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Ratiometric electrochemiluminescence detection of circulating tumor cells and cell-surface glycans

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ABSTRACT

Ultrasensitive quantitative detection of circulating tumor cells (CTCs) and the profile of glycan expression are active and rapidly advancing fields in cancer research. This work designed a dual-potential ratiometric electrochemiluminescence (ECL) system for highly sensitive detection of CTCs and simultaneous evaluation of glycan expression on cell surface. Two kinds of ECL nanoemitters, carbon nitride nanosheets and luminol-reduced gold nanoparticles as cathodic and anodic nanoemitters respectively, were prepared to successfully achieve the dual-potential ratiometric ECL. The carbon nitride nanosheets were decorated with gold nanoparticles to conjugate aptamer for specific capture of CTCs, and the anodic nanoemitters were functionalized with lectin for specific recognition of surface glycans. Using MCF-7 CTCs as model target, the proposed ratiometric ECL sensor exhibited excellent analytical performance with a wide linear detection range and a detection limit down to 2 cells. This method could be further used for profiling the variation of glycans such as *N*-acetylglucosamine, *N*-acetylneuraminic acid and mannose on cell surface upon treatment. The proposed strategy realized highly sensitive determination of cell concentration, and accurate analysis of glycan expression on cell surface, showing promising application.

1. Introduction

Circulating tumor cells (CTCs) that are shed from tumor tissues into the peripheral blood stream or lymphatic system have been considered as an important factor in the cancer growth and metastasis [1–3], and become potential biomarkers for early cancer detection, diagnosis, prognostic, predictive, stratification and pharmacodynamics evaluation [4]. Since the amount of CTCs in the peripheral blood is extremely rare, ranging between 1–10 cells per 10 mL in most cancer patients [5], highly sensitive methods for quantitative detection of CTCs has been an important challenge in CTCs research [6–9].

The majority detection methods for CTCs include immunomagnetic separation [10], size-based filtration techniques [11] and microfluidic-based CTC devices [12–14]. In order to address the problems of insufficient capture efficiency and low purity [15], different nanomaterials have been introduced into this area due to their high surface-area-to-volume ratio, including carbon nanotubes [16], nanowire [17], nanopillar [18], nanofiber [19], and nanoscale dendrimer [20]. The EpCAM antibody functionalized graphene oxide nanosheets has been used to sensitively capture rare CTCs on a gold pattern [21] due to the overexpression of EpCAM on CTCs [9]. This two-dimensional (2D) graphene oxide chip not only enhances the capture efficiency of CTCs (73 ± 32.4% at 3–5 cells per ml blood), but also promotes the cell proliferation. However, previous studies usually focused on 2D nanomaterials with high surface-area-to-volume ratio, easy biofunctionalization and good biocompatibility, the combination of the electrical and optical properties of 2D nanomaterials for improving capture efficiency and providing the detectable signal is still an urgent need.

This work presented a specific capture strategy of CTCs on a 2D nanomaterial by using aptamer to functionalize carbon nitride nanosheets (CNNS) decorated with gold nanoparticles (AuNPs@CNNS). The AuNPs@CNNS could act as a cathodic probe to construct a ratiometric electrochemiluminescence (ECL) sensor for sensitive detection of CTCs, by coupling with luminol-reduced gold nanoparticles (LuAuNPs) as the anodic probe. Due to the steric effect of cells and consumption of the same coreactant H_2O_2, the cathodic ECL intensity of AuNPs@CNNS decreased, while the anodic ECL signal of LuAuNPs appeared. The ratio of anodic to cathodic ECL peak intensity showed good relativity with the amount of CTCs. Compared with single-signal method, this dual-potential ratiometric ECL cytosensing approach could normalize the variation from environmental changes and provide more accurate measurement results in analytical application, especially for cell and other complex biological samples [22,23]. After the anodic probe was further functionalized with lectin, the sensor could be used to simultaneously monitor cell-surface glycan expression through the specific recognition between lectin and glycan on cell surface and produced anodic ECL signal, providing a highly sensitive methodology for profiling the variation of glycans on CTC surface.

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Glycans on cell surface play crucial roles at various pathophysiological stages, including the regulation of tumor proliferation, invasion, haematogenous metastasis and angiogenesis. Tumor cells often aberrantly express certain terminal glycans. Thus profiling the changes of glycans may provide a new perspective for the study of pathologies [24–27] and useful information for screening of tumor biomarkers. Some mass spectroscopic methods have been developed for profiling the surface glycans of CTCs [28,29]. This work proposed another kind of candidates for evaluation of glycan expression on CTCs. These proposed methods showed promising application in both highly sensitive detection of CTCs and elucidation of biological functions of glycans on CTCs.

2. Experimental

2.1. Materials and reagents

Melamine (99%), luminol, tris (2-carboxyethyl) phosphine hydrochloride solution (TCEP, 0.5 M), 6-Mercapto-1-hexanol (MCH), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Co., Ltd. (Shanghai, China). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and sodium borohydride ($NaBH_4$) were purchased from Fuchen Chemical Reagent Co. (Tianjin, China). Lectins and fluorescein-labeled lectins, including *Lens culinaris* agglutinin (LCA), fluorescein-labeled LCA, *Sambucus nigra* agglutinin (SNA), fluorescein-labeled SNA, succinylated wheat germ agglutinin (WGA) and fluorescein-labeled WGA, were purchased from Vector Laboratories (USA). Glycan exonucleases, including mannosidase, neuraminidase and *N*-acetylglucosaminidase, were purchased from New England Biolabs (UK).

Thiolated anti-EpCAM aptamer and FITC (fluorescein isothiocyanate) labeled anti-EpCAM aptamer were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequence of the aptamer was 5′-HS-CAC TAC AGA GGT TGC GTCTGT CCC ACG TTG TCA TGG GGG GTT GGC CTG-3′ [30]. Phosphate buffer saline (PBS, 0.01 M, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4 and 1.41 mM KH_2PO_4. For lectin-glycan recognition, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ were added in PBS. All reagents were of analytical grade. Ultrapure water obtained from a Millpore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all assays.

2.2. Apparatus

Transmission electron microscopic (TEM) images were acquired on JEM-2100 transmission electron microscope (JEOL Ltd., Japan). X-ray photoelectron spectroscopic (XPS) analysis was carried out on an ESCALAB 250 X-ray photoelectron spectrometer (Thermo-VG Scientific, USA) with an ultrahigh vacuum generator. The UV-vis absorption spectra were obtained using a Nanodrop-2000C UV-vis spectrophotometer (Thermo, USA). The cell images were acquired on a TCS SP5 laser scanning confocal microscope (Leica, Germany). Flow cytometric analysis was performed on a Coulter FC-500 flow cytometer (Beckman Coulter, USA). Electrochemical experiments were performed on a CHI 660B electrochemical workstation (CH Instruments Inc., USA). Electrochemiluminescence measurements were carried out in a self-made cell on a MPI-E multifunctional electrochemical and chemiluminescent analytical system (Xi'an Remex Analytical Instrument Co., Ltd. China).

2.3. Cell culture and capture

MCF-7 and HEK-293 T cell lines were purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). MCF-7 cells were cultured in a flask in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS, Sigma), penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2. HEK-293T cells were cultured in high glucose (4.5 g/L) version of Dulbecco's Modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal calf serum, penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells at the exponential growth phase were collected and separated from the medium by centrifugation at 1000 rpm for 5 min, and then washed twice with a sterile PBS (pH 7.4). The sediment was resuspended in the PBS containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+} to obtain a homogeneous cell suspension. Here, the divalent cations Ca^{2+} and Mn^{2+} were used to ensure the effective binding between lectins and cell surface glycans. Cell number was determined using a Countess® II Automated Cell Counter (Invitrogen, USA).

2.4. Preparation of AuNPs@CNNS

The bulk g-C_3N_4 was prepared by polymerization of melamine molecules under high temperature [31]. In brief, melamine was heated at 600 °C for 2 h under air condition with a ramp rate of about 3 °C/min for both of the heating and cooling processes. The obtained yellow product was the g-C_3N_4 powder.

The ultrathin g-C_3N_4 nanosheets was obtained by liquid exfoliating of as-prepared bulk g- C_3N_4 powder in water [32]. In detail, 100 mg of bulk g-C_3N_4 powder was dispersed in 100 mL water, and then ultrasound for 6 h in ultrasonic cleaner (210 W, 53 kHz) and another 1 h (600 W) with the aid of ultrasonic cell disruptor. The initial formed suspension was then centrifuged at about 5000 rpm to remove the residual unexfoliated g-C_3N_4 nanoparticles and large-area nanosheets before used for further study.

The preparation of AuNPs@CNNS was referred in the previous report [33,34]. As shown in (Fig. 1A), 16.7 μL of 1 wt% $HAuCl_4 \cdot 4H_2O$ solution was added to 2 mL of the above prepared g-C_3N_4 nanosheets suspension (2 mg·mL^{-1}). After adding additional 5 mL water, the suspension was vigorously stirred for 10 min. Then, 50 μL of 0.01 M freshly prepared $NaBH_4$ solution was added quickly into the suspension to reduce the $AuCl_4^-$ and the color of suspension turned from milky white to pink immediately. After continuously stirring for 20 min, 10 μL of 0.01 M trisodium citrate solution was added dropwise into the above suspension, and kept stirring for 30 min. To remove excess $NaBH_4$, trisodium citrate and unbound gold nanoparticles, the obtained hybrid materials were separated by centrifugation (12,000 rpm, 10 min), washed thoroughly with ultrapure water, and finally redispersed into 2 mL water. The obtained Au@CNNS was stored in 4 °C for future use.

2.5. Preparation of lectins@LuAuNPs

The synthesis of LuAuNPs was prepared by the reduction of $HAuCl_4$ with luminol [35]. All glass containers used for synthesis were immersed in freshly prepared aqua regia (HNO_3/HCl = 1:3, v/v) for 12 h, then washed with ultrapure water and dried before use. 100 mL of 0.01 wt% $HAuCl_4$ was heated to boiling point with stirring slowly. 1.6 mL of 0.01 M luminol solution was added rapidly into boiling $HAuCl_4$ solution for 30 min. The solution turned to wine-red color from pale yellow, indicating the formation of AuNPs. Then, the heating source was removed and cooled down to room temperature. The obtained LuAuNPs was stored at 4 °C for the following use.

Lectin functionalized LuAuNPs were prepared through direct physical adsorption by adding 1 mL of LCA (5 mg mL^{-1}) into as-prepared LuAuNPs solution (1 mL) [36] (Fig. 1B). After being adjusted to pH 6.0 with 1 M HCl, the resulting mixture was vibrated for 30 min at room temperature. Then, the LCA@LuAuNPs was purified by centrifugal washing (10,000 rpm, 5 min, 4 °C) with 10 mM Tris-HCl, and redispersed in probe buffer (0.01 M PBS containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+}, pH 7.4). Conjugation with other lectins (SNA and WGA) to LuAuNPs was followed the same procedure. All obtained lectin@LuAuNPs were stored in 4 °C for future use.

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