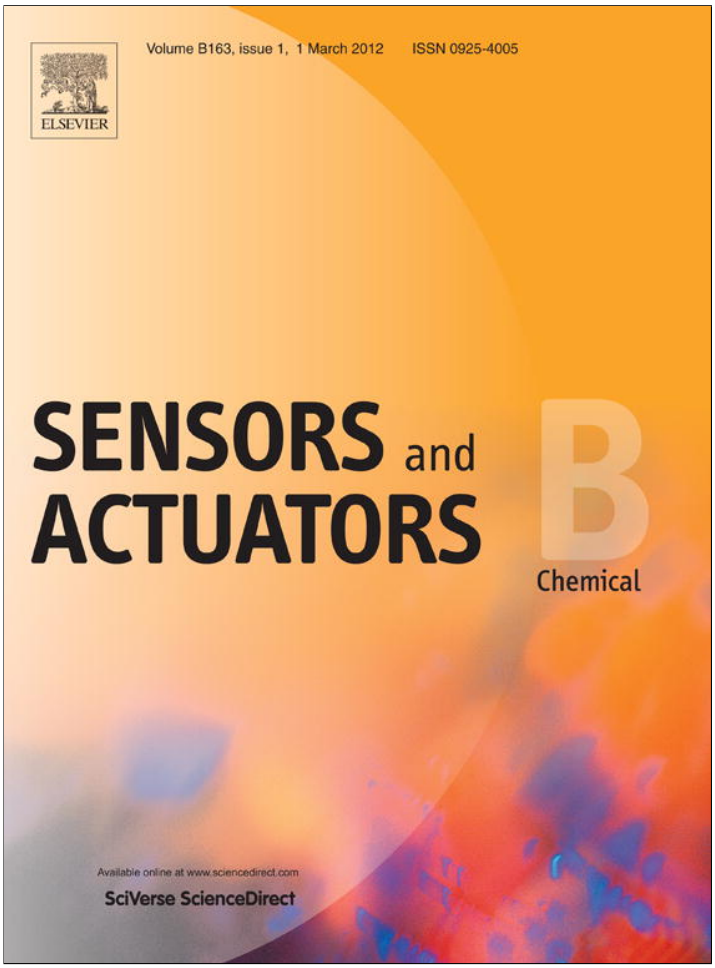

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An electrochemiluminescent biosensor based on polypyrrole immobilized uricase for ultrasensitive uric acid detection

Haihong Chu, Xiuhua Wei, Meisheng Wu, Jilin Yan, Yifeng Tu *

Institute of Analytical Chemistry, Department of Chemistry, Soochow University, Suzhou Industrial Park, Suzhou 215123, PR China

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ABSTRACT

An electrochemiluminescent (ECL) biosensor based on immobilized uricase has been developed for uric acid detection with luminol as signaler. The uricase has been embedded in polypyrrole (PPy) matrix on platinum electrode during the electropolymerization of pyrrole monomer at potential of 0.80 V versus Ag/AgCl. This ECL-based biosensor responds to uric acid due to yielded hydrogen peroxide during its catalytic oxidization by uricase with potassium ferricyanide acted as an electron receptor to promote the enzymatic reaction. The so-generated hydrogen peroxide enhanced the ECL intensity of luminol. The kinetic parameters of enzymatic reaction as maximum reaction rates (V_{max}) and Michaelis–Menten constants (K_m) are also evaluated for 1.42×10^{-3} A s^{-1} and 3.4×10^{-7} M in the presence of potassium ferricyanide, which were all greatly improved. The resulting biosensor showed excellent analytical performance for determination of uric acid as more than 50 times sensitivity than a bare electrode. It gives a 75 pM of detection limit and a relative standard deviation of 4.4% for 6.25×10^{-9} M uric acid ($n = 6$). This ECL-based biosensor has been successfully applied for determination of uric acid in porphyra and kelp samples.

1. Introduction

The biosensor is a kind of analytical devices for the detection of analytes that combines a biological component with a physicochemical detector component. It provides the selective response toward the target analyte based on the bioactive recognition of those bio-species as enzyme, antibody, DNA, receptor, tissue or microbe, etc. [1]; meanwhile the physicochemical transducer such as electrochemical electrode, photon counter, thermosensor, quartz-crystal microbalance, semiconductor device or surface plasmon-resonant device, etc. transfers the content of target analyte into electric output rapidly, accurately and reliably [2]. In nowadays, it displays great importance in the fields of medicine, agriculture, food safety, national security, environmental and industrial monitoring.

To immobilize those bio-species with feasibly affinitive and chemically inert matrix is a pivotal procedure in biosensor constructing [3]. There are conventional methods as physical/chemical adsorption, covalent bonding, cross-linking and entrapment in gels or polymer matrices [4,5]. In all of these means, polymers are advantaged because of their flexibility, corrosion-resistivity, high chemical inertness and facility of processing [6–9]. Polypyrrole (PPy) or its derivatives are very versatile for such purpose. Often times, the enzymes, especially oxidases, have been considered to be immobilized by entrapment in PPy matrix.

In this paper, an electrochemiluminescence (ECL) based biosensor was created with PPy as matrix to immobilize the enzyme and the ECL of luminol for signaling. ECL, a chemical luminescent phenomenon where the luminescence is created by the electrolysis or with further concomitant chemical reactions, has attracted increasing interests in analytical chemistry in recent years [10–15]. Its advantages include high sensitivity, good reproducibility and controllability, etc. Recently, some ECL-based biosensors had been developed for detection of bioactive species [16–19].

With uricase as a bio-recognition species, the created ECL biosensor selectively responds to uric acid (UA). There the uricase catalyzes the oxidation of UA to yield the products as allantoin, H_2O_2 and CO_2 under the promotion of potassium ferricyanide. The catalytically yielded hydrogen peroxide enhanced the ECL of luminol, therefore reflected the content of UA. Here an extremely low detection limit of 75 pM was obtained.

UA is a final product of protein degradation. Its content in blood is an important index of proper renal function for clinical diagnosis [20]. A recent report from Rock and colleagues proposed its importance in cytoplasm for immune system [21]. The sensor developed here with so extreme sensitivity, compared with those reported methods [22–27], is potentially applicable in some practical cases such as cytoplasm researches.

* Corresponding author. Tel.: +86 138 12768378; fax: +86 512 65101162.
E-mail addresses: tuyf@suda.edu.cn, Tuyf_1@hotmail.com (Y. Tu).

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2. Experiment

2.1. Instruments and reagents

A BAS-100A electrochemical analyzer (Bioanalysis System Inc., USA) combined with a custom-built RST300 electrochemiluminescent workstation (Suzhou Risetest Instrument Co. Ltd, PR China) was used for ECL test with a R212 photomultiplier (Hamamatsu, Japan) as the detector which was powered at −800 V. A three-electrode system was used in all experiments. Here the working electrode and the auxiliary electrode were made of platinum, a silver chloride coated silver wire worked as the reference electrode. The absorption spectra were recorded with a TU-1810 UV–Vis spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., PR China).

Luminol was purchased from Fluka (Buchs, Switzerland). Uric acid was purchased from Alfa Aesar (Tianjin) Chemicals Co. Ltd. (Beijing, PR China). Uricase is purchased from Sigma (St. Louis, MO, USA). Pyrrole, lithium perchlorate, potassium ferricyanide, all other chemicals were of analytical grade. Double-distilled water was used in all experiments.

2.2. The fabrication of UA ECL biosensor

By potentiostatic electrolysis at the potential of 0.80 V (versus Ag/AgCl) in 1 mL of phosphate buffered electrolyte solution (pH 7.0) containing 1 μL pyrrole, 8×10^{-2} M $LiClO_4$ and 0.05 g L^{-1} uricase for several minutes, the polypyrrole film would be deposited onto the surface of platinum electrode; meanwhile the uricase could be entrapped into the PPy film. The prepared biosensor was then washed with water and stored in a refrigerator.

2.3. Pre-treatment of the samples

Porphyra and kelp samples were adopted as the model for evaluation of analytical performance of resulting biosensor. The samples were washed, dried at 120 °C for 6 h, and then ground to powder in an agate mortar. Then 0.05 g of the prepared sample was extracted with 8 mL of methanol under ultrasonic for 30 min. The extracts were dried by N_2 blowing, and then dissolved with 1 mL of 0.01 M NaOH. After centrifugation, the supernates were preserved for subsequent determination.

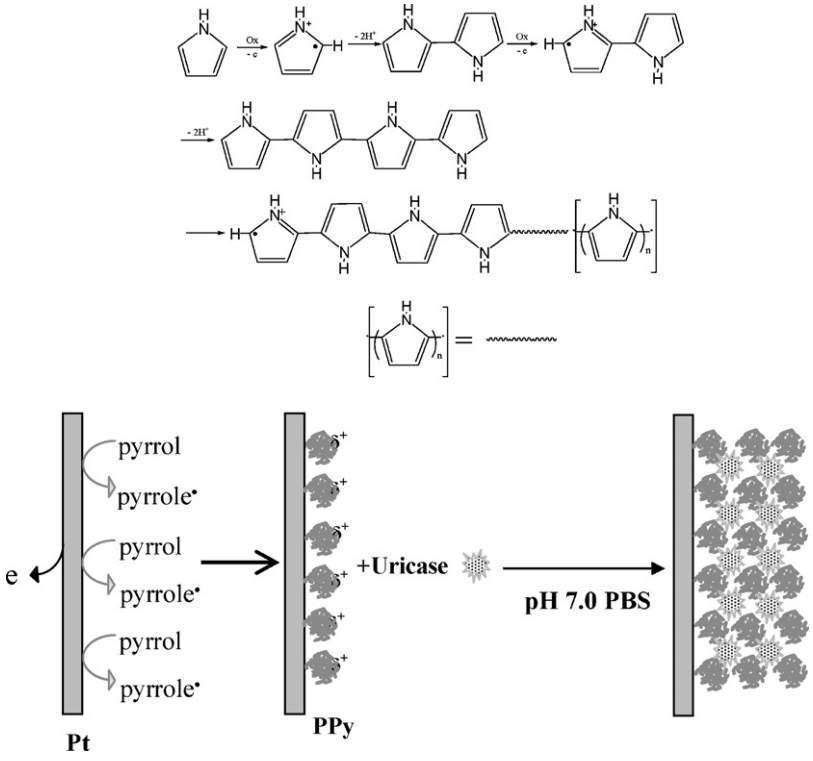
2.4. The detection procedure of uric acid

For best response, all of the conditions included the composition of supporting electrolyte, pH value, concentration of luminol, temperature and electric parameters for ECL would be optimized. The ECL signal was detected during the electrolysis, which strongly depended upon the concentration of uric acid in solution. With the ECL intensity of a blank solution as background, the sensing signal increased linearly upon the escalating concentration of uric acid. The uric acid contents in real samples are therefore calculated from as-obtained regression equation.

3. Results and discussion

3.1. The mechanism of uricase immobilization

As can be seen in Scheme 1, the pyrrole monomers were oxidized to free radicals on the surface of the electrode and linked each other to form the dimer, tetramer and so on. The so formed hydrophobic clews during a chain propagation reaction resulted in the deposition of the PPy onto the electrode surface to form a film. During the growing of the PPy film, the uricase would be entrapped. The uricase could be embedded tightly in the PPy film is mainly due to the electrostatic attraction between the positively charged PPy and the negatively charged enzyme, the hydrogen bonds, and also the stochastic twist PPy chains. Also the PPy film provides good perviousness, which was beneficial for the maintenance of enzyme's activity and the diffusion of the molecules including the substrates.



Scheme 1. The immobilizing procedure of uricase by PPy to create the biosensor.

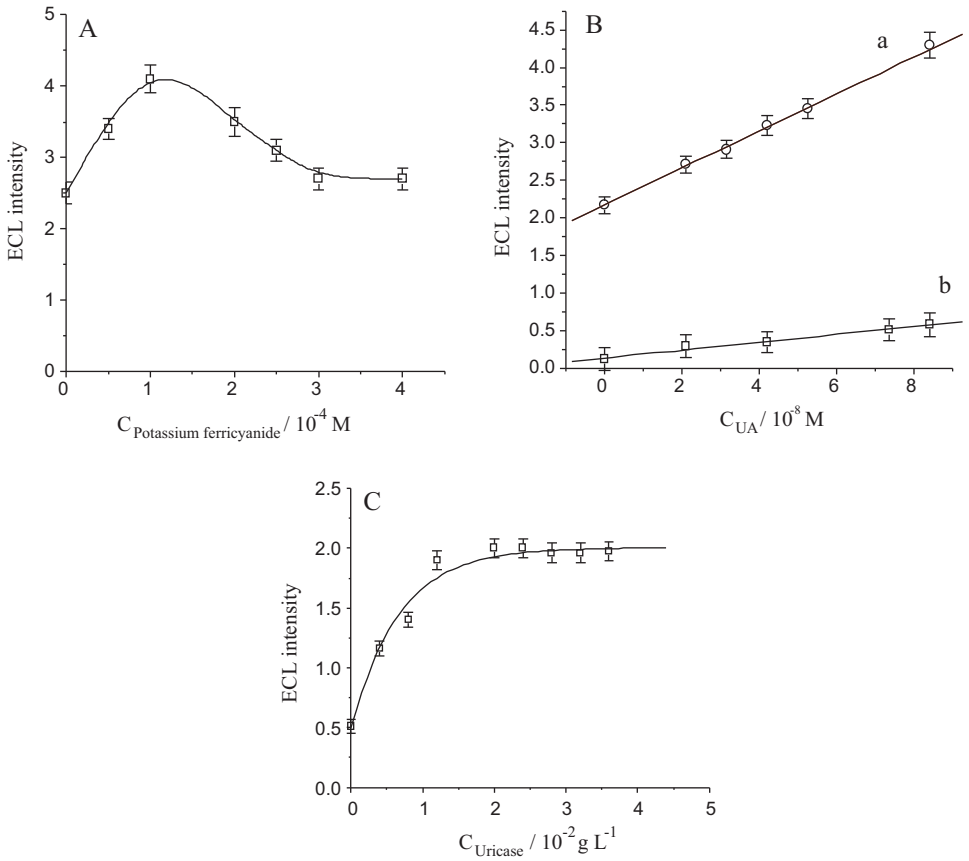


Fig. 1. The influence of (A) potassium ferricyanide, (B) dissolved oxygen, here (a) for oxygen atmosphere and (b) for nitrogen atmosphere and (C) uricase amount on the ECL response on UA.

3.2. The homogeneous ECL response upon UA by enzymatic catalysis

In homogeneous phosphate buffer of pH 8.0, which counterbalanced the conflict from high pH condition which was favorable for luminol's ECL but resulted in the deactivation of enzyme, UA could be detected with luminol's ECL in the presence of uricase. Potassium ferricyanide displayed important promotion to this ECL process. The effect of potassium ferricyanide (from 0 to 9.5×10^{-4} M) on the ECL intensity was studied. As shown in Fig. 1A, the ECL intensity reached the maximum at its concentration of 1.0×10^{-4} M. The existence of oxygen also influenced both the ECL of luminol and the enzymatic reaction. As can be seen in Fig. 1B, the ECL response of UA in oxygen saturated solution was higher than in N_2 purged solution. So the detection was carried out in air-saturated solution for higher sensitivity. The content of uricase greatly affected the ECL intensity. As shown in Fig. 1C, the ECL intensity increased at first and then turned stable when uricase content was higher than 1.4×10^{-2} g L^{-1}.

Temperature is always an important factor for ECL response and bio-catalytic system. As shown in Fig. 2, the curve "a" presents the variation of enzymatic activity upon the temperature, it reached to maximum above 35 °C. Curve "b" presents the influence of temperature on ECL response on UA if there was no uricase in solution. The result suggested that UA was a quencher toward luminol ECL and the quenching degree was temperature related. The counterbalanced result from above-mentioned effects has been shown as curve "c", it showed the maximal ECL intensity at about 23 °C. Generally, it could be regarded as the room temperature, so no thermostatic apparatus was required through the experiments. Under aforementioned optimal conditions, the ECL signal responded upon the concentration of UA within the range from 1.62×10^{-8} to 8.3×10^{-6} M (regression equation: $I = 0.27 + 6.20 \times 10^{7}\, C_{UA}$) with correlation coefficient of 0.9943.

3.3. The performance of resulting ECL-biosensor

3.3.1. The condition optimization of uricase immobilization

The performance of the immobilized enzyme film is significantly influenced by the pH during the electro-polymerization

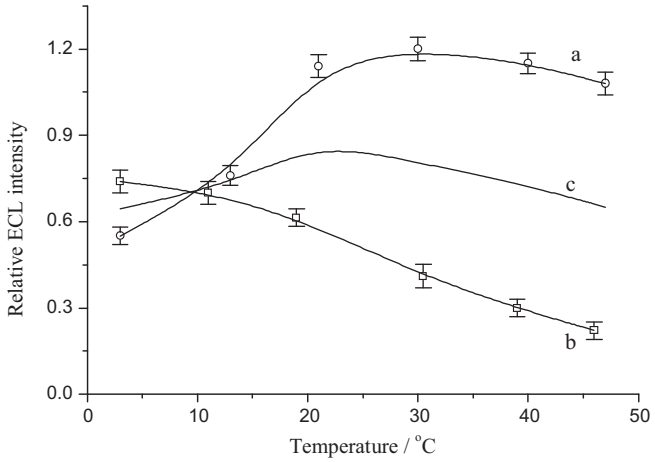


Fig. 2. The influence of temperature on ECL intensity here (a) presents the influence for enzyme activity, (b) for quenching degree from uric acid and (c) for integrated ECL response.

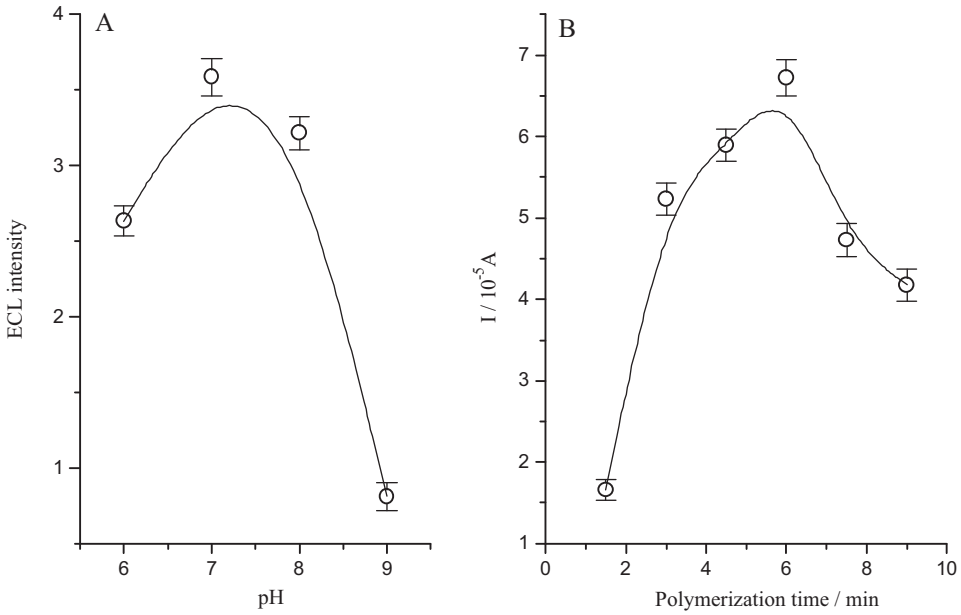


Fig. 3. The effect of (A) pH of electrolysis solution and (B) electro-polymerization time on the response of resulting ECL biosensor.

of pyrrole. As shown in Fig. 3A, pH 7.0 is a best condition for ECL response of resulting biosensor. Fig. 3B shows that the prolonged duration of electro-polymerization resulted in increasing response up to 6 min. The longer time is not beneficial for response. Thus a polymerizing period of 6 min was decided for the deposition of PPy-uricase film for creating the biosensor.

3.3.2. The performance of resulting ECL-biosensor

Under the conditions optimized in Section 3.2, the ECL intensity of the biosensor responded upon UA with a higher sensitivity than in homogeneous mode. Within a wide linear range from 7.5×10^{-11} to 8.3×10^{-6} M, there is a regression equation of $I = 1.16 + 3.15 \times 10^{9}\, C_{UA}$ with correlation coefficient of 0.9892. And it provided an analytical sensitivity (the slope of regression equation) for 50.8 times higher than in homogeneous mode. Compared with some reported results, the UA sensor developed in present work provides the highest sensitivity for UA detection (see Table 1). The relative standard deviation (RSD) of the response was 4.4% for six successive measurements of a 6.25×10^{-9} M UA solution, which testified the good reproducibility of the biosensor.

The selectivity of the resulting biosensor was evaluated by measuring the ECL responses of 2.5×10^{-9} M UA standard solution contained varied amount of interferents. The interferents were considered to cause no disturbance if they made a relative error of less than ±10% on the signal of the UA. The tolerance ratios were found as 10,000-fold for K^+ and Fe^{3+}, 5000-fold for alanine, 1000-fold for I^-, Ca^{2+} and Mn^{2+}, 500-fold for Vitamin C and Mg^{2+}, 200-fold for glutamic acid.

3.4. The kinetics and response mechanism of the resulting ECL biosensor

The UA could be oxidized by oxygen under the catalysis of uricase to yield the H_2O_2 as following equation:

$$\text{uric acid}\,(C_5H_4N_4O_3) + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{allantoin}\,(C_4H_6N_4O_3) + CO_2 + H_2O_2$$

According to our previous investigations [17,28–32], the yielded H_2O_2, one form of reactive oxygen species (ROSs), is the origination of ECL response of resulting biosensor. It could be further involved in the ECL reaction of luminol as an efficient intensifier. The evidence to verify the production of H_2O_2 during the sensing response could be obtained from the cyclic voltammetric tests (Fig. 4). In the presence of uricase, there was a couple of redox peaks showed up around −0.50 V when UA was added into the air-saturated buffer solution, which was attributed to H_2O_2.

The potassium ferricyanide plays an indispensable role in response of resulting biosensor due to its function as an electron receptor to promote the enzyme reaction [33]. The Michaelis–Menten curves [34] in the absence or presence of potassium ferricyanide were detected by UV–vis spectrometry (see Fig. 5). According to Lineweaver–Burk method, V_{max} values are 5.07×10^{-5} A s^{-1} and 1.42×10^{-3} A s^{-1}, K_m values are 1.00×10^{-6} M and 3.4×10^{-7} M for absence and presence of potassium ferricyanide, respectively. Namely, V_{max} promoted for 28 times and K_m decreased to one third in the presence of potassium ferricyanide.

Table 1
The comparison of detection limit with reported methods.

Author(s)	Method	Sample	Detection limit	Ref.
J.M. Zen, J.J. Jou, G. Ilangovan	Square wave voltammetry	Urine	10 nM	[22]
T. Nakaminami, S. Ito, S. Kuwabata, H. Yoneyama	Amperometric sensor	Human blood	0.1 mM	[23]
J. Wang, T. Golden	Amperometric detection HPLC, FIA	Urine	0.10 ng	[24]
Hs. C. Hong, Hs. J. Huang	FIA-CL	Serum, urine	50 nM	[25]
P. Kannan, S. J. Abraham	Amperometry	Serum, urine	50 nM	[26]
Z.F. Chen, Y.B. Zu,	ECL	Urine	1 μM	[27]
Present work	ECL sensor	Sea food	75 pM	

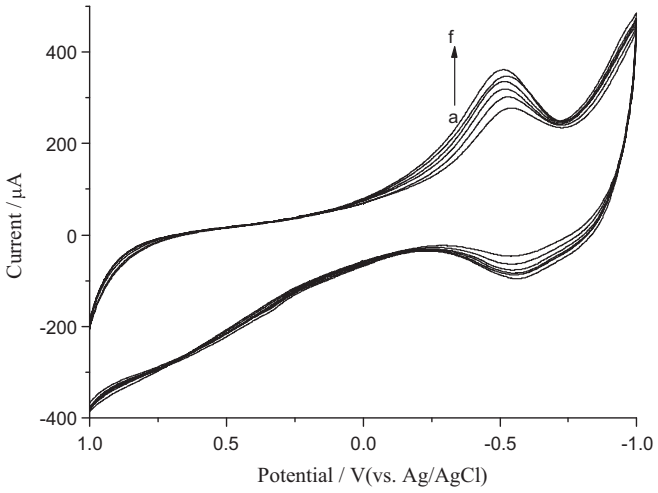


Fig. 4. Cyclic voltammetric curves of the sensor in different concentrated uric acid in air-saturated phosphate buffer (pH = 8.0). C_{UA} = a. 1.25, b. 1.87, c. 2.50, d. 12, e. 37.5, f. 43.7×10^{-9} M.

Meanwhile, another experimental result was also obtained to support the above discussion. If there was no potassium ferricyanide presented, the ECL intensity decreased noticeably when UA was added into the solution ($I = 0.868 - 4.93 \times 10^4\ C_{UA}$). This proved that ECL intensity of luminol would be suppressed by the reducibility of UA in this case, which accorded with the results of Fig. 2b. So there must be a competition between the enzymatic reaction and the reducibility of UA for luminol. It is absolutely necessary to promote the enzymatic reaction by potassium ferricyanide to offset the reducibility of UA to get the positive response on this ECL-based biosensor.

After all of discussions, we can conclude that those enzymatically generated H_2O_2 were the essential of ECL response. They will be further oxidized on electrode surface to yield the $O_2^{\bullet -}$. Then, a Haber–Weiss reaction would occur between them to form the $OH^{\bullet}$, and thereafter via many ways to generate the singlet oxygen ($^1O_2$) [32,35]. The $^1O_2$ is a high energic molecular oxygen with 92 kJ mol^{-1} entropic energy than triplet oxygen ($^3O_2$) [36]; therefore, it can transfer the energy toward the oxidized intermediate of luminol during its deexcitation to result in higher ECL emission. The response mechanism was therefore illustrated as Scheme 2.

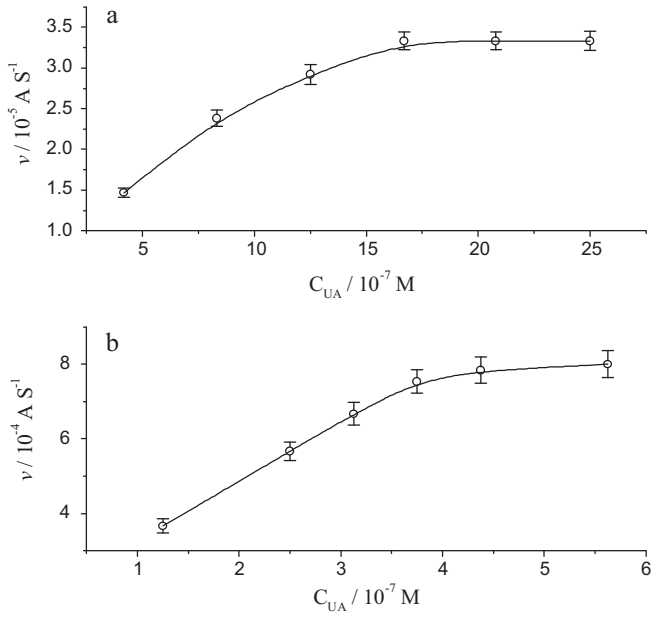
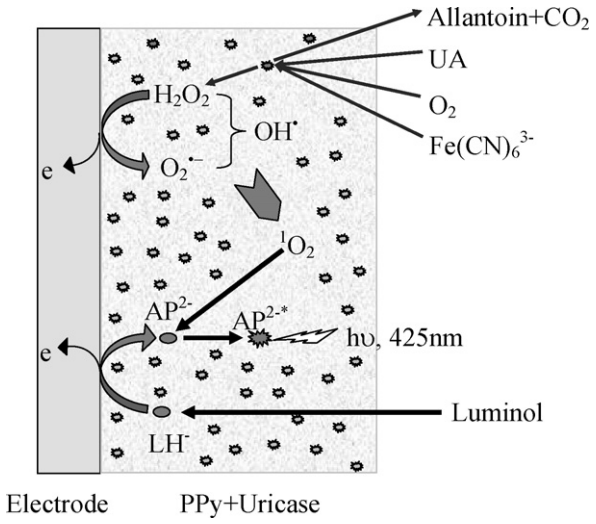


Fig. 5. The kinetic characters of enzymatic reaction (A) without and (B) with potassium ferricyanide in the presence of 10 mg L^{-1} uricase.



Scheme 2. The sensing mechanism of created ECL biosensor.

Table 2
The results of uric acid in real samples and the recovery tests.

Sample	Determined (μg/g)	Added (μg/g)	Determined (μg/g)	Recovery (%)
Porphyra	3.78	2.10	5.78	95.2
			5.74	93.3
			5.68	90.5
Kelp 1	7.35	3.50	11.46	117.4
			10.59	92.6
			10.92	102.0
Kelp 2	5.57	3.50	9.47	111.4
			8.94	96.3
			9.80	120.9

3.5. Quantitative analysis of UA in real samples

Considering the ultra-sensitivity, the urine and serum samples are not appropriate for validation of applicability of developed biosensor because of high contents of UA. Suitable real samples which contained trace amount of UA such as porphyra and kelp are in demand for this purpose. 0.3 μL of prepared sample solution was injected into the supporting solution and was then tested with the developed sensor. The results of UA contents in samples and the recoveries are listed in Table 2, which shows satisfactory results from the so-created ECL-biosensor.

4. Conclusion

An ECL-based biosensor with immobilized uricase in polypyrrole matrix has been successfully constructed and applied to determine the uric acid. It exhibits a fast response to uric acid within the linear range from 7.5×10^{-11} to 8.3×10^{-6} M with good selectivity and reproducibility. The excellent analytical performances of the resulting biosensor in this work have been proved to be attributed to the enhanced ECL by H_2O_2 which was yielded in a uricase catalytic reaction, and the promotion of the enzymatic reaction by potassium ferricyanide.

Acknowledgements

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Biographies

Haihong Chu received her Ph.D. degree in Soochow University under the supervision of Professor Yifeng Tu. Her research focuses on the ECL sensors.

Xiuhua Wei is a Ph.D. candidate in Soochow University under the supervision of Professor Yifeng Tu. Her research focuses on the ECL detectors and applications.

Meisheng Wu received her M.S. degree in 2009 in Soochow University under the supervision of Professor Yifeng Tu. Her research focuses on the ECL biosensors.

Jilin Yan received his Ph.D. degree from Changchun Institute of Applied Chemistry, the Chinese Academy of Sciences in 2006. Then he worked as a postdoctoral research associate in the chemistry department in University of Florida. Currently he is an associate professor at the Chemistry Department in Soochow University, China. His research interests include ECL sensing and molecular recognition.

Yifeng Tu received his PhD degree from Department of Chemistry, Nanjing University, PR China in 2001. Presently, he is a professor in the Department of Chemistry, Soochow University, PR China. His current interests are to develop electrochemical biosensors and electrochemiluminescent detectors for biomedical applications.