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A sandwich-type label-free electrochemiluminescence immunosensor for neurotensin based on sombrero model with graphene-hyaluronate-luminol composite

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ABSTRACT

An ultrasensitive sandwich-type label-free electrochemiluminescence (ECL) immunosensor based on sombrero model for the detection of neurotensin (NT) in human serum and urine was developed. Graphene-hyaluronate-luminol (G-HY-luminol) composite was coated on the surface of the glassy carbon electrode to build the ECL immunosensor, which gave in-situ ECL signals by luminol. A secondary NT unlabelled antibody was used to form the final sandwich immunocomplex as a sombrero model, facilitating reduction of the ECL intensity to improve the sensitivity of detection significantly. Results showed that the inhibition of ECL intensity increased with the logarithm of NT concentration within a wide linear range from 0.001 pg cm^{-3} to 100 pg cm^{-3}. In addition, specificity, stability, reproducibility, regeneration and application were satisfactory. Therefore, this developed ECL immunosensor has a potential for practical detection of NT and NT-like peptides with small molecular weight in the clinical diagnostics.

1. Introduction

Neurotensin (NT) is a 13 amino acid neuropeptide, which was first isolated from the extracts of bovine hypothalamus [1] and was later prepared by solid-phase synthesis procedures [2]. It was intimately involved in a number of important biological processes, including dopamine transmission [3], analgesia [4], hypothermia [5,6], etc. Recent reports suggest that NT has an impact on the proliferation of normal cells and neoplastic cells in breast, pancreas, colon, lung, thyroid and prostate tissues [7–11]; and the level of NT in serum and urine can correctly identify patients at high risk [12]. Therefore, it is crucial to develop diagnostic tools for the detection of very low concentrations of NT and NT-like peptides in sub-healthy humans or early-stage patients in order to identify the risk at an early stage.

Lots of methods and strategies have been developed for the detection of NT, including enzyme-linked immunosorbent assay (ELISA) [13,14], radioimmunoassay (RIA) [15,16], mass-spectrometry (MS) [17], high performance liquid chromatography (HPLC) [18–20], HPLC-MS [21], electrochemical immunoassay (ECIA) [22,23], etc. Despite their high reliability, the detection performance of those methods still has some problems such as: (1) radioactive or toxic markers are needed, (2) detection time is long, (3) experimental procedures are cumbersome, (4) instruments used are expensive, and (5) the most important, the detection sensitivity is not high enough. The detection limit of 3.3 pg cm^{-3} [24] achieved using the radioimmunoassay for NT was the lowest among the detection methods presented above. However, the concentration of NT in human plasma and urine ranges from several pg cm^{-3} to tens of pg cm^{-3} [8,12–14], and decreases down to lower levels in some diseases [25,26]. Therefore, it is very important to develop more sensitive detection method for NT to meet the detection demand.

Electrochemiluminescence (ECL) has received increasing attention due to its acknowledged advantages such as versatility, low-cost, simplified optical setup, very low background signals,

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wide dynamic range and easy operation [27–32]. ECL immunoassay (ECLIA) that combines the advantages of ECL and specific recognition of immunoassay provides a fast, sensitive and selective method for determining disease-related proteins with short assay time and simplified optical setup [33,34]. In many cases, a sandwich-type is the commonly used mode of ECLIA.

In this work, a sensitive ECL immunosensor to determine NT based on a sombrero model using graphene-hyaluronate-luminol (G-HY-luminol) composite film as supporting matrix and luminol as luminophore was developed. Graphene (G) has drawn much attention owing to its unique properties such as large surface area, low manufacturing cost, excellent mechanical properties and high electrical conductivity [35–37]. Due to these excellent properties, G has been widely applied in the fabrication of immunosensors [38]. Luminol is a popular ECL reagent with a high light efficiency. Hyaluronate (HY) gel matrix is an ideal biopolymer to immobilize special subjects on solid substrates with an aqueous microenvironment [39–43]. Significant interest is evident on the development of HY films and coatings for biomedical applications [39,42]. However, to the best of our knowledge, use of HY in building a stable composite film to develop an immunosensor has not been reported yet.

Compared with the traditional model, this proposed sombrero model using a secondary unlabelled antibody to form the final sandwich immunocomplex could inhibit the ECL reaction and block the ECL signal more effectively to improve the detection sensitivity, especially for proteins with small molecular weight. Benefitted from the sombrero model of inhibiting ECL signals efficiently, ultrasensitive detection of NT was achieved with a quantitation limit of 0.001 pg cm^{-3}. It also possesses other advantages such as simple instrumentation, high specificity, good stability, excellent reproducibility and regeneration, etc. Therefore, this novel strategy could provide a potential tool for detecting small molecular weight NT-like proteins in clinical application.

2. Experimental

2.1. Apparatus

A laboratory-built ECL detection system, as described previously [44] was used in this study. A three-electrode system, including a bare or modified glassy carbon electrode (GCE, Φ = 3 mm), a platinum wire electrode and a Ag/AgCl/3 M KCl electrode as working electrode, counter electrode and reference electrode, respectively, was used. Electrochemical impedance spectroscopy (EIS) analysis was performed with a CHI 660B electrochemistry workstation (Chenhua Instrument Company, Shanghai, China). The transmission electron microscope (TEM) images were obtained using a FEI Tecnai G2 F20 transmission electron microscope (FEI, Hillsboro, Oregon, USA).

2.2. Reagents and materials

Bovine serum albumin (BSA, 98–99%), 3-aminophthalhydrazide (luminol), N-hydroxysuccinimide (NHS), neurotensin (NT) and polyclonal anti-neurotensin antibody (anti-NT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hyaluronate (HY) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Pierce (Rockford, IL, USA). Graphite powder (8000 mesh, 99.95%) and hydrazine monohydrate were purchased from Aladdin (Shanghai, China). All other reagents were of analytical grade, and purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Carbonate buffer solution (CBS, pH 9.78) containing 0.015 mol dm^{-3} sodium carbonate and 0.035 mol dm^{-3} sodium bicarbonate, was used as the working solution for the ECL measurement. Ultra-pure water (18 MΩ cm), obtained from a Heal Force PW ultrapure water system (Heal Force, Hong Kong, China), was used throughout the experiment.

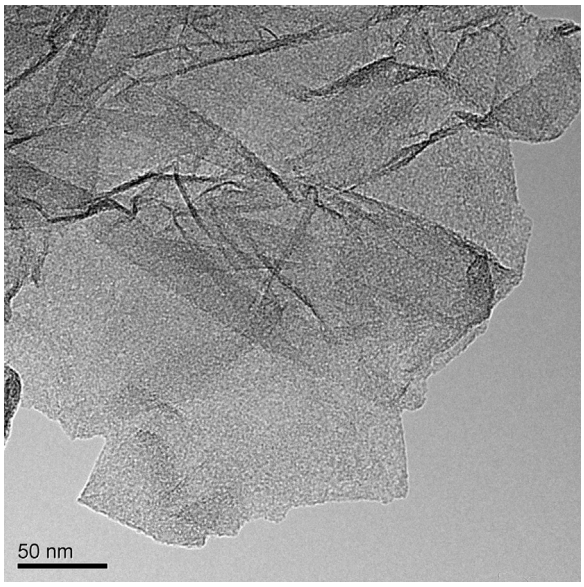

Fig. 1. TEM image of GCE coated by G-HY-luminol composite.

2.3. Synthesis of graphene-hyaluronate-luminol (G-HY-luminol) composite

Graphene (G) was prepared as described in our previous paper [45] while the G-HY-luminol composite was prepared as follows: 15 mm^3 of 2 mg cm^{-3} G solution was added into 35 mm^3 of 0.5 wt% HY and vortexed for 10 min to obtain a homogeneous solution; and then 50 mm^3 of 1 mmol dm^{-3} luminol solution was added and vortexed for 30 min to obtain the G-HY-luminol composite. The TEM image of GCE coated by G-HY-luminol composite is shown in Fig. 1. It could be found that G was immobilized onto the surface of GCE by HY efficiently.

Homogeneous G-HY-luminol composite could be obtained conveniently, and it could emit intense and stable ECL, due to the noncovalent π-π interaction between luminol molecules and G. Luminol containing a small aromatic ring is a weak electronic delocalization system, and G contains large π bonds. The noncovalent interaction based on π-π interactions as the binding force between luminol and G, not only immobilized luminol into G-HY composite tightly but also provided the opportunity to improve the electronic properties of G-HY-luminol composite [46–50].

2.4. Preparation of the ECL immunosensor

Fig. 2A presents the fabrication protocol of the ECL immunosensor. Firstly, the bare glassy carbon electrode was polished with 1.0, 0.3 and 0.05 μm Al_2O_3 slurries in sequence, ultrasonicated in ethanol and ultrapure water, and allowed to dry. Subsequently, 10 mm^3 of G-HY-luminol composite was added onto the electrode surface and dried for 2 h at 37 °C. Then, the electrode was soaked in 45 mm^3 of mixed solution containing 20 mg cm^{-3} EDC and 10 mg cm^{-3} NHS for 2 h at 4 °C to form a stable active ester layer, rinsed with water, and incubated in 40 mm^3 of 1:5000 anti-NT (Ab_1) at 4 °C for 12 h to form GCE/G-HY-luminol/Ab_1 and rinsed with water. Finally, the electrode was incubated in 2 wt% BSA solution for 1.5 h at 4 °C to block non-specific binding sites and the ECL immunosensor, ready for the immunoassay, was obtained.