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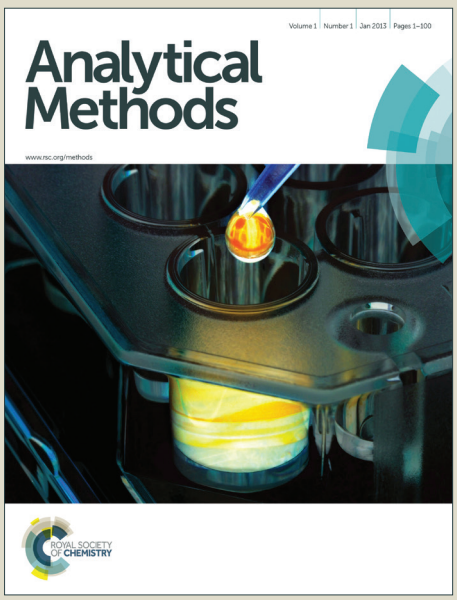

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A label-free morphine immunosensor based on electrochemiluminescence of luminol on nano-Au functionalized indium tin oxide coated glass

Yang Ya[1,2], Wang Xiaoshu[2], Da Qing[2], Jiang Lin[1] and Tu Yifeng[1,3]*

1 Institute of Analytical Chemistry, Soochow University,

2 Department of Forensic Medicine, Soochow University,

3 The Key Lab of Health Chemistry and Molecular Diagnosis of Suzhou.

Dushu Lake Campus of Soochow University, Suzhou Industrial Park,

215123, Suzhou, P. R. China

To whom the correspondence should be addressed; Tu Yifeng, Tel: 86-13812768378;

email: tuyf@suda.edu.cn

Abstract

This paper reports an electrochemiluminescence (ECL)-based morphine (MO) immunosensor using luminol as probe. By the function of hydrolyzed 3-amino-propyltrimethoxysilane, the gold nano-particles had been attached onto the surface of indium tin oxide coated glass. It was hence acting as the matrix to directly immobilize the MO-antibody as sensing host via the adsorption. With a totally label-free mode, the ECL intensity of luminol on this sensor decreased along with the concentration of target MO solution after the immuno-incubation. There is a linear regression upon the logarithm of MO concentration within the range of 2 to 200 ng/mL and a detection limit of 0.82 ng/mL (S/N=3). It has been used to analyze the MO in spiked urine samples with satisfactory recoveries to check its applicability. This label-free sensor has the potential possibility as a disposable, portable and mass-producible device because of the simplicity for preparation and utilization.

Keywords: Immunosensor; Electrochemiluminescence; Luminol; Au nanoparticles; Morphine

1. Introduction

Morphine (MO), an opium alkaloid, is often clinically used as an analgesic agent. Although its use is recommended by the World Health Organization[1], the excess use or abuse will result in toxic symptoms such as respiratory depression, seizures even death[2, 3]. So, the monitoring of morphine content in blood or urine to ensure the safe usage is necessary. Besides, in forensic cases, it is an indicator of heroin taking as its major metabolite. To date, there are several methods for morphine determination, such as radioimmunoassay (RIA)[4], ELISA[5], GC-MS[6, 7], HPLC[8, 9] and electrochemical methods[10, 11]. These methods are all sensitive but require delicate instruments, and are generally time-consuming. In clinical or forensic cases, a rapid, selective, portable and disposable device for spot or mobile detection is in expectation.

Electrochemiluminescence (ECL) holds the merits including high sensitivity, wide dynamic range and good stability with simple and facile operation[12-15]. In recent years, it has been proved as a powerful and important signaling tool in immunoassay[16], food or environmental detection[17], clinical diagnosis[18], DNA sensing[19, 20] and pharmaceutical analysis *etc* [21], there the ECL immunoassay has received considerable attention. Luminol is one of the most popular ECL reagents with high efficiency; thus the luminol-based ECL biosensors have been developed[22, 23].

Gold nanoparticles (AuNPs) have been used as an outstanding sensing matrix owing to its excellent electric conductivity, catalytic activity and biocompatibility[24-26] and also a considerable signal amplifier of luminol-based ECL devices. The sensors can be constructed on AuNPs by the techniques such as electropolymerization[27], layer-by-layer[28], self-assembly[29] or biological template[30]. Recently, the indium-tin oxide (ITO) coated glass, with good conductivity and transparency[31], has been used acting as the electrode in electrochemistry and ECL[32, 33]. These technical findings

have established a great foundation for fabricating the immunosensing ECL device.

In this work, we develop an MO immunosensor with the ECL of luminol as the sensing host. The AuNPs were attached onto the surface of ITO by hydrolyzed 3-aminopropyltrimethoxysilane (APTMS) [34], thereafter to construct the sensor by direct immobilization of MO-antibody onto them by adsorption. After the specific immuno-conjugation of MO with the antibody, in totally a label-free mode, the ECL intensity of luminol on resultant sensor decreased along with the MO concentration. With this very simple methodology, we not only proposed a new route with low-cost, convenient procedure and facile craftwork for sensor preparation, but also provided a possibility about the simplest way to use the sensor.

2. Experimental

2.1 Reagents and materials

Morphine antibody was obtained from Fankel Co. Ltd (Shanghai, China). MO injection was purchased from Northeast Pharmaceutical Group Co. Ltd. (Shenyang, China). The luminol was purchased from Fluka (Buchs, Switzerland). 3-aminopropyl-trimethoxysilane (APTMS) was purchased from Chengdu Aikeda Chemical Reagent Co. Ltd (Chengdu, China). Potassium carbonate (K_2CO_3, 99%), chloroauric acid ($HAuCl_4 \cdot 4H_2O$), bovine serum albumin (BSA, 96-99%), phosphate ($NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$), acetone were obtained from Sigma-Aldrich Chemical Co. (USA). All reagents were used as received without further purification. The ultrapure water was used throughout. ITO glass was purchased from Suzhou Nippon Sheet Glass Electronics Co. Ltd. (Suzhou, China).

2.2 Apparatus

The ECL measurements were carried out with an RST600 ECL workstation

(custom-built, Risetest, Suzhou, China) as reported in our previous paper [35] with a conventional three-electrode system including the AuNPs functionalized ITO acting as working electrode, a platinum wire acting as counter electrode and a saturated calomel electrode acting as reference electrode. An R212 photomultiplier tube (PMT) (Hamamatsu, Japan) was used as a detector powered by a -800V bias potential. The ECL-potential curves were recorded on an MPI-A multifunctional electrochemical analytical system (Xi'An Remax Electronic Science & Technology Co. Ltd, Xi'An, China) in potential sweep mode. The electrochemical impedance spectroscopic (EIS) investigations were carried out on an RST5200 electrochemical workstation (Risetest, Suzhou, China). Scanning electron microscopy (SEM) was performed with an S-4700 scanning electron microanalyzer (Hitachi, Japan). Transmission electron microscopy (TEM) investigations were taken using a 4000 EX microscope (JEOL, Japan). The AFM micrographs were obtained with a Dimension Icon Atomic Force Microscope (Bruker AXS Inc., Madison, WI, USA).

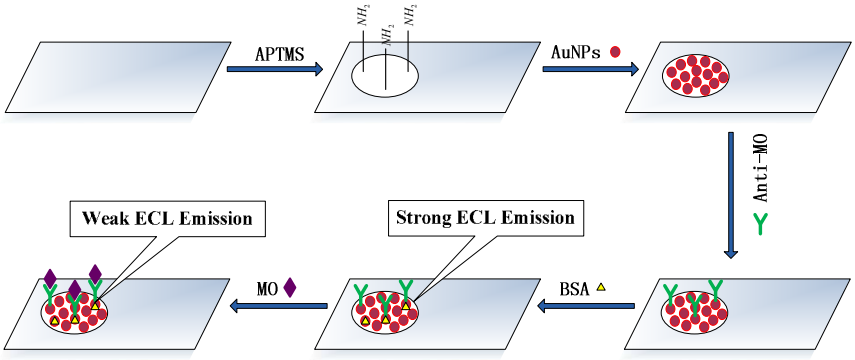
2.3 Preparation of basal working electrode

The ITO glass was cleaned with acetone, anhydrous ethanol, ethanol/1 M NaOH (1:1, v/v), and ultrapure water ultrasonically for 20 min in turn, and dried by nitrogen blowing. 10μL of 0.1% APTMS (in anhydrous ethanol, v/v) was dropped onto its surface, and laid for full volatilization of ethanol in a desiccator. It was then put into a humid environment at 35°C for 4h to ensure the complete hydrolysis of APTMS.

By adding 4.5 mL of sodium citrate solution (1%) into 100 mL of boiling $HAuCl_4$ solution (0.01%) with vigorous stirring, the gold nanoparticles had been prepared[36]. Then, after refinement, some AuNPs dispersoid was dropped onto the surface of APTMS/ITO to obtain an AuNPs/APTMS/ITO matrix.

2.4 Fabrication of immunosensor

Via electrostatic interaction, the antibody protein can be adsorbed onto the surface of AuNPs directly. 20μL of morphine antibody (8 μg mL^{-1}) was dropped onto the AuNPs/APTMS/ITO matrix in wet environment at 4°C for 12h. At last, 20 μL of BSA (1%) solution was dropped on it and placed at room temperature for 60 min to block the residual active sites. The prepared sensor was then flushed with PBS (pH 7.4), dried by nitrogen blowing and stored at 4°C. The whole procedure of the immunosensor preparation is shown in Scheme 1.



Scheme 1 The schematic diagram of fabrication procedure of the ECL immunosensor

2.5 The detection of morphine

The ECL measurements were carried out in 5 mL of PBS solution (pH=8.0) containing 1×10^{-4}M of luminol. The period of pulsed electrolytic potential is 3s. The upper and lower limiting potentials are 1.1 V and -0.1V respectively.

After incubated in differently concentrated MO solution, washed thoroughly with PBS (pH=7.4), the ECL emission on the immunosensor was detected for calibration. The simulate samples were detected to check the practicability of developed sensor using human urine as the succedaneum.

3. Results and discussion

3.1 The characteristics of the developed immunosensor

The AuNPs were attached on ITO by hydrolyzed APTMS with homogeneous dispersion (see Fig. 1). The preparation of uniformly sized AuNPs is the crux to ensure the sensing performance. Here the refinement by centrifugation had been carried on to eliminate those small or agglomerated particles[37]. Thus it is in well distribution with the size of 16±2 nm (see inserted "a, b" in Fig. 1). The experimental results revealed that the size distribution of AuNPs is important for the reproducibility of resulted sensor. The refined AuNPs gave a 2.8% of the RSD for 5 paralleled electrodes, but the crude product gave the result as 8.3%. After the immobilization of MO-Ab, densely stacked soft clumps were obviously found on electrode surface by AFM (shown as insert "c" in Fig. 1) sized for 30 to 50 nm. These evidences clearly demonstrate the fulfillment of sensor constructing.

Fig. 1 should be placed here

The EIS curves of electrode in different stages revealed the change of surface status during the process of sensor preparation[38] (see Fig. 2). The APTMS/ITO (curve b) showed a larger radius of Nyquist diagram than the bare ITO glass (curve a), meant a more difficult electron transporting on APTMS covered ITO glass. When the AuNPs were attached on, the radius of Nyquist diagram (curve c) reduced greatly, indicates an easier electron-transfer owing to the excellent electric conductivity of AuNPs. When MO antibody proteins were immobilized, the EIS showed a largely increased impedance (curve d), forcefully suggests the presence of protein molecules on the electrode surface which greatly inhibited the electron transfer. Finally, when the antigen molecules specifically bounded onto the antibody, the impedance became

larger (curve e), demonstrates the function of developed immunosensor for antigen.

Fig. 2 should be placed here

3.2 Optimization of experimental conditions

The loading quantity of antibody on sensor surface importantly functions affecting the sensing performance. As shown in Fig. 3A, too small or large quantity of Ab will both lead to low response. Low loading of Ab provides very few active sites for immuno-conjugation that dropped down the sensing capacity, and there must be a multilayered accumulation of Ab proteins on the sensor surface to fritter their efficacy when excessively loading. Thus, a balanced response during the static incubation with target solution in a limited period is related to Ab quantity, and will get the greatest at loading quantity of MO-Ab around 160 ng.

Normally, we excited the ECL with a pulsed potential for its high sensitivity. But, the potential sweep mode is better to reveal the relation between ECL emission and the potential, as presented in Fig. 3B. Clearly the ECL intensity is dependent upon the potential on every electrode. With the pulsed mode, the upper and lower limiting potential of electrolytic pulse obviously effect the ECL intensity too, as shown in Fig. 3C. With increasing upper limiting potential, the ECL intensity increased and then could reach a plateau (curve “a”). With decreasing lower limiting potential, the ECL intensity increased till to -0.1V and then decreased (curve “b”). Thus 1.1V of upper limiting potential and -0.1V of lower limiting potential were selected.

Fig. 3 should be placed here

3.3 The analytical performance of the immunosensor toward MO

Within the concentration range of MO from 2 to 200 ng/mL, there is a calibration of ECL intensity upon the logarithm of MO concentration. The linear regression equation is ECL=2.47-0.52logC_{MO} (R^2=0.998, as shown in Fig. 4A). According to the rule of S/N=3, a detection limit (LOD) of 0.82 ng/mL is acquired.

Fig. 4 should be placed here

The stability of resultant sensor was investigated. After the immuno-conjugation with 100 ng/mL of MO (stored in a refrigerator of 4ºC after each test), Fig. 4B displays its relative response during a period of 9 days. Clearly, there is no significant variation in at least 5 days (less than 5% of relative error). It is absolutely believable that the 5 days stability was perfect for disposable use.

The reproducibility of the sensor preparation had also been evaluated, with 8 chips of parallel prepared sensor to detect the MO (100 ng/mL). The tests give an acceptable result as 5.8% of RSD.

Some conceivable coexisted interfering substances including glucose, urea, uric acid and Vitamin C are the familiar components in body fluids such as blood or urine. Their responses on developed sensor have been examined to evaluate the specificity of this immunosensor toward target MO. The sensing values on resulted sensor toward those compounds are listed in Table 1A, suggests the tolerance of resultant sensor for those compounds for at least 32 times. Of course, for consideration of there might be more concentrated interferences in real samples, the simplest way is to dilute the sample. The results indicated (see Table 1B) that the disturbance for ECL signal from urine components would decrease to less than 2% if it was diluted for 1200

times with PBS. Thus, the possible interference of urine components can be avoided successfully without any other pretreatment, beneficial to simplify the procedure of sensor application. As we known, about 10% of MO is excreted via urine as its prototype. So, there might be the concentration at the level of $\mu g \cdot mL^{-1}$ in urine (the consumers generally take the drug for dozens of milligrams). Thus, although the samples are diluted for 1200 times, the sensitivity of the sensor is still generally balanced for this concentration.

Table 1 should be placed here

3. 4 Determination of MO in spiked urine samples

Urine from volunteer was collected to be employed as the sample. After spiked with differently concentrated MO and diluted for 1200 times, the ECL emission of every solution on immunosensor were recorded. The quantified results and recoveries are listed in Table 2.

Table 2 should be placed here

4. Conclusion

In this work, a label-free morphine immunosensor has been developed based on direct adsorption of antibody onto the surface of gold nano-particles functionalized ITO glass. The ECL signal of luminol on this sensor decreased linearly upon the logarithm of MO concentration within a dynamic range from 2 to 200 ng/mL with a detection limit of 0.82 ng/mL (S/N=3). The preparing process and application of this immunosensor is very simple. It might be useful for morphine surveillance in clinical

or forensic cases. We believe that the developed sensor had the prospect of mass-production for disposable and portable usage because of its feasibility of industrial scale fabrication and cheap cost.

Acknowledgements

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Table 1 A: The interference of potential coexisted compounds on sensing response (MO: 100 ng/mL, others: 500 ng/mL); B: The interfering degree of urine at different dilution multiple.

A					B		
Compound	ECL emission	Response	Relative response %	Tolerance times	Dilution multiple	ECL emission	Relative error %
blank	3.70	-	-	-	0	0.96	25.9
MO	1.43	2.27	-	-	400	0.511	13.8
glucose	3.50	0.20	8.8	57	800	0.216	5.8
urea	3.42	0.28	12.3	41	1200	0.043	1.2
uric acid	3.32	0.38	16.7	32	1600	0.023	0.6
Vitamine C	3.42	0.28	12.3	41			

Table 2: The detected results and the recoveries.

Sample	Spiked (ng/mL)	Detect average (ng/mL), n=5	RSD (%)	Recovery (%)
1	8.0	7.29	7.1	91.1
2	15.0	15.4	5.8	102.7
3	25.0	27.2	4.4	108.8
4	40.0	36.4	5.7	91.0
5	80.0	85.5	5.1	106.9
6	160	152	6.9	95.0

Figure Captions:

Fig. 1 The SEM image of AuNPs/APTMS/ITO. Here inserted "a" is the TEM image of AuNPs, "b" is the size distribution of refined AuNPs and "c" is the AFM image of obtained MO immunosensor.

Fig. 2 The EIS curves of (a) bare ITO, (b) APTMS/ITO, (c) AuNPs/APTMS/ITO, (d) obtained MO immunosensor and (e) responded toward MO, in 0.05 M KCl solution containing 2 mM $[Fe(CN)_6]^{3-}$.

Fig. 3 (A) The effect of loading amount of MO-Ab on ECL intensity; (B) the ECL-potential curves of (a) bare ITO, (b) APTMS/ITO, (c) AuNPs/APTMS/ITO, (d) obtained MO immunosensor and (e) responded toward MO; (C) the effect of (a) upper limiting potential and (b) lower limiting potential on sensor performance.

Fig. 4 (A) The calibration curve for ECL signal to logarithm of MO concentration; (B) the stability of the obtained immunosensor.

Fig. 1

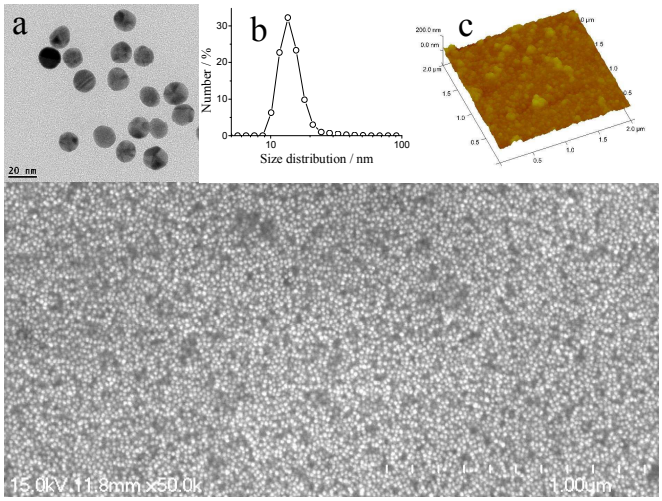

Fig. 2

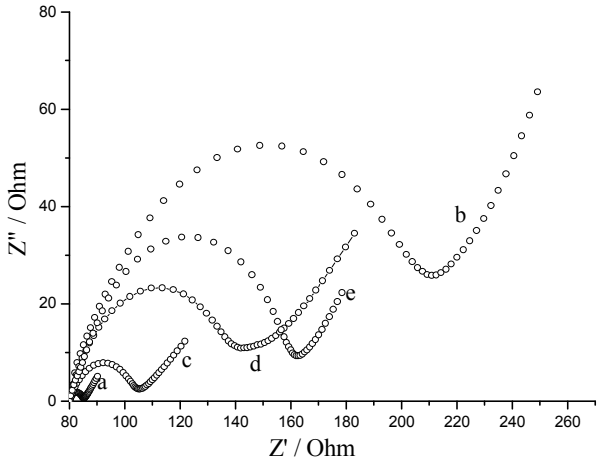

Fig. 3

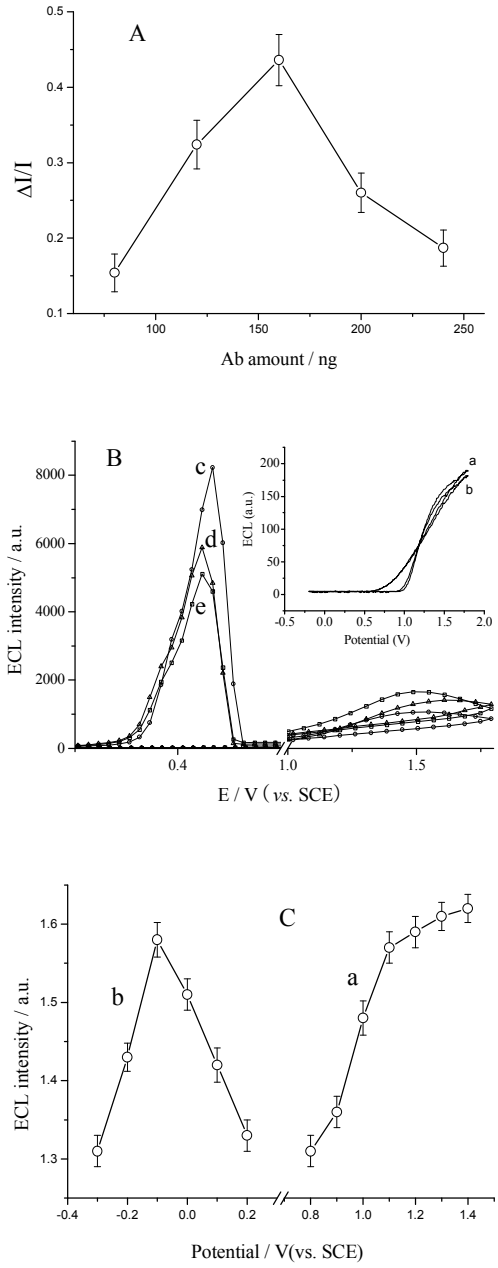

Fig. 4