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Highly sensitive electrogenerated chemiluminescence biosensor for galactosyltransferase activity and inhibition detection using gold nanorod and enzymatic dual signal amplification

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As one of the glycosyltransferase involved in protein glycosylation, β-1,4-galactosyltransferase (Gal T) plays an important role in the cellular process and progression of cancer. Here, using the bovine serum albumin conjugated *N*-acetylglucosamine (GlcNAc-BSA) as a receptor to fabricate bioelectrode interface, a sensitive electrochemiluminescence (ECL) biosensor was constructed for Gal T activity analysis based on the recognition between artocarpus integrifolia lectin (AIA) and galactose, integrating with a dual signal amplification strategy from the xanthine oxidase (XOD) and AIA multi-labeled gold nanorod nanoprobes. The gold nanorods promoted the electron transfer on the electrode interface and were also employed as carriers of AIA and XOD due to their large surface area. Furthermore, both the gold nanorods and XOD catalyzed the ECL reaction, which dramatically amplified the ECL signal of luminol in the presence of hypoxanthine (HA) and oxygen. The as-proposed ECL biosensor exhibited high sensitivity on the detection of Gal T activity and a detection limit of 9×10^{-4} U mL^{-1} was obtained. This assay was successfully applied for the analysis of Gal T activity expression in different cell lines and inhibition detection, showing great potential for glycosyltransferase activity analysis and inhibitors screening in clinic diagnostics.

1. Introduction

Carbohydrates on the surface of eukaryotic cells play significant roles in a broad range of crucial biological processes, including cell growth and differentiation, cell adhesion and signaling, immune response, and progression of cancer [1–4]. Galactosyltransferase is a type of glycosyltransferase which catalyzes the transfer process of galactose to form the cellular glycoconjugates [5]. Previous studies found that the enzyme β-1,4-galactosyltransferase (Gal-T), which catalyzes the transfer of a galactose residue from UDP-galactose (UDP-Gal) to *N*-acetylglucosamine, is closely associated with some vital cellar processes such as cell adhesion [6], and diseases such as lung cancer [7]. Developing rapid and sensitive biosensor to evaluate the Gal-T activity in biological samples is important in clinic diagnostics and biomedical research.

Various approaches for the determination of Gal T activity have been developed, such as chromatography [8], radiochemical assay [9], fluorescence [10], and colorimetry [11]. However, these methods often require costly labeling, sophisticated sample pretreatment and complicated instruments. Electrogenerated chemiluminescence (ECL), which involves electron-transfer reactions and light-emitting process of the luminophores on the electrode, combines the electrochemical and luminescent techniques [12,13]. In comparison to the conventional methods, ECL is a powerful analytical tool with its advantages of low cost, low background noise, wide dynamic concentration response range and high sensitivity [14]. By integrating some biomolecular recognition strategy, ECL biosensors have been widely applied in DNA analysis [15], immunoassay [16,17], protein analysis [18,19], cell analysis [20,21], clinical diagnosis [22] and environment monitoring [23].

Recently, the developments of nanostructures and nanomaterials have greatly enhanced the performance of electrochemical biosensors owing to their remarkable electrocatalytic activity, large surface area, and good biocompatibility [24,25]. These excellent optical, electrical, and electrochemical properties allow the nanomaterials to promote the surface area and improve the electron transfer at the electrode interface [26–28]. Also, they can be used as carriers to load more active biomolecule ligands for target recognition and ECL labels for signal amplification. Owing to their unique properties, some nanomaterials such as gold nanoparticles [29,30], and gold nanorods [31] have been extensively used in ECL biosensors in previous works.

Herein, a sensitive sandwich type ECL biosensor for Gal T activity and inhibition detection is designed. The glycosylation receptor *N*-

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Acetylglucosamine-BSA (GlcNAc-BSA) was immobilized onto the gold electrode to form the bioelectrode interface. After the glycosylation catalyzed by Gal T, galactose was conjugated to the GlcNAc-BSA. And the galactose was then specifically recognized by the artocarpus integrifolia lectin (AIA) on gold nanorods (GNRs), resulting the absorption of the AIA and xanthine oxidase (XOD) conjugated GNR (AIA-XOD@GNR) nanoprobes. The ECL signal was markedly enhanced due to the gold nanorod and enzymatic dual amplification of the luminol ECL signals. The ECL biosensor showed high sensitivity toward Gal T activity detection with a low detection limit, and further was applied for cell lysate detection and inhibition evaluation.

2. Experimental section

2.1. Reagents

Artocarpus integrifolia lectin (AIA) was obtained from BioSun Sci&Tech Co. (Shanghai, China). *N*-Acetylglucosamine-BSA (GlcNAc-BSA) was received from Professor Lokesh Joshi, National University of Ireland, Galway, Ireland. Luminol, $HAuCl_4 \cdot 3H_2O$, hypoxanthine (HA), β-1,4-galactosyltransferase, and uridine 5′-diphosphogalactose disodium (UDP-Gal) were purchased from Sigma-Aldrich. Cetyltrimethyl Ammonium Bromide (CTAB), poly(sodium 4-styrenesulfonate) (PSS, molecular weight = 7000), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysulfosuccinimide sodium salt (NHSS) were obtained from Alfa Aesar Co. (Ward Hill, MA, USA). 11-mercaptoundecanoic acid (MUA) and 6-mercapto-1-hexanol (MEH) were purchased from J&K Scientific Ltd. (Beijing, China). Xanthine oxidase (XOD) was purchased from Yuanye Biotech. Ltd. (Shanghai, China). Bovine serum albumin (BSA), 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and bicinchoninic acid (BCA) protein assay kit were from Dingguo Biological Products Co. (Beijing, China). Other regents of analytical grade were obtained from Beijing Chemical Co. (Beijing, China).

2.2. Preparation of AIA and XOD conjugated gold nanorods

The gold nanorods (GNRs) are synthesized according to previous works [32–34]. In brief, 0.25 mL $HAuCl_4 \cdot 3H_2O$ (10 mM) was mixed with 0.6 mL of 10 mM $NaBH_4$ solution and 5 mL cetyltrimethylammonium bromide (CTAB) (100 mM) to get a pale brown gold seed solution. Then, 0.25 mL of 10 mM $AgNO_3$ and 0.27 mL of 100 mM ascorbic acid solution were added to a mixed solution of 40 mL CTAB (100 mM) and 1.7 mL $HAuCl_4 \cdot 3H_2O$ (10 mM) successively to get the growth solution. Finally, 0.42 mL of gold seed solution was added to the growth solution. The mixture was incubated for 15 h at 28 °C before centrifugation. To remove the extra free CTAB, the fresh GNRs solution was centrifuged at 14,000 rpm for 10 min, and the products at the bottom were then re-dispersed in PBS solution. The prepared GNRs were characterized by transmission electron microscopy (TEM) and UV–Vis spectra (Fig. S1 in Supporting information). Statistical analysis of them shows that the GNRs had an average diameter of ~10 nm and an average length of ~36 nm with an aspect ratio of ~3.5. And a strong adsorption at 750 nm was observed for the gold nanorods.

Then, 40 μL of 5 mg mL^{-1} PSS was incubated with 1 mL of the re-dispersed GNRs solution for 40 min to get a PSS functionalized GNRs. The mixture was centrifuged at 10,000 rpm to get rid of excessive PSS and the pH value was adjusted to 7.5 with 10 mM PBS buffer. To obtain the AIA and XOD conjugated GNRs, 1 mL colloidal solution of PSS caped GNRs was mixed with the solution of 100 μL of 5 mg mL^{-1} AIA and 6 μL of 1 mg mL^{-1} XOD. The mixture was incubated for 60 min under shaking, and was centrifuged to remove extra AIA and XOD and re-dispersed in PBS solution containing 1 mM Ca^{2+} and Mn^{2+}.

2.3. Fabrication of ECL biosensor

Prior to fabrication of ECL sensors, a gold electrode (diameter of 3 mm) was successively polished with 0.3, and 0.05 μm α-Al_2O_3 powder and ultrasonically cleaned with ethanol and water. After being dried with a nitrogen flow, the electrode surface was immersed into 100 μL solution of 0.01 mM MUA and 0.09 mM MEH in 4:1 ethanol/H_2O overnight at room temperature. To activate carboxyl groups, the electrode was then immersed in 30 μL of a mixture aqueous solution containing 50 mM EDC and NHSS at 37 °C for 1 h. For GlcNAc-BSA immobilization, the electrode was incubated with 5 mL of 0.2 mg mL^{-1} GlcNAc-BSA at 37 °C for 80 min. Next, the electrode was washed with PBS and followed by incubation in solution of 1 mg mL^{-1} BSA for 1 h at 37 °C to block the additional active groups.

The solution for glycosylation reaction was prepared by mixing 75 mL of 50 mM HEPES, 5 mL of 20 mM UDP-Gal, 10 mL of 20 mM Mn^{2+} and 5 mL Gal T at a certain concentration. The GlcNAc-BSA modified electrode was soaked in 25 mL of reaction solution with Gal T and UDP-Gal at 37 °C for 3 h to conjugate galactose to the GlcNAc-BSA. After being thoroughly rinsed with PBS, the electrode was incubated with 5 μL solution containing AIA-XOD@GNR at 37 °C for 1 h to capture the nanoprobes via the specific binding between galactose and AIA. The resultant electrode was washed with PBS to remove the extra nanoprobes before being used for ECL assays.

ECL measurements were performed in a 0.1 M PBS (pH 8.5) containing 5 mM HA and 100 μM luminol using Ag/AgCl electrode with saturated KCl solution and platinum wire as the reference electrode and counter electrode, respectively. The ECL measurements were performed from 0 to 0.6 V with scan rate of 100 mV s^{-1}. The experiments for Gal T activity measurements of cell lysate protein were the same as described above except for substituting 50 μg of cell lysate protein for 5 mL Gal T.

2.4. Cell culture and lysis

The HeLa cells, CCRF-CEM cells, SMMC-7721 cells and HL-7702 cells were kindly provided by the Medicine School of Tsinghua University, Beijing, China. HeLa cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Logan, UT, USA). CCRF-CEM cells and SMMC-7721 cells were cultured in RPMI 1640 medium (Dingguo Biological Products Co., Beijing, China). Each medium was supplemented with 10% fetal calf serum (Zhejiang Tianhang Biological Technology Co., Ltd., Zhejiang, China), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. And the HL-7702 cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) supplemented with 20% fetal calf serum (Zhejiang Tianhang Biological Technology Co., Ltd., Zhejiang, China). The cells were grown to midlog phase and then collected and separated from the medium by centrifugation at 1000 rpm for 5 min. Then the cells were washed with sterile phosphate buffer saline (PBS, pH 7.4) twice. The cells were added with cell lysis buffer containing 1 mM PMSF (Beyotime biotechnology Co., Shanghai, China) at 4 °C for 30 min. The suspension was centrifugated by refrigerated centrifugation at 14,000 rpm for 5 min, and the supernatant was retained and stored at 80 °C for further Gal T analysis.

Before Gal T analysis, the proteins in each cell lysate sample were quantified with the bicinchoninic acid (BCA) protein assay kit (Dingguo Biological Products Co., Beijing, China). Cell lysate containing 50 μg proteins was added in the glycosylation reaction solution for each ECL test.

2.5. Apparatus and characterization

Scanning electron microscope (SEM) images were obtained with an SU8010 Scanning transmission electron microscope (Hitachi, Japan). UV–vis experiments were carried out with a UV-3900 spectrophotometer (Hitachi, Japan). The cyclic voltammetry was conducted on a CHI 660b instrument (CH Instrument Co., USA). Electrochemical impedance

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