A Convenient Electrochemiluminescent Immunosensor for Detecting Methamphetamine Antibody

Wang Xia\textsuperscript{a}, Zhai Suyan\textsuperscript{b}, Liu Chao\textsuperscript{b}, Wang Xiaoshu\textsuperscript{b}, Yang Ya\textsuperscript{b} and Tu Yifeng\textsuperscript{a}*

\textsuperscript{a}: College of Chemistry, Chemical Engineering and Materials

\textsuperscript{b}: Department of Forensic Medicine, Institute of Forensic Science

Dushu Lake Campus, Soochow University, Industrial Park, 215123, Suzhou, P. R. China

*Corresponding Authors: Prof. Tu Yifeng, Tel: 86-13812768378; Email: tuyf@suda.edu.cn; Dr. Yang Ya, Email: yangya@suda.edu.cn
Abstract

An antibody-based immunotherapy for Methamphetamine (MA) addictive treatment is getting more and more attention in recent years. However, the study about methamphetamine antibody (Anti-MA) immunodetections are rare, owing to the lack of immunogenicity of small molecule MA. This study provides a simple and effective approach to develop a convenient electrochemiluminescent (ECL) immunosensor for the test of Anti-MA. In short, the synthetic holoantigen of MA is immobilized on a homemade gold nanoparticles modified electrode as the sensing host for the specific recognition and detection of Anti-MA. The research suggested, under optimal experimental conditions, the ECL intensity on resultant immunosensor has a nice wide-linear regression toward the Anti-MA quantity within the range from 0.03 ng to 3.07 ng with a detection limit of 2.32 pg. It responded the dosage of Anti-MA in spiked blood samples with satisfactory recovery. According to the research, the developed sensor promised a portable Anti-MA fast seized device which performs quickness and convenience, and will be helpful for forensic identification and clinic treatment.

Keywords: Electrochemiluminescence; Immunosensor; Methamphetamine; Methamphetamine antibody; Drug addiction therapy
Introduction

Methamphetamine (MA) has gradually become one of the major abused drugs worldwide, which results cognitive dysfunction, psychological problem, infections, and numerous complications for drug users and induces significant problems about social security, legal action and moral issues. MA effects heavily on parts of the central nervous system. A lot of research results show that MA may induce dopaminergic neurotoxicity in the animal and human striatum. The mechanisms include effects on dopaminergic signaling and dopamine oxidation, glutamate induced excitotoxicity, oxidative stress and inflammatory cytokines, disruption of mitochondria. It is found by the researches of clinical and drug abuse cases that people can get a series of symptoms soon after injection of MA. The drug users will be overexcitement and behave strangely, such as head swinging and limbs twisting. MA can not only cause obvious biological damage to the human body, but also sociological damages to the families of addicts, and furthermore bring in the disturbance and destruction to society. Therefore, how to treat MA addicts has received more and more attention in recent years.

As we all know, when virus or bacteria invaded human, the body owns the ability to engender those "antibodies" or "lymphocytes" to fight those pathogens. Medically, this defense agency was aroused to launch such a confrontation is called an "immune response". As learned knowledge, the methamphetamine antibody (Anti-MA) has high affinity with MA, can be used to reduce the drug effect with no adverse actions of the neurons by isolating the drug molecules from the brain. The quantity of Anti-MA has an important influence on therapeutic effect. So, quantitative analysis of Anti-MA is an important task in those cases including drug enforcement, addiction treatment, drugged driving and so on. Basically, current
methodologies for the measuring of Anti-MA are mainly the enzyme-linked immunosorbent assays (ELISA), liquid chromatography - mass spectrometry (LC-MS) and gas chromatography - mass spectrometry (GC-MS)\textsuperscript{12-14}, which have the advantages of high accuracy and sensitivity with only a little amount of samples\textsuperscript{15,16}. However, the high cost of equipment and the immobility of above methods have limited their real application\textsuperscript{17,18}. And also, those methods are impossible to be applied for identification of the drug abusers on-site because of their large equipment. For all the Anti-MA detection applications, the cost-effective and accelerating judicial procedures have become increasingly important\textsuperscript{19-21}. So there is a great value to develop a simple, rapid, low-cost and high sensitive method for detection of Anti-MA in drug users’ samples.

Currently, optical assay systems have been intensely focused, because of their high ability of monitoring those interactions between the substances and sensor substrate\textsuperscript{22-25}. As a matter of fact, there are many studies about applications of optical methods, like Raman spectroscopy, reflectrometric interference spectroscopy and so on. In a variety of optical sensing techniques, electrochemiluminescence (ECL) is widely used especially as a useful tool for detecting biomolecules in liquid media\textsuperscript{26-28}. There have been several reports on ECL biosensors for detection of various proteins\textsuperscript{29-31}. Considering the good conductivity and huge surface area of gold nanoparticles (GNPs), we deposited GNPs on indium tin oxide glass (ITO) to increase the luminous background signal\textsuperscript{32,33}. This research may provide a new insight for Anti-MA detectors.

In this paper, we proposed an optimal qualitative and quantitative sensing device by integrating ultrasensitive electrochemiluminescence and high selective immune response to detect Anti-MA. Compared to those traditional methods, it has the
advantages of low cost, easy operation, portability, high sensitivity and accuracy. The sensor was produced by immobilizing the synthesized MA-BSA holoantigen onto the GNPs decorated ITO glass surface. With the ECL signal of this blank sensor in luminol solution as base value, the response toward different concentrated Anti-MA was obtained as the result in different degrees of inhibition of luminescent signal after incubation with Anti-MA. The quantifying property of developed sensor was then demonstrated by testing the Anti-MA in spiked simulative blood serum.

Experiments

Reagents and solution

Methamphetamine antibody was obtained from Gene Tex Inc. (Shanghai, P. R. China). Methamphetamine-BSA was supplied by Guangzhou Wondfo Biotech Co., Ltd. (P. R. China). Luminol was obtained from Fluka Chem. Co. (USA). APTMS (3-aminopropyltrimethoxysilane) was bought from Aladdin Industrial Co., Ltd. (Shanghai, P. R. China). BSA (Bovine serum albumin), chloroauric acid (HAuCl₄·4H₂O), trisodium citrate and other major chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, P. R. China). All chemicals and reagents are analytical grade, or better. 10 mM phosphate buffer solution (pH=7.4) was used as supporting solution for MA and Anti-MA. Luminol was diluted with 0.2 M phosphate buffer solution (pH=8.0) and to serve as electrolyte in ECL analysis. Unless otherwise stated, all the solutions were prepared with 18.2 MΩ water from an ALH-6000-U water purification system (Aquapro, P. R. China).

Apparatus

All experiments were performed in a conventional three-electrode system with a lab-built ECL workstation, as exposited in our previous papers 34, 35. The ITO glass
was provided by Guluo Glass Electronics Co., Ltd. (Luoyang, P. R. China), used as the substrate electrode after being cut into 5.0 cm × 1.0 cm sheets.

SEM (Scanning electron microscope) images were supplied by as S-4700 scanning electron microanalyzer (Hitachi, Japan) and GNPs’ size and shape were observed by the TEM (transmission electron microscope) with an accelerating voltage of 200 KV (FEI, USA). RST-5200 Electrochemical Workstation (Risetest Instruments Co. Ltd., Suzhou, P. R. China) was used to take tasks of CV (cyclic voltammetry) and EIS (electrochemical impedance spectroscopy).

Preparation of the GNPs

The GNPs are obtained by reducing chloroauric acid (HAuCl₄) with trisodium citrate according to previous paper 36. Briefly, 500 μL of 4.86×10⁻² M HAuCl₄ solution was thoroughly mixed with 99.5 mL of ultra-pure water into a round-bottom flask by heating stirring, and then 4.5 mL solution containing 45 mg trisodium citrate was injected into the flask at boiling. The above solution was continuously stirred until the color of reaction mixture changed to wine red. In order to obtain gold nanoparticles with uniform size distribution, 4.0 ml of acquired gold sol was taken out and low speed centrifugation for 10 min to wipe out these oversize particles and then centrifuged at 10000 rpm for 0.5 h to get the condensed sol. Finally, the condensed sol was dispersed with 0.25 mL of water to get a homogeneously sized GNPs sol. It was stored at 4 °C in case of not in using.

Fabrication of GNPs/ITO basal electrode

In our experiment, ITO (indium tin oxide) glass was used as the fundamental substrate of immune sensor, and the subsequent modifications and reactions were performed on it. The GNPs/ITO basal electrode was made by following steps: the carved ITO glass was ultrasonically washed with NaOH/EtOH and acetone
respectively to remove impurities and organics from the surface. Then the ITO glass was steeped in 30% (V/V) NH$_3$·H$_2$O overnight to obtain a hydroxylated surface with abundant -OH groups. Next, the APTMS (3-aminopropyl trimethoxysilane) was added on this surface to act as linker. Theoretically the aldehyde group and carboxyl group can be fixed on the surface of ITO by condensation reaction, and the amino group can bind with GNPs via Au-N bond. In this way, the gold nanoparticles are fixed firmly on the surface of ITO$^{37,38}$. In this research, 50 μL of GNPs sol was added at ITO surface, put in a petri dish for a period of 4 h to deposit the GNPs to prepare full functionalized substrate.

Synthesis of methamphetamine holoantigen

This immunosensor is arranged for antibody test based on the recognition of immobilized antigen protein$^{39-41}$. Since MA is a small molecule and does not have immunogenicity, we need to combine MA molecule into biological macromolecule without losing antigenic activity. Firstly a hapten was formed by introducing a carboxyl group onto the second amino group of MA, then the holoantigen (MA-BSA) was prepared by conjugation of carrier protein (BSA) via a carbodiimide (EDPC) method. The final product was identified by UV spectroscopy and SDS-PAGE$^{42,43}$. Compared to the reference material (the standard MA-BSA, supplied by Guangzhou Wondfo Biotech Co., Ltd.), the synthesized antigen is not only effective but also advantageous of low cost and easy acquisition.

Fabrication of the Anti-MA immunosensor

Apply 10 μL of solution containing 200 ng of MA-BSA to the GNPs/ITO fundamental electrode and incubated overnight at room temperature to capture MA-BSA on GNPs via Au-N bond and electrostatic interaction$^{44}$. Furthermore, the nonspecific binding sites were sealed with 10 μL of solution containing 0.2 mg of
BSA for 1 h at 25 °C. The sensor was thus obtained after being washed by phosphate buffer solution at pH 7.4 to get rid of uncombined substances on the surface. The final sensor was saved in the refrigerator at the temperature of 4 °C.

_The ECL determination of Anti-MA with the immunosensor_

In this research, a platinum foil and a silver wire were served as the auxiliary electrode and reference electrode, the GNPs functionalized electrode or obtained sensor was used as the working electrode. The three-electrode system is immersed in the buffer solution (pH 8.0) with 5.5×10⁻⁷ M luminol in it. The ECL signal gets excited by a pulsed potential. Then a photomultiplier tube (PMT) converted the ECL intensity to an electrical signal. The main influence factors on the output signal of the sensor were all optimized. Immune complex was formed when the MA antibody combined with the MA-BSA antigen, it will lead to weaker ECL emission of luminol on the sensor. Under the optimal state, the accurate measurement of Anti-MA was achieved.

**Results and discussion**

In this study, luminol is selected as the luminescent probe. It can emit ECL under a wide range of experimental conditions and has the advantages of high quantum yield, good water solubility and low cost. Luminol has been widely used in inorganic analysis, organic analysis and immunoassay. More significantly, the use of luminol is sufficiently permissive without any limit of patent right as other luminescent probe as tris(2,2-bipyridyl) ruthenium. The ECL mechanism of luminol has already been clearly discussed, there was a two steps oxidation of its anion to yield an intermediate and final product (3-amino-benzenedicarboxylicacid) by an
open-ring reaction, in an alkaline medium. Thus, the excitons will be generated by the energy transfer between those triples of this product, and to emit the light \(^{47}\).

**The fabrication process and detection mechanism of the sensor**

As we can see from Scheme 1, ITO glass was used as the platform of immune sensor after pretreated to acquire a hydrophilic surface (a). With APTMS acting as linker after its hydrolysis on ITO surface (b), GNPs were combined on ITO via the Au-N bonds (c). GNPs have the characteristics of facilitating the electron transfer, catalyzing oxidation of luminol, so it further amplifies the signals of response. Thereafter, each one of the sensing components will lead to lowered intensity of ECL output when they were combined onto the electrode successively. Such being the case, the MA-BSA or BSA was directly connected with GNPs via Au-N(S) bonds (d), which will impede luminol to contact with the electrode surface, then to inhibit the ECL signal own to the spatial steric hindrance and the obstacle to electron transfer. Thus there would be a maximized balanced output (I), which viewed as the reference value of this sensor. When Anti-MA combined with the holoantigen to generate immune complex (e), a significant decrease of ECL intensity (ΔI) will happen. The relative value (ΔI/I) has a positive correlation to the amount of Anti-MA, which demonstrated the result to enable its quantitative detection.

**Scheme 1 should be placed here**

**The optimization and properties of GNPs substrate electrode**

For obtaining a better ECL signal on the GNPs functionalized ITO electrode, the preparation conditions are optimized, covering the dosage of APTMS, the deposition
of GNPs, soaking time of GNPs and the decoration temperature. Fig. 1A reveals that
the proper dosage of ATPMS is 5 μg. In Fig. 1B, it is obvious that the ECL intensity
related with the depositing amount of GNPs, the highest ECL is obtained at a
moderate quantity. Taking count of the number of GNPs by ImageJ analysis of SEM
micro-image (the inserted picture in Fig. 1B), we can calculate the optimal dosage of
GNPs on electrode surface is 8.5 μg. Fig. 1C demonstrated that the ECL signal was
enhanced significantly along with the deposition period of GNPs, 4 hours is the best.
Fig. 1D reveals the change of electrode performance at different temperature for
GNPs deposition. Higher temperature (from 5 to 25 °C) is favorable for gaining
higher ECL output, but there is a threshold. The ECL emission on those electrodes
that prepared under the temperature higher than 25 °C will decrease on the contrary.
The primary causation of this variation could be attributed to the thermo-dynamics of
those nanoparticles. They move faster at higher temperature, thus to induce larger
amount of deposited nanoparticles on electrode surface to intensify the ECL, which
can be evidenced by the thicker color of electrode surface (see attached pictures in
this figure). However, when the stack-up of nanoparticles getting to a certain point,
they will agglomerate, it can be demonstrated by the color of electrode surface which
changed to blue-purple from pink. As we all known, this will result in the failure of
nano-functionalization, thus to give a poor ECL performance.

Fig. 1 should be placed here

The period and upper/lower limiting potentials of electrolytic pulse can all
influence the properties of the sensor for its ECL emission. As illustrated in Fig. 2A, 3
s as the best pulse period, and in Fig. 2B -0.3 V and 1.2 V as the optimal lower
limiting potential and upper limiting potential. Here the upper limiting potential is a crucial factor for usage of ITO glass because of its safe potential window, too high potential (>1.5V) would cause its damage. The reason of why it can be safely applied in this sensor to act as a basal substrate is totally thanks to the function of those decorated NGPs, which reduced the oxidation potential of luminol. More detailed discussion will be presented in next part.

The pH condition of medium for best ECL response and to preserve inherent biological activity of biomolecules is also required to be discussed. Although there is a spontaneous dependence of luminol’s ECL upon higher pH value (no matter curve a or b in Fig. 2C), but the effect of GNPs for enhancing its ECL only presented in the range between pH 7.5 to 9.0, dominant in 8.0 to 8.5, as the inset curve in this figure. Considering that the weak alkaline solution is benefit to hold the activity of those biological molecules, a pH value of 8.0 corresponding to largest attribution of GNPs is just what we seeking.

Thus, as the result (presented in Fig. 2D), the ECL intensity of the GNPs electrodes (curve c), which showed 17-fold stronger than on bare ITO (curve a), even more than ATPMS covered one (curve b), demonstrated that GNPs has good catalysis for oxidation of luminol. And just agree with the discussion in sensing mechanism, the ECL output decreased along with the loading of sensing components (see the real maps of ECL output, curves c, d, e in Fig. 2D).

It can be seen from the particle size distribution map (Fig. 3A) that the diameter of synthesized GNPs is within the range of 12 nm to 16 nm. The electrochemical

Fig. 2 should be placed here
impedance spectroscopy (EIS) was applied to study the variations of the multistage electrode surface. As shown in Fig. 3B (all of the experiments were carried on in a 100 mM KCl solution containing 5 mM K₃Fe(CN)₆, with the frequency from 0.1 Hz to 100 kHz), the electrochemical impedance of APTMS/ITO electrode (curve b) is larger than bare ITO glass (curve a). But a much smaller radius (Rₑ) of GNPs decorated electrode (curve c) was observed, indicating the better conductivity of GNPs. Thereafter, hence the holoantigen immobilization (curve d) and immune complex formation (curve e), the impedance greatly increased successively due to their steric resistance and the non-conductivity. These evidences proved that GNPs, MA-BSA and Anti-MA are well fixed onto electrode surface.

In order to further examine the modification on the electrode surface, the cyclic voltammetry curves (CV) of Fe(CN)₆⁴⁻/³⁻ on multistage electrodes were also revealed in Fig. 3C. A well-shaped CV of Fe(CN)₆⁴⁻/³⁻ is presented on bare ITO glass (curve a). But the peak current reduced to a great extent (curve b) after modifying with APTMS, for the reason there was a polymeric silicon rubber of hydrolyzed APTMS which was nonconductive and electrochemically inactive. Thus the effective area of electrode would be reduced when it coated on the surface of ITO, to result in a decreased current. However, after the deposition of GNPs, the redox currents increased significantly (curve c) even greater than that on bare ITO. The promotion of electricity is due to the function of GNPs based on its catalytic ability. Fig. 3D displays that two remarkable oxidation peaks of luminol appeared at the potential of about 0.56V (greater than normal Au electrode (curve b in Fig. 3D)) and 0.92V on GNPs modified electrode (curve a in Fig. 3D), other than indistinctive peak on bulk Au electrode (curve b in Fig. 3D). It is clear there the peak potentials of the oxidation of the luminol were obviously decreased. The results have revealed that the
decoration of GNPs not only promoted the ECL emission of luminol, but also reduced
the risk of destroying the ITO under high potential, the voltage of 1.2 V guarantees
sufficient oxidation level of luminol to trigger a satisfactory ECL signal. All above
consequences indicated that GNPs/ITO electrode surface owns an excellent
electrochemical activity.

 GNPs not only greatly accelerates the electron transfer rate but also enlarge the
area of electrode surface. To characterize the active electrochemical surface areas of
the modified electrode and bare ITO electrode, their electrochemical behaviour
toward [Fe(CN)₆]⁴⁻/³⁻ at different scan rates was investigated (see Fig. 4A and Fig.
4B). Refer to Randles-Sevcik equation, \( I_p = 2.72 \times 10^5 n^{3/2} A D^{1/2} C \nu^{1/2} \), by to calibrate the
\( I_p \) values toward \( \nu^{1/2} \) to get the slope (inserted in Fig. 4A and 4B)⁴⁹-⁵¹, we can find that
the GNPs electrode has a larger surface area for around 1.14 multiple than bare ITO
electrode. So, it is remarkable that the enhanced redox current of luminol on GNPs
electrode was contributed simultaneously from larger surface area and higher
catalytical activity.

 The reconfirmation of immunosensor design

 SEM can take useful insight to the microstructure of the prepared electrode.
Compared with the clean SEM image of bare ITO glass, the gold nanoparticles were
evenly distributed on electrode surface (as shown in Fig. 5A and Fig. 5B). The size
and shape of GNPs were presented by TEM image (the smaller picture) inserted in Fig. 4B, displayed a diameter ranging from 12 to 16 nm. The SEM of the Anti-MA immune sensor (Fig. 5C) shows that a layer of sol of MA-BSA evenly decorated on the surface of GNPs electrode. This proves the successful preparation of the sensor from another perspective.

Fig. 5 should be placed here

The analytical properties of resultant immunosensor

Another important factor is the dosage of fixed MA-BSA on GNPs/ITO surface, which affect the properties of obtained sensor. Either too much or too little MA-BSA quantity will result in the de-sensibility of the sensor. We can see from Fig. 6 A, the highest responding value of the ECL intensity was achieved at 200 ng of MA-BSA. Meantime, we fixed MA-BSA on the GNPs/ITO surface over 8 hours with the temperature of 4 °C in order to stabilize the antigen well on the electrode.

When all the experimental conditions are optimized, an assessment about the sensitivity of the result sensor was made. It is clearly shown by Fig. 6B, the response value ($\Delta I/I$) of the immune sensor closely related to the amount of Anti-MA. The Fig. 6C displayed that the answer signal of the sensor shown a linear positive correlation toward the logarithm of Anti-MA quantity ranged from 0.03 ng to 3.07 ng with the equation of $\Delta I/I = 0.682 + 0.432 \log[\text{Anti-MA}]$ (R = 0.996) and a detection limit of 2.32 pg ($10^{3S/R'+1}$).

The so-produced ECL immune sensor has been achieved a lower level of detection limit, and is proved to be a reliable, simple and convenient method. Referring to another comparable immune system (as anti-ampicillin monoclonal
antibody, 50 µg/kg in animal tissue)\textsuperscript{52}, the sensor would meet the detection requirements of Anti-MA in drug users although there was no accurate reference value.

**Fig. 6 should be placed here**

In order to evaluate the detection performance of the sensor we developed, we integrated some other detection methods, as shown in Table 1. Compared with other methods, our sensor currently shows better sensing ability represented by higher sensitivity.

**Table 1 should be placed here**

The stability test of the immune sensor was studied by detecting 0.06 ng Anti-MA sample with only one sensor for successive five electrochemical pulse signals, and then RSD value of 4.06 % was obtained. After storing at 4 °C for 17 days, the ECL signal on the same sensor barely changed, indicating that the stability of the sensor is excellent. And the reproducibility of the sensor was also researched with three parallel sensors, and then the final RSD is 4.16 %, suggesting a good performance of the sensor.

The selectivity of the result sensor is depended upon the specific recognition between antigens and antibodies, but a number of existing active sites on the sensor may cause occasional adsorption by chemical or physical interfering substances. BSA blocking is a frequently-used and valid mean for interference elimination. With 10 µL solution containing 15 ng albumin acted as the interferent, we investigated the
function of BSA for a sealant. Fig. 7A, revealed a very strong interference without employing BSA. Nevertheless, the interference will reduced along with the introduction of BSA to the surface of the sensor. The results shown that 200 ng BSA was the best choice. Furthermore, the BSA blocked sensor was inspected by responding to 0.06 ng Anti-MA and potential interferents (6 ng glucose (GLC), 6 ng uric acid (UA), 6 ng albumin (ALB) and 6 ng ascorbic acid (AA)). As illustrated in the Fig. 7B, less than 5 % of the ECL signal change was found, implying that at least two-thousand times of the tolerance for these substances. In order to avoid some interferences from other retaining impurities in serum sample, we take the dilution method. As illustrated in Fig. 7C, the ECL response of blank serum sample of four healthy people (two men and two women of different ages) decreased along with the dilution, and it is clearly found that it would not be reduce furtherly (less than 10 %) after diluting the serum sample to 500 multiples. This proof implies a convenient method to eliminate those interferences from those unknown coexistences. According to related report, there will be greater or equal amount of monoclonal antibody as drug taking when they were injected for therapy (45-108 μg/mL)\textsuperscript{56}, it is believable that the sensor is bearable for this 500 multiple dilution because of its sensitivity.

To test the simulated serum samples

To investigate the practicability of the immunosensor, the sensor was tested by verifying the recovery of the detection of Anti-MA in simulated serum samples. A four-sample test was carried on by adding different content of Anti-MA into blank serum. The results of recovery rates ranged from 88.0 % to 108 %, implying good
accuracy of the sensor, as shown in Table 2. It proves once again that our sensor has
the characters of highly accuracy and selectivity.

Table 2 should be placed here

Conclusion

In this paper, we have achieved a simple and label-free ECL immune sensor on
GNPs/ITO for detecting Anti-MA. Under the best detection conditions, the declining
degree of the ECL signal was closely correlated with the logarithm of the Anti-MA
quantity from 0.03 ng to 3.07 ng, with a detection limit of 2.32 pg. The immunosensor
was prepared in a simple and cost-effective way, which displayed an expected
performance with an ultra-low detection limit. The obtained immunosensor colligated
the high sensitivity of ECL and excellent specific recognition of immunoreaction,
which could be taken for a promising approach for Anti-MA detection. It would be a
great value to serve the need of forensic identification and clinical treatment and
provides an auxiliary approach to determine effectiveness of the drug treatment.

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Figure Captions

Scheme 1. The fabricating procedure and sensing mechanism of the immunosensor.

Fig. 1 The optimal conditions for preparation of GNPs/ITO electrode: (A) The content of APTMS; (B) The amount of GNPs (inserted is the map of SEM count of GNPs); (C) The soaking time of GNPs deposition and (D) the temperature of GNPs decoration (the insets are the color change of obtained GNPs/ITO at different temperature).

Fig. 2 The optimization of ECL detection conditions of (A) pulse period; (B) supplied voltages and (C) pH of the buffer solution on (a) bare ITO and (b) GNPs/ITO, $C_{\text{luminol}} = 1.0 \times 10^{-6}$ M (inserted one is the difference of b to a). (D) The ECL maps of (a) ITO, (b) APTMS/ITO, (c) GNPs/ITO, (d) immunosensor and (e) as “d” but after immune-reaction, 3 s of pulse period, -0.3/1.2 V of lower/upper limiting potential, pH = 8.0, $C_{\text{luminol}} = 1.0 \times 10^{-6}$ M.

Fig. 3 (A) The DLS of GNPs, (B) The EIS (in 0.1 M KCl solution containing 5 mM $K_3Fe(CN)_6$, with the frequency range from 0.1 Hz to 100 kHz, and a perturb voltage of 5 mV) and (C) CVs (in 0.1 M KCl solution containing 5 mM $K_3Fe(CN)_6$, 50 mV s$^{-1}$ of scan rate) of (a) ITO, (b) APTMS/ITO, (c) GNPs/ITO, (d) immunosensor and (e) responded toward Anti-MA. (D) The CVs of $5.0 \times 10^{-3}$ M luminol on (a) GNPs/ITO and (b) a commonly bulk Au electrode, with scan rate of 50 mV s$^{-1}$ in phosphate buffer solution (pH = 8.0).
Fig. 4 The CVs of (A) bare ITO, (B) GNPs/ITO electrode in 0.1 M KCl solution containing 5 mM Fe(CN)$_6^{3-/4-}$ at scan rates from 0.025 to 0.2Vs$^{-1}$, the inserted are the corresponding calibration of peak currents vs. (scan rate)$^{1/2}$.

Fig. 5 The SEM images of (A) ITO, (B) GNPs/ITO, here the inserted one is the TEM image of GNP, (C) resultant immunosensor.

Fig. 6 (A) The optimal dosage of MA-BSA. (B) The ECL response for differently concentrated Anti-MA, $C_{\text{Luminol}}=5.5 \times 10^{-7}$ M, pH=8.0. (C) The relationship between the respond signals and the logarithm of Anti-MA concentration. Other conditions are same as in Fig. 2.

Fig. 7 (A) The effect of BSA blocking on anti-interference ability of immunosensor. (B) The disturbance of different interferent on resultant immunosensor. (C) The ECL response of serum samples along with the diluted multiple. All conditions are same as in Fig. 6.
Scheme 1

[Diagram of the scheme]
Fig. 1
Fig. 2

A

\[ \text{ECL Intensity} \]

Time/s

1.2

1.4

1.6

1.8

2.0

2.2

2.4

2.6

B

\[ \text{ECL Intensity} \]

E/N

0.4

0.6

0.8

1.0

1.2

1.4

C

\[ \text{ECL Intensity} \]

PH

6.0

6.5

7.0

7.5

8.0

8.5

9.0

D

\[ \text{ECL Intensity} \]

Time/s

0

0.5

1.0

1.5

2.0

2.5

3.0

3.5

4.0

4.5

5.0

a

b

c

d

e
Fig. 3
Fig. 4

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)
Fig. 5
Fig. 6
Fig. 7

A.

B.

C.
Table 1 The comparison of detection limit of different Anti-MA test methods.

| Detection method | Volume of sample | Detection limit quantity | Detenction limit concentration | References |
|------------------|------------------|--------------------------|-------------------------------|------------|
| ELISA            | 100 μl           | --                       | 410 nM                        | [53]       |
| ELISA            | 1 mL             | --                       | 0.1 mg/mL                     | [54]       |
| ELISA            | --               | --                       | 1:50000                       | [55]       |
| ECL Biosensor    | 10 μL            | 2.32 pg                  | --                            | Present work |
Table 2 The detection results of Anti-MA in spiked serum samples (n=4)

| Measurement No. | Anti-MA in sample (ng) | Spiked (ng) | Found (ng)  | Recovery (%) | RSD (% n=5) |
|-----------------|------------------------|-------------|-------------|--------------|-------------|
| 1               | 0                      | 18.0        | 19.3±0.30   | 107          | 2.86        |
| 2               | 0                      | 36.0        | 36.7±0.42   | 102          | 2.14        |
| 3               | 0                      | 54.0        | 48.9±0.18   | 90.5         | 3.24        |
| 4               | 0                      | 90.0        | 79.2±0.42   | 88.0         | 3.55        |