared well with those obtained by the routine method.

1 Introduction

Electrochemiluminescence (ECL), also known as electrogenerated chemiluminescence, is probably best described as chemiluminescence (CL) produced directly or indirectly as a result of electrochemical reactions. As a powerful analytical technique, ECL has become an important detection method in analytical chemistry in recent years.[1–4]

Bis-[3,4,6-trichloro-2-(pentyloxycarbonyl)-phenyl] oxalate (BTPPO) is one of the most efficient chemiluminescent reagents among the aryl oxalate ester compounds.[5] Our preliminary study showed that BTPPO could give the ECL signal in the presence of hydrogen peroxide at glassy carbon electrode.[6]

In recent years, there were extensive interests in the field of polymer modified electrode.[7] The polymer modified electrode possesses a lot of advantages. One of the most significant advantages is that the multilayer polymer coating yields a three-dimensional reaction zone at the electrode surface, which results in an increase in the rates of reaction occurring at the surface of the electrode. Therefore, the response sensitivity of the electrodes can be greatly improved. Polymeric materials can be attached to the substrate electrode by different ways, such as adsorption, physical mixing, and covalent bonding. In general, electropolymerization, compared with other methods, seems to have more advantages because it is a simple, clean and efficient route for polymeric synthesis. When electrochemical methods are adopted, the rate and extent of the electropolymerization process, as well as the chemical and physical properties of the resulting polymer, can be carefully controlled.[8]

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† The HTML version of this article has been enhanced with colour images.

Metallophthalocyanine complexes are well known as the electrocatalysts for many reactions. Ni phthalocyanine has shown interesting electrocatalytic properties, and there were some reports about using Ni phthalocyanine as electrocatalysts to prepare modified electrodes and these modified electrodes have also been used for analytical applications.[9–15]

In this work, we present the results of the ECL of BTPPO at an indium tin oxide (ITO) electrode coated with a film of poly Ni-tetrasulfophthalocyanine (polyNiTSPc/ITO). NiTSPc electropolymerized on ITO electrode apparently retains phthalocyanine structure in which the Ni atoms play a role similar to that in $Ni(OH)_2$ films.[16] It has been found that polyNiTSPc could catalyze the ECL reaction of BTPPO in the presence of trace H_2O_2, and the enhanced ECL intensity would be increased with increasing H_2O_2 concentration. It is well known that uric acid can react with uricase to produce hydrogen peroxide;[17] therefore, uric acid can be detected by measuring the ECL signal *via* hydrogen peroxide. Based on this fact, an ECL biosensor can be developed for detection of uric acid. Since the enzyme reaction has high selectivity, the developed biosensor showed its selectivity, sensitivity, simplicity and low cost for determination of uric acid in the real serum samples.

2. Experimental

2.1. Apparatus

The experimental equipment for ECL measurement includes a BPCL Ultra-Weak Chemiluminescence Analyzer controlled by a personal computer with BPCL program (Institute of Biophsics, Academia Sinica, China) and an electrochemical analyzer (CHI660a, Shanghai Chenhua Instrument Co., China). A conventional three-electrode system was used as the electrolytic system, which was composed of an ITO electrode as the working electrode, a platinum wire as the counter electrode and an

Ag/AgCl (sat. KCl) electrode as the reference electrode. The ITO electrode area in contact with the electrolyte was 0.5 cm^2. A commercial 5 mL cylindroid's glass cell was used as an ECL cell. Before each measurement, the working electrode was fixed in the same position and directly faced the window of the photomultiplier tube. The ECL cell was washed with 0.2 mol L^{-1} nitric acid and water in sequence before use. All experiments were performed at 25 °C.

2.2. Chemicals

BTPPO, uric acid and uricase were obtained from Fluka. A stock solution of 1.0×10^{-3} mol L^{-1} BTPPO was prepared by dissolving the solid in ethanol, and it was stored at 4 °C in a refrigerator, to minimize exposure to light and air. A working solution of H_2O_2 was prepared fresh daily from 30% (v/v) H_2O_2 (Xinke Electrochemical Reagent Factory, Bengbu, China). Nickel(II) phthalocyanine tetrasulfonic acid tetrasodium salt (NiTSPc, Fluka) was used without further purification. Working solutions were made by dilution of these stock solutions. All other chemical reagents were of analytical grade or better, double-distilled water was used throughout the experiments. The serum samples were obtained from the Hospital of Fuzhou University, Fuzhou, China, and the concentrations of uric acid in the samples were examined by SYSMEX CHEMIX-180 automated biochemistry analyzer (Japanese SYSMEX Corporation). The samples were stored at −20 °C. Before determination, an appropriate amount of serum was diluted by the buffer solution.

2.3. Modification of ITO working electrode

ITO conductive glass was obtained from Weiguang Corporation (Shenzhen, China), and was cut into a 2 cm × 1 cm electrode. The electrode was cleaned in an ultrasonic cleaner sequentially with the following solutions: detergent solution (15 min), deionized water (2 min, twice), acetone (5 min), isopropanol (5 min) and deionized water (10 min, twice).

A film of polyNiTSPc was electrodeposited onto the ITO electrode surface (0.5 cm^2) by using cyclic voltammetry with a scanning rate of 0.10 V s^{-1} in the potential range of 0.2–0.8 V (*versus* Ag/AgCl) in a 0.20 mol L^{-1} NaOH solution containing 5.0×10^{-3} mol L^{-1} NiTSPc under argon deoxygenated conditions. When the electropolymerization was finished, the electrode was rinsed with deionized water, and then transferred to the buffer solution for preservation. The film thickness could be controlled by varying the cycling number of the electropolymerization scanning.

2.4. Static ECL measurement

A three-electrode electrochemical system was use for electrochemical and ECL measurement simultaneously. BTPPO and hydrogen peroxide were mixed just before the experiment in phosphate buffer solution (pH = 10.0). A potential in the range of 0.0–1.1 V was applied to the working electrode and the ECL signal was recorded simultaneously. The emission at 0.82 V was used for the quantitative analysis.

3. Results and discussion

3.1. Cyclic voltammetric behavior of polyNiTSPc/ITO electrode

Fig. 1 shows the typical cyclic voltammograms recorded during the electropolymerization of NiTSPc on ITO electrode. It can be seen from Fig. 1 that a pair of redox peaks, P_I and P_{II}, appeared at E_c = 0.43 V and E_a = 0.56 V, respectively. This can be assigned to the Ni^{III}–Ni^{II} process.[18,19] The continuous increase in the amplitude of the voltammetric peaks indicates that the film has been formed as a result of the conductive polymer film formation. The peak separation increased with the increasing of the thickness of the film. These results are the same as those of the report of Bedioui *et al*.[19]

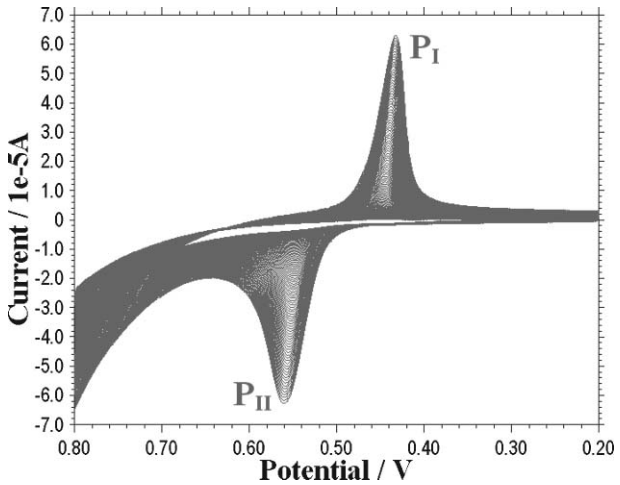

Fig. 1 Cyclic voltammograms of the electrodeposition of NiTSPc film on an ITO electrode by consecutive potential cycling in a fresh solution containing 5.0×10^{-3} mol L^{-1} NiTSPc and 0.20 mol L^{-1} NaOH in the potential range of 0.20–0.80 V (*versus* Ag/AgCl) at a scan rate of 100 mV s^{-1}.

The mechanism of electrooxidative polymerization of the nickel complex is still not clear. There are a lot of papers concerned on this kind of study.[18–23] It is generally accepted that NiTSPc complex is attached to the electrode surface *via* an –O–Ni^{II} bond at first, and during the further scans the attached nickel(II) complex undergoes oxidation to Ni(III).[20,23]The anodic peak at 0.56 V for ITO electrode can be attributed to the Ni(II)/Ni(III) process, whereas the cathodic peaks at 0.43 V in the negative scan corresponds to the reverse process.

3.2. Electro-oxidation and enhanced ECL of BTPPO on polyNiTSPc/ITO electrode

The film thickness could be controlled by the number of the cyclic voltammetry scans in the electropolymerization process, and the effect of film thickness on the BTPPO oxidation was investigated in phosphate buffer solution at pH 10.0. Results showed that the faradic current increased with the increasing of the film thickness. Fig. 2 shows the correlation between the thickness of the polymeric NiTSPc film and the amperometric signal of BTPPO under linear sweep voltammetry (LSV) mode.

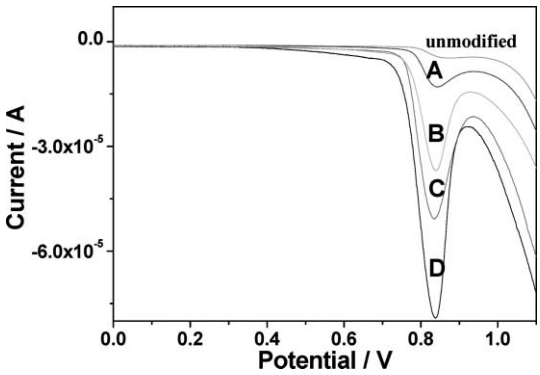

Fig. 2 Linear sweep voltammograms obtained using the polyNiTSPc modified ITO electrode with different thicknesses in a phosphate buffer (pH = 10.0) in presence of BTPPO and H_2O_2 The polymers with different thicknesses were obtained by controlling the number of the electropolymerizing scans: A = 20 cycles, B = 40 cycles, C = 60 cycles, D = 80 cycles. [BTPPO] = 2×10^{-6} mol L^{-1}; [H_2O_2] = 8×10^{-5} mol L^{-1}.

In the current–potential curve, an obvious catalytic oxidation of BTPPO could be observed. The oxidation current of BTPPO increased with the growing of the polymer film. Compared with the response to the oxidation of BTPPO on the bare ITO electrode, the poly-NiTSPc/ITO electrode exhibited a higher current response (see Fig. 2). As for the peak potential, there was a slight shift toward the negative direction (about 20 mV) on the polymeric electrode comparing with that obtained on the unmodified electrode.

Fig. 3 shows the ECL curves of BTPPO in the presence and absence of H_2O_2. If there is no H_2O_2, BTPPO produces no ECL signal on the ITO electrode. We also found that the ECL intensity increased with increasing concentration of H_2O_2. These results are the same as our earlier study.[6] Fig. 4 shows the ECL intensity obtained by using the polyNiTSPc/ITO electrode with different thicknesses of the electropolymerization film. The light emission on the unmodified ITO electrode was quite small in comparison with polyNiTSPc/ITO electrode. The maximum ECL obtained at polyNiTSPc/ITO electrode was about 25 times higher than that obtained at the bare ITO electrode. These results indicated that polyNiTSPc/ITO electrode could greatly enhance the ECL of BTPPO.

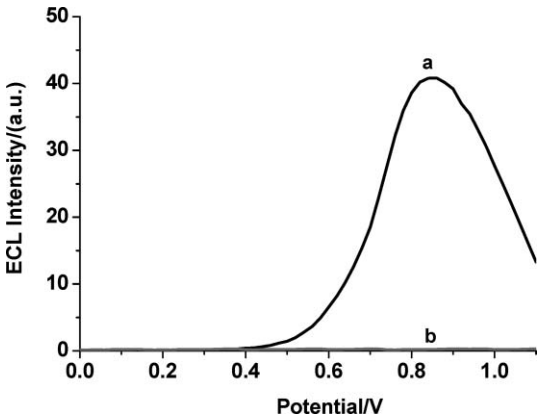

Fig. 3 ECL response of 2.0×10^{-6} mol L^{-1} BTPPO on the bare ITO electrode in the presence (a) and absence (b) of 8.0×10^{-5} mol L^{-1} H_2O_2.

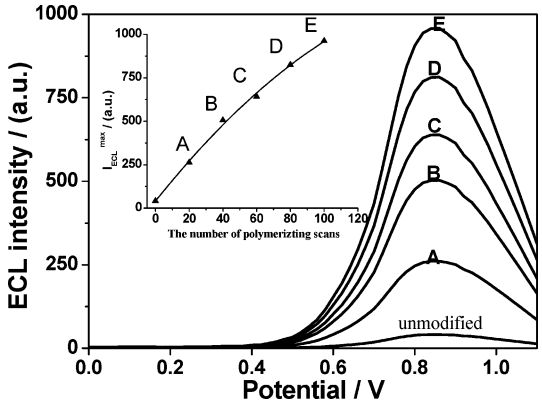

Fig. 4 ECL response of BTPPO on the polyNiTSPc modified ITO electrode with different thicknesses. The polymers with different thicknesses were obtained by controlling the number of the electropolymerizing scans: A = 20 cycles, B = 40 cycles, C = 60 cycles, D = 80 cycles, E = 100 cycles; [BTPPO] = 2.0×10^{-6} mol L^{-1}; [H_2O_2] = 8.0×10^{-5} mol L^{-1}.

3.3. Reproducibility of polyNiTSPc modified ITO electrode

In order to check the reproducibility of the polyNiTSPc/ITO electrode, replicate measurements of ECL for the solution containing 1.5×10^{-6} mol L^{-1} of BTPPO and 8.0×10^{-5} mol L^{-1} of H_2O_2 were carried out. The results showed that the relative standard deviation of the ECL intensity was 3.3% ($n = 7$).

It is also very important for a sensor to be stable for a prolonged time. It was found that there was no significant change of response for the poly-NiTSPc/ITO electrode after it was stored in phosphate buffer for 7 days. The electrode retained 94% of its initial response. Such stability is acceptable for most practical applications.

3.4. Mechanism for the ECL of BTPPO in the presence of H_2O_2 on polyNiTSPc/ITO electrode

The possible mechanism for the ECL of BTPPO in the presence of H_2O_2 in alkaline solution is shown in Fig. 5. BTPPO is oxidized to produce an intermediate, which would react with the hydrogen peroxide in the diffusion layer to form 1,2-dioxetanedione. 1,2-dioxetanedione is then decomposed into a singlet excited state carbon dioxide CO_2* and a ground state carbon dioxide CO_2; when CO_2* returns to the ground state it emits light at 460 nm.[6]

The reason for the enhancement of the ECL of BTPPO on the polyNiTSPc/ITO electrode is the effective generation of oxidation products of BTPPO. It is known that the electrocatalytic activity of the NiPc complex is dependent on the Ni(III)/Ni(II) couple for many reactions.[16,24] When the electrode potential was more positive than the redox potential of Ni(II)/Ni(III), the Ni(III) catalyzed the oxidation of BTPPO, and the produced Ni(II) was re-oxidized to Ni(III) on the modified electrode immediately. The oxidation process of BTPPO was accelerated at the polyNiTSPc/ITO electrode. Then, the oxidation products of BTPPO reacted with hydrogen peroxide to produce emitted light.

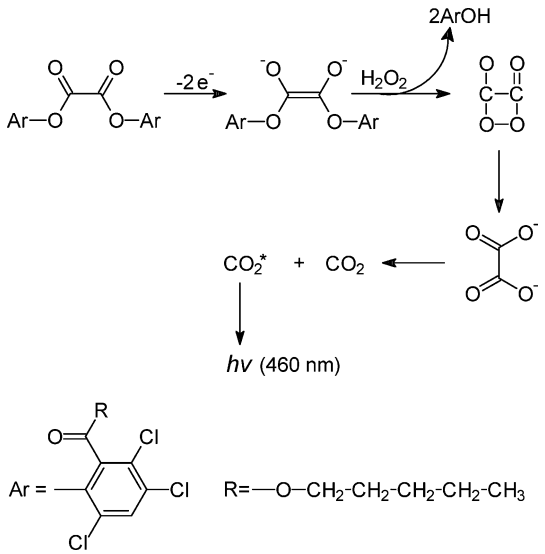

Fig. 5 The possible mechanism for the ECL of BTPPO in the presence of H_2O_2 in alkaline solution.

3.5. Development of an ECL biosensor for uric acid

In our work, we found that the hydrogen peroxide played an important role in the ECL behavior of BTPPO and we also knew there were enzymatic reactions that can produce hydrogen peroxide, such as glucose and uric acid in the presence of glucose oxidase and uricase, respectively.[25,26] Based on which, the BTPPO/uricase/uric acid/polyNiTSPc/ITO ECL system can be used as the sensing system for detection of uric acid.

As pH plays a very important role in the biosensor system, we have investigated the effect of pH on the ECL behavior of BTPPO/H_2O_2 system in our previous paper.[6] It was found that BTPPO electrochemiluminescent reaction is more efficient in pH 10.0. But the higher pH value may lead to the enzyme losing its activity. We investigated the effect of pH on the performance of the biosensor in the range of 6.0–10.0. It was found that the strongest ECL signal could be obtained at pH 8.0, so pH 8.0 was chosen as the optimum pH for subsequent uric acid assays.

3.6. Linearity and detection limit

We investigated the ECL behavior of BTPPO in the presence of uric acid, dissolved oxygen and uricase (the concentration is 10 U mL^{-1}), and found that BTPPO did not give an ECL signal in the presence of uricase. However, when uric acid was added, an ECL signal would be observed. It can be seen from Fig. 6 that the light emission of BTPPO in the presence of uricase was increased with increasing uric acid. The enhanced ECL intensity has a linear relationship between the logarithm of ECL intensity and the logarithm of concentration of uric acid in the range of 6.0×10^{-5}–6.0×10^{-4} mol L^{-1}. The regression equation is

$$\mathrm{Log}(y) = 3.57 + 0.47\mathrm{Log}(x);\ R = 0.9982$$

Where y is the net intensity of ECL, x is the concentration of uric acid. The detection limit was 8.0×10^{-6} mol L^{-1} (defined as $S/N = 3$). The RSD was 3.8% for 2.0×10^{-4} mol L^{-1} uric acid ($n = 11$).

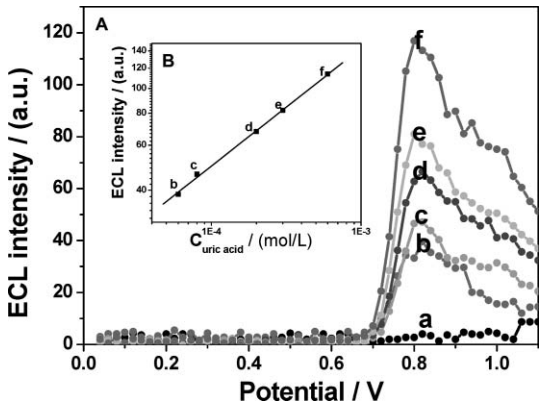

Fig. 6 ECL of BTPPO elicited by the uric acid and uricase reaction in the presence of dissolved oxygen. A: ECL curves with various concentrations of uric acid: (a) 0.0 mol L^{-1}; (b) 6.0×10^{-5} mol L^{-1}; (c) 8.0×10^{-5} mol L^{-1}; (d) 2.0×10^{-4} mol L^{-1}; (e) 3.0×10^{-4} mol L^{-1}; (f) 6.0×10^{-4} mol L^{-1}; B: linear relationship between ECL intensity and the concentration of uric acid. Reaction mixtures contained 10 U mL^{-1} uricase and 1.0×10^{-5} mol L^{-1} BTPPO in pH 8.0 phosphate buffer solution.

3.7. Interference study

It is known that in clinical samples, uric acid is not the only component that could interact with the sensor electrode. Some species common in those samples that could possibly interfered with uric acid determination were tested. Uric acid was placed in the solution and increasing amounts of foreign species were added into the solution. The foreign species were considered not to have interference if it causes a relative error of <5% during the measurement of 8.0×10^{-5} mol L^{-1} uric acid. The tolerated ratio of foreign substances to uric acid was 800 for Cl^- and K^+; 350 for NH_4^+; 200 for Mg^{2+}; 150 for glucose, sucrose, lactose, glutamic acid, urea, maltose and 100 for ascorbic acid. Comparing with electrochemical detection, we found that the interference of ascorbic acid is very light.[27,28]

3.8. Detection of uric acid in the real serum samples

The developed ECL biosensor has been used to detect the uric acid in real serum samples, and the results are shown in Table 1. It can be seen from Table 1 that the proposed method is compares well with the routine method.

In order to evaluate the reliability of the proposed method, the recoveries of some real samples were examined, and the results were shown in Table 2. It can be seen from Table 2 that the recoveries were in the range of 91.4–104.5%.

4. Conclusions

Our experimental results showed that BTPPO gave an ECL signal at a bare ITO electrode in the presence of H_2O_2, when the electrode was modified by NiTSPc the ECL intensity was greatly enhanced, and the enhanced ECL intensity was related to the concentration of H_2O_2. Based on which, an ECL biosensor for determination of uric acid has been developed. We have provided optimal analytical conditions and data reproducibility, linearity, and sensitivity. The established method has the character of high selectivity and low detection limit, which provides new

Table 1 Detection of uric acid in real serum samples

Sample	ECL method/10^{-4}mol L^{-1}	Reference method[a]/10^{-4}mol L^{-1}
1	3.48 ± 0.11	3.56
2	2.05 ± 0.061	2.00
3	3.12 ± 0.098	3.09
4	2.23 ± 0.082	2.19
5	3.54 ± 0.115	3.61
6	3.03 ± 0.111	3.06
7	2.76 ± 0.089	2.73
8	2.67 ± 0.088	2.63
9	2.39 ± 0.085	2.35
10	3.21 ± 0.109	3.29
11	2.41 ± 0.085	2.35
12	3.80 ± 0.123	3.86
13	3.33 ± 0.109	3.42
14	3.15 ± 0.092	3.12
15	2.50 ± 0.089	2.48
16	3.55 ± 0.106	3.58
17	2.87 ± 0.098	2.83
18	2.55 ± 0.081	2.51
19	4.28 ± 0.119	4.33
20	4.32 ± 0.112	4.40
21	6.15 ± 0.103	6.06

[a] Determined by Sysmex CHEMIX-180.

Table 2 Recoveries of the proposed method

Sample	Added/ 10^{-4}mol L^{-1}	Detected/ 10^{-4}mol L^{-1}	Recovery (%)	RSD (%)[a]
1	0.50	0.488	97.6	3.4
2	1.10	1.15	104.5	3.7
3	1.80	1.86	103.3	3.5
4	2.80	2.56	91.4	3.6

[a] $n = 7$.

possibilities to improve the selectivity for detection of uric acid. Moreover, more ECL biosensors can be developed based on this mechanism to detect bioactive compounds which produce H_2O_2 through an enzymatic reaction.

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