A label-free electrochemiluminescent immunosensor for glutamate decarboxylase antibody detection on AuNPs supporting interface

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ABSTRACT

In this paper, we will describe a novel lable-free electrochemiluminescent (ECL) immunosensor for the detection of glutamate decarboxylase antibody (GADA) in which Au nanoparticles (AuNPs) had pre-functionalized the indium tin oxide (ITO) glass to support the sensing interface. The preparation of the basal electrode only need a simple two-step drop coating, a thin polymer of hydrolyzed 3-aminopropyl trimethoxysilane, and the AuNPs gel on the ITO substrate. The AuNPs not only enhanced the ECL signal of luminol, but also acted to immobilize the glutamate decarboxylase (GAD) to build the sensing host. This immunosensor exhibits excellent specificity, reproducibility and stability. On resultant sensor, after the direct immunoreaction, the decreased ECL intensity has a good linear regression toward the logarithm of GADA concentration in the range of $0.30\,\mathrm{ng\,mL^{-1}}$ to $50\,\mathrm{ng\,mL^{-1}}$ with a detection limit of $0.10\,\mathrm{ng\,mL^{-1}}$. The proposed senor has prospective capability for the clinical detection of GADA in human serums, which had important value for diagnosis and precaution of type-1 diabetes or latent autoimmune diabetes in adult (LADA).

1. Introduction

Diabetes mellitus is a chronic endocrine disease with the characters of hyperglycemia and glucose metabolism disorder. The incidence of type 1 diabetes mellitus (T1DM), a genetically immune-mediated form of diabetes which was caused by dysfunction of β-cell for insulin secretion [1,2], has doubled in last 20 years [3,4]. The latent autoimmune diabetes in adult (LADA) [5,6] is also resulted by chronic β-cell auto-immune injury. These two types of diabetes are both insulin-dependent, thus the early screening and symptom monitoring is very important for survival [7,8]. Some autoimmune markers [9–14] including glutamate decarboxylase antibody (GADA), islet cell antibody (ICA), insulin autoantibody (IAA), protein tyrosine phosphatase antibody (anti-IA₂), and zinc transporter 8 autoantibody (ZnT8A) *etc* can be found in serum of those persons whom are in risk of autoimmune injury for their pancreatic islet cells or ingredients [15]. The level of their positive rate reflects the prognosis of islet cell disabling. The abnormal rise of GADA is most closely related with T1DM, and can be employed to distinguish LADA from Type II diabetes, will appear in an early phase, exist for a long duration and has highly positive rate. Since the 1990s, the glutamate decarboxylase (GAD, catalyzes the decarboxylation of glutamate,

an inhibitory neurotransmitter in central nervous system, to yield 4-aminobutyric acid) has been confirmed universally as a vital target antigen in pancreatic β-cell immune injury, thus, the GADA has attracted intensive attention in recent years [16,17]. Its assay is of great value for early diagnosis, monitoring and prevention of T1DM and LADA [18].

The familiar methods as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) have already been maturely used for GADA detection. On account of the requirement of labeled enzymes or fluorescent reagents, these methods are costly and time-consuming. The techniques as electrochemistry [19,20] or surface plasmon resonance (SPR) [21,22] have been demonstrated as prospective signaling tunnel for immunosensors with remarkable advantages including the possibility of real-time analysis without the labeling procedure. The nano-materials provide proper substrate for privileged immobilization of the bio-recognition molecules as antibodies, enzyme, peptide, DNA or RNA. Here the gold nanoparticles, displays excellent conductivity, catalytic activity, and good biocompatibility, has exhibited a significant potential application in chemical sensors or biosensors [23–25].

In this investigation, commenced with indium tin oxide glass (ITO), the Au nanoparticles (AuNPs) were assembled on it via the adhesion of

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hydrolyzed polymer of (3-aminopropyl)trimethoxysilane (APTMS) to construct a basal electrochemical electrode. The GAD can be directly immobilized on it to obtain a label free immunosensor for GADA. With luminol as the probe for electrochemically luminescent signaling, the GADA was quantitatively analyzed according to the quenching effect for this electrochemiluminescence (ECL). Opposite the general mode of immunosensors, use to immobilize antibody on the electrode to detect antigen, this is a label-free ECL immunosensor to detect antibody. It not only reduces the tedious labeling process, but also develops a new method for the fixation of proteic antigen without any binder. In addition, the sensor preparation needs only simple drop coatings with low cost. So, the technics is considerable an alternative approach for massive production of disposable devices, just meet the demand of clinical use. This sensor possesses great specificity, high sensitivity and fast signaling, suitable for high-throughput immunoassay [26–32].

This sensing device can be easily built in the detection cell of flow-injection analysis (FIA) system. Followed the proposal of Ruzicka and Hansen [33] in 1975 which first induced the dynamic mode into chemical analysis, Dr. G. D. Christian [34], one of the pioneers, has tremendously contributed to the development of FIA technique. In recent years, the FIA is feckly to be employed as standard configuration in many applications in the fields of industrial, environmental monitoring and clinical chemistry, *etc*. A series of ECL-FIA cell [35–38] configured with a pump/sampling unit have been developed for this purpose in our research and exhibit excellent performance. On this platform, the high efficient detection is enable no matter under dynamic or stopped-flow mode (Table 1).

2. Experiments

2.1. Reagents

Synthetic GAD_{65} peptides (HPLC purified) and rabbit GADA (Rabbits Polyclonal lgG) were supplied by Qiyi Biological Technology Co., Ltd (Shanghai, P. R. China). The ELISA kit for GADA (EUROIMMUN, Medizinische Labordiagnostika AG). Luminol was purchased from Fluka Chem. Co. (USA). 3-aminopropyltrimethoxysilane (APTMS) was purchased from Aladdin Industrial Co., Ltd. (Shanghai, P. R. China). Bovine serum albumin (BSA) and other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, P. R. China). All reagents are of analytical grade and used as received without further purification. The buffer and supporting solutions employed in this work are: 0.01 M phosphate buffer solution (pH = 7.4) for GAD_{65} peptides and GADA; 0.2 M phosphate buffer solution (pH = 8.0) containing 1×10^{-6} M luminol for ECL detection.

2.2. Apparatus

The ITO glass was purchased from Suzhou Nippon Sheet Glass Electronics Co. Ltd. (Suzhou, P. R. China), cut into 1.0 cm × 5.0 cm pieces, are used as substrate electrode with certain area. The ECL

experiments were carried out on a lab-built system as described in our previous papers [35,39]. All experiments were carried out by a three-electrode system.

Scanning electron microscope (SEM) (S-4700 scanning electron microanalyzer, Hitachi, Japan) and transmission electron microscope (TEM) (FEI, USA, at an accelerating voltage of 200 kV) were used to observe the size and morphology of nanomaterials. The measurements of electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were performed on an RST-5200 Electrochemical Workstation (Risetest Instruments Co. Ltd., Suzhou, P. R. China).

2.3. Synthesis of the AuNPs

Citrate-stabilized AuNPs were prepared by water-phase reduction of $AuCl_4^-$ by trisodium citrate referred to previous papers [40]. In brief, 1 mL of 2.43×10^{-2} M $HAuCl_4$ solution was added into a glass flask containing 99 mL of ultrapure water with vigorous stirring, and then, until to boiling, 4.5 mL of 1% trisodium citrate solution was added into it, and continued stirring until the color changed to wine red. Taken 4 mL of this AuNPs colloid to centrifuge at 3000 rpm for 10 min to eliminate those larger or agglomerated particles, and to condense the supernatant by centrifugation again at 10,000 rpm for 30 min, the expectant AuNPs gel was obtained.

2.4. Preparation of AuNPs/ITO basal electrode

The preparation procedure of the immunosensor can be illustrated by Scheme 1.

The first step is to decorate the AuNPs on ITO glass. Primarily, the ITO glass was ultrasonically washed with 1:1 (V/V) ethanol / NaOH (1 M) mixture, acetone, ultrapure water (30 min each) in sequence, then immersed in 30% $NH_3 \cdot H_2O$ for 12 h to acquire a hydrophilic surface with dense -OH groups. Thereafter, the 0.05% APTMS anhydrous ethanol solution was scattered on it. After the volatilization of ethanol in a saturated ethanol atmosphere, it was then transferred into a hygrothermostat to ensure the complete hydrolysis of APTMS at 55 °C. Subsequently, 50 μL of condensed AuNPs gel was dropped onto its surface, laid in quiescence for a period of 0.5 h to deposit the AuNPs. Finally, it was washed with ultra-pure water and dried with nitrogen blowing.

2.5. Fabrication of the immunosensor

This immunosensor is designed as an antibody recognizable sensing matrix based on immobilized antigen protein, GAD. It presents two different isoforms as GAD_{65} and GAD_{67} with molecular weight of 65 or 67 kDa, encoded by two distinct genes respectively. Here GAD_{65} is more related to T1DM which expressed in human islet tissue. The majority of GADA is GAD_{65} antibody in serum of diabetic patients, meanwhile the GAD_{67} antibody also can bind with GAD_{65}, so the GAD_{65} is logical to be used in this work for GADA sensing.

On account of the good biocompatibility of AuNPs, which can directly fix protein molecules, it ensures a strong interaction between them via Au-N or Au-S bonds or electrostatic interaction [41–43]. 10 μL 50 μg/mL GAD_{65} solution was dropped onto the AuNPs/ITO basal electrode and incubated for overnight at 4 °C, then to block non-specific binding sites with 10 μL of 2% BSA for 2 h at room temperature, the sensor is acquired after thoroughly rinsed with phosphate buffer solution (pH = 7.4) to remove non-chemically bonded substances on the electrode. The resultant sensor was stored at 4 °C when not in use.

2.6. The ECL investigations of electrodes and the determination of GADA with so-obtained immunosensor

In experiments, the prepared AuNPs decorated electrode or resultant sensor served as working electrode, while a silver wire as quasi-

Table 1
Comparison of linear range and detection limit of different GADA sensors.

Types of biosensor	Linear range (ng mL^{-1})	Detection limit (ng mL^{-1})	Reference
SAMs/SPR biosensor	3.3×10^4 $- 2.6 \times 10^5$	–	[22]
3-MPA/11-MUA/SPR immunosensor	1.0×10^3 $- 5.2 \times 10^4$	–	[44]
SAMs/SPR immunosensor	$78 - 4.6 \times 10^4$	–	[45]
AuNPs/anti IgG-HRP/SPR immunosensor	~100	0.03	[46]
ELISA	$7.4 - 2.9 \times 10^3$	–	[47]
ECL immunosensor	$0.30 - 50$	0.10	This study