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A sensitive electrochemiluminescent biosensor based on AuNP-functionalized ITO for a label-free immunoassay of C-peptide

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ABSTRACT

The C-peptide is a co-product of pancreatic β-cells during insulin secretion; its content in body fluid is closely related to diabetes. This paper reports an immune-sensing strategy for a simple and effective assay of C-peptide based on label-free electrochemiluminescent (ECL) signaling, with high sensitivity and specificity. The basal electrode was constructed of an indium tin oxide (ITO) glass as a conductive substrate, which was decorated by Au nanoparticles (AuNPs) with hydrolysed (3-aminopropyl)trimethoxysilane as the linker. The characteristics of the fabricated electrode were investigated by electron microscopy, cyclic voltammetry, and electrochemical impedance spectroscopy. After immobilizing the C-peptide antibody, which takes great advantage of AuNPs' binding capacity, this immunosensor can quantify C-peptide using luminol as the ECL probe. By measuring ECL inhibition, calibration can be established to report the C-peptide concentration between 0.05 ng mL^{-1} and 100 ng mL^{-1} with a detection limit of 0.0142 ng mL^{-1}. As a proof of concept, the proposed strategy is a promising and versatile platform for the clinical diagnosis, classification, and research of diabetes.

1. Introduction

C-peptide, also known as the connecting peptide, is a component of blood secreted by pancreatic cells. Human C-peptide consists of 31 amino acids with an isoelectric point of approximately pH 3.0 [1]. Because it is produced by the proteolytic dissociation of proinsulin, its amount is equimolar to that of insulin. C-peptide is barely absorbed by the kidneys, resulting in the excretion of at least 85% in urine [2]. The half-life of C-peptide in plasma (20 to 30 min) is longer than that of insulin (3 to 5 min), making it five times more concentrated than insulin [3]. Human C-peptide concentration in urine (reference interval, 40 to 150 ng mL^{-1}) [4,5] or in plasma (0.5 to 10.0 ng mL^{-1}) [6,7] can indicate the early failure of insulin secretion in the preclinical stages of diabetes. As a result, considering its constancy and persistence, the blood C-peptide content has become an important marker for diabetic patients and provides a means to distinguish type 1 and type 2 diabetes [8,9].

Several methods have been developed for C-peptide detection. Kippen et al. first reported a method for serum C-peptide measurement that utilized a solid phase extraction cartridge and immunoaffinity purification using an immobilized antibody column [6]. Others have reported chromatography-based methods such as two-step solid phase extraction and two-dimensional chromatographic methods [10,11]. Although these methods function over a wide range of serum C-peptide concentrations, their lack of sensitivity requires large amounts of serum resulting in high detection cost and sometimes reduced reliability.

The measurement of C-peptide has been routinely performed by immunoassays [2]. Immunoassay is a detection method based on specific immune recognition. It established the standard for biodetection that can be used in clinical laboratories for diagnosis, the food industry, and environmental contamination monitoring [12–17]. Compared to conventional immunoassays, electrochemical immunosensors based on highly specific immune recognition have received increasing attention due to their simple construction, lower detection limit with small sample quantities, fast procedure, ease of mass-production, and potential for miniaturization [18]. C-peptide biosensors, with their intrinsic biorecognition ability, also have benefits including high specificity, simple operation, and low cost [19].

The most crucial step in the construction of immunosensors is the immobilization of the immune component (antibody or sometimes antigen) on a solid supporting surface for target capturing [20]. Gold nanoparticles (AuNPs), one of the most widely used nanomaterials in electrochemical immunosensors, have excellent abilities for binding

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biomolecules (such as proteins, enzymes, and antibodies) due to their unique physicochemical properties such as large surface-to-volume ratio, non-toxicity, good conductivity/catalysis, and biocompatibility [21,22]. However, controlling the size, dispersity, morphology, surface chemistry, and assembly of AuNPs on an electrode surface remains a great challenge in real-life applications [23,24]. Therefore, it is crucial to explore novel strategies, both materials and approaches, for stabilizing AuNPs in order to enhance the immobilization efficiency of biomolecules and improve the overall performance of immunosensors.

In recent years, ECL analysis has greatly progressed due to its distinct advantages such as high sensitivity, spatio-temporal controllability, simple set-up, and low background noise [25–27]. Because of its nontoxicity, low cost, and high quantum yield, luminol is one of the most widely used luminescent reagents and has been used in ECL detection for many biomarkers [28–31]. It has also already been demonstrated that ECL biosensing via an immune-strategy on nanomaterial-functionalized indium tin oxide (ITO) glass performed well in these instances [32].

In this study, the AuNPs nanomaterials were assembled on the surface of ITO via the adhesion of a hydrolysed polymer of (3-aminopropyl)trimethoxysilane (APTMS). After that the C-peptide antibody was loaded onto the surface of electrode using the AuNPs. Utilizing the inhibited ECL of luminol as sensing signal, a label-free direct ECL immunosensor was built for C-peptide detection via specific immune recognition. Its ease of operation, low cost, excellent reproducibility, and potential for industrial mass-production makes it suitable for clinical use as a disposable device for in point-of-care tests (POCTs).

2. Experiments

2.1. Reagents and materials

ITO glass was provided by Suzhou Nippon Sheet Glass Electronics Co. Ltd. (Suzhou, China) and was used as a working electrode after being cut into 1.0 cm × 5.0 cm pieces. Luminol was purchased from Fluka (Sigma-Aldrich China, Shanghai, China). C-peptide and its antibody (Anti-C-peptide, $M_W = 33$ kDa, 1 mg mL^{-1}) were obtained from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China). 3-Aminopropyltrimethoxysilane (APTMS), chloroauric acid ($HAuCl_4 \cdot 4H_2O$), trisodium citrate, bovine serum albumin (BSA, 96–99%), and phosphate ($NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All chemicals and reagents were of analytical grade without further purification. Phosphate buffer solution (0.2 M, pH 8.0) containing 5×10^{-8} M luminol served as the electrolyte in ECL analysis. Ultrapure water was used throughout the experiments.

2.2. Instruments

The ECL experiments were carried out on our lab-built apparatus as reported in a previous paper [33] using a three-electrode system (with a Pt wire as auxiliary electrode and Ag/AgCl as reference electrode). An RST-5200 Electrochemical Workstation (Risetest Instruments Co. Ltd., Suzhou, China) was used for electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) measurements. Scanning electron microscopy (SEM) (S-4700 scanning electron microanalyzer, Hitachi, Japan) was used to observe the surface morphology of AuNPs/ITO and resulting immunosensor, and transmission electron microscopy (TEM) (FEI, USA, at an accelerating voltage of 200 KV) was used to measure the form and size of the AuNPs.

2.3. AuNPs preparation

AuNPs were prepared according to a previous report [34]. Briefly, 4.5 mL of 1% sodium citrate solution was added to 100 mL of boiling 0.01% $HAuCl_4$ solution, and the reaction was allowed to proceed until the colour of the solution changed to wine red. The size of the obtained AuNPs was approximately 15 nm. Next, 4 mL of freshly prepared AuNPs sol was centrifuged at 3000 rpm for 10 min and then at 10000 rpm for 30 min. Then, the concentrate was re-dispersed with water to 250 μL and a homogeneously sized AuNPs sol was acquired.

2.4. Biosensor preparation

Prior to each experiment, a flake of ITO was ultrasonically washed sequentially with a 1:1 (v/v) ethanol/NaOH (1 M) mixture, acetone, and ultrapure water (20 min each) then immersed in 30% $NH_3 \cdot H_2O$ for 12 h to create a hydrophilic surface with dense -OH groups (first step in Scheme 1). After drying with a nitrogen flow, the APTMS solution of anhydrous ethanol (0.05%, v/v) was dropped onto the ITO flake, followed by waiting for the complete volatilization of ethanol. Then, this obtained ITO flake was placed in a wet environment at 55 °C to ensure the complete hydrolysis of APTMS (second step in Scheme 1). Subsequently, 50 μL of prepared AuNPs sol was dropped onto the ITO surface and left undisturbed for 3 h to deposit the AuNPs (third step in Scheme 1). Finally, it was rinsed with water and dried under nitrogen flow.

The anti-C-peptide had high affinity for AuNPs via electrostatic interaction, thus the resulting AuNPs/ITO electrode can provide a substrate for immobilizing the antibody with good bioactivity and stability. Then, 10 μL of anti-C-peptide solution was dropped onto the AuNPs/ITO electrode, incubated for 2 h at 30 °C (step four in Scheme 1), and blocked by BSA solution (2%) for 1 h at 25 °C to avoid non-specific binding (step five in Scheme 1). The resultant sensor was then washed with PBS (pH 7.4) to remove non-chemisorbed species. The sensor was dried under a nitrogen gas environment after every step and finally stored at 4 °C.

2.5. The formality of ECL detection

On each electrode containing the as-prepared immunosensor, the ECL emission of luminol is triggered by a pulsed voltage that induces luminol oxidation, yielding excitons that then radiate the light during de-excitation. The upper/lower limiting potentials and pulse period of the pulsed voltage, which greatly affect the ECL performance, require optimization. Under optimal conditions, the synchronized pulsed ECL signal pulse can be recorded, depending on the electrode surface status or concentration of some participant. The sensing output can then be detected by measuring the average of a cluster of pulses and quantified by correlating the response to the target concentration.

3. Results and discussion

3.1. Optimization of AuNPs functionalized ITO electrode performance

The AuNP-functionalized substrate electrode plays an important role in sensor construction. Therefore, some design parameters were optimized to obtain higher performance. Through analysis of the performance with multiple parameter values as listed in Fig. 1, we chose 10 μL of 0.05% APTMS for hydrolysis under 55 °C for 3 h and decoration with 1.93×10^{-6} mol (380 μg) of AuNPs over a 3 h time period for optimal performance.

Under those optimized conditions, 85.3% surface coverage of ITO by AuNPs was obtained. This result was determined by a cyclic voltammetric scan of the $K_3Fe(CN)_6$ probe to calculate the real active area of the electrode according to the Randles-Sevcik equation. The active area of the electrode is 1.71 times larger after AuNPs decoration. The covering of hydrolysed APTMS on ITO might hinder the electron transfer of electrode without deposited AuNPs. The weight of the spherical Au nanoparticles causes them to penetrate the thin film and to come into direct contact with the ITO, enabling electron transfer. When controlling the thickness of APTMS to less than half size of AuNPs, these results