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Protein-inorganic hybrid nanoflowers as ultrasensitive electrochemical cytosensing Interfaces for evaluation of cell surface sialic acid

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ABSTRACT

The identification of biocompatible nanomaterials with high conductivities as sensing interfaces is important in developing novel electrochemical cytosensors. We prepared a novel protein-inorganic nanomaterial-bovine serum albumin (BSA) incorporated Ag nanoflowers with three-dimensional porous architectures, using a simple biomimetic method. The BSA-incorporated Ag nanoflowers were modified on a glassy carbon electrode (GCE) surface and conjugated with a targeting lectin molecule, i.e., *Sambucus nigra* agglutinin (SNA), for sensing DLD-1 human colon cancer cells. The BSA-incorporated Ag nanoflowers were a suitable platform, and showed improved cell-immobilization capacity, and good biocompatibility, with retention of activity of the immobilized cells. These properties are attributed to the large surface area of the porous structure and the natural BSA layer acting as a biocompatible support. The attachment of DLD-1 cells to the GCE increased the electron-transfer resistance, with a good correlation with the logarithm of the concentration from 1.35×10^2 to 1.35×10^7 cells mL^{-1}, with a low detection limit of 40 cells mL^{-1}. Based on the affinity between SNA and sialic acid (SA), the UV–vis absorption spectrum of the one-step reaction between SA and acidic ninhydrin indicated that the average number of SA molecules on a single living DLD-1 cell surface was approximately 2.16×10^{12}. This proposed cytosensing strategy had good reproducibility, acceptable precision, and high specificity for SA-over-expressed cells, indicating that it has potential applications for the early monitoring of tumor cells and convenient evaluation of SA on living cells.

1. Introduction

Glycoproteins on cell surfaces play essential roles in a wide variety of cell activities, including cell proliferation and differentiation, cell–cell communication, and immune response modulation (Raman et al., 2005). Sialic acid (SA), a clinically approved tumor marker, is a glycoprotein containing a nine-carbon backbone, and is commonly found at the terminal positions of sugar chains (Liu et al., 2011; Matsumoto et al., 2009). Accumulating evidence shows that over-expression of SA on cell surfaces is closely associated with the malignant and metastatic phenotypes of many cancers such as colorectal, breast, liver, and ovarian cancers (Ang et al., 2006; Chen et al., 2007a; Wang et al., 2003; Zhang et al., 2010). Therefore, the study of SA on the cell surface is critical both to understand its role in disease development and to provide effective diagnostic tools to help prompt therapy.

Currently, there are various methods for SA analysis, including fluorescence analysis, mass spectrometry (MS), and liquid chromatography (LC) (Shen et al., 2007; Wang et al., 2014a, 2014b; Xiong et al., 2013). However, these techniques need long analytical times and stringent laboratory conditions, and are not suitable for living cell analysis. The design and development of rapid, non-invasive, highly sensitive, and specific techniques for evaluating SA expression level are therefore important. In recent years, electrochemical impedance spectroscopy (EIS) has become a popular approach in a broad range of fields, including semiconductor batteries, corrosion, fuel cells, and biosensors (Chang and Park, 2010; Sherif and Park, 2006; Venkatanarayanan et al., 2013; Wang et al., 2014c). In particular, EIS has been widely applied in living cell measurements; for example, Zhu et al. reported a highly sensitive impedance sensor for HL-60 cells, based on carboxylic single-walled carbon nanotube/Au composites (Zhang et al., 2009), Ju et al. used electrochemical impedance detection of K562 leukemia cells (Ding et al., 2007), and Wang et al. prepared folate conjugated-polyethylenimine carbon nanotubes for HeLa cell detection using EIS (Wang et al., 2013). Thus, we intend to fabricate EIS cytosensor to evaluate SA expression level of cell membrane.

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However, interfacial effects play a key role in EIS measurements, therefore selecting appropriate and biocompatible interface materials is crucial in fabricating EIS cytosensors. Moreover, cell immobilization based on nanomaterials with good biocompatibility also provides a favorable platform for evaluating SA expression on the living cell surface.

Recently, protein-mediated synthesis of inorganic nanomaterials for the sensing interfaces of electrochemical cytosensors is attractive for a number of reasons: the synthetic conditions are mild, i.e., near room temperature, in aqueous solutions, and at neutral pH; the sizes, shapes, morphologies, and crystal structures are complex and controllable; and the resulting products are multifunctional and biocompatible. In our previous work, we synthesized a series of metal@protein nanocomposites via a protein-mediated method, and used them as sensing platforms for the sensitive detection of cancer cells (Hu et al., 2013a, 2013b). These hybrid nanocomposites have a range of advantages such as excellent conductivities, large surface areas, and nontoxicity. Considering the effects of the substrate morphology and structure on the cytosensor performance, we prepared flower-like Ag nanomaterials using bovine serum albumin (BSA) as a template. Compared with previously reported Ag@BSA microspheres (Huang et al., 2011), the BSA-incorporated Ag nanoflowers have larger surface areas and open nanoporous structures, providing an electrochemical sensing matrix that is better for cytosensor fabrication for the following reasons. First, the three-dimensional (3D) porous architecture promotes cell anchoring and increases the number of cells that adhere to the substrate. Secondly, the incorporated BSA molecules provide a multifunctional interface for conjugation of targeting molecules, improving the water solubility and maintaining the biomolecule bioactivity and reducing nonspecific interactions. Finally, Ag has excellent electrical conductivity and can amplify the electrochemical signal, improving the cytosensing ability. Because of these advantages, BSA-incorporated Ag nanoflowers are expected to provide an effective sensing platform for monitoring SA change on tumor cells surface. However, the designed substrate should achieve specific target-cell recognition, while preventing nonspecific adhesion of non-target cells, therefore modification of specific recognition molecules is required. *Sambucus nigra* agglutinin (SNA) with a highly specific binding affinity with SA (Chen et al., 2007a) is an excellent candidate for biosensor fabrication, because of its ease of production and labeling, and good stability during long-term storage (Cho et al., 2014).

Herein, we report the synthesis of novel BSA-incorporated Ag nanoflowers as biomimetic 3D nanoporous sensing interfaces for electrochemical measurements of tumor cells and SA on the DLD-1cells surface. Human colon cancer DLD-1 cells, which have SA expression patterns on the cellular surface, were used as model SA-over-expressed cells. Lectin (SNA), which was successfully conjugated with the sensing platform using glutaraldehyde (GA), was employed as the targeting molecule for specifically recognizing SA groups on cell surfaces. The DLD-1 cells were captured on the cytosensor surface by specific binding between the SA groups on the cell surfaces and SNA, i.e., the amount of cells captured on the cytosensor surface was related to the expression of SA groups on the cell surfaces. On the basis of this, we developed a novel approach for conveniently and sensitively quantifying the SA expression value on the DLD-1 cell surface by combining a simple UV–vis measurement with one-step reaction. Owning to achieving highly specific recognition and utilizing ultrasensitive interfaces, the proposed cytosensor had a broad detection range, with a lower detection limit, than other reported electrochemical cytosensors. Our proposed EIS cytosensing therefore offers a potential protocol for tumor cells detection and quantitative evaluation of the cell surface SA in clinical diagnosis. Furthermore, our results highlight the importance of nano-bio interfaces in optimizing biosensor performances.

2. Experimental

2.1. Chemicals and materials

$AgNO_3$, ascorbic acid, and ethanol GA solution (25%) were purchased from the Sinopharm Chemical Reagent Co., Ltd. (China). SNA, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, ultrapure grade), lyophilized 99% BSA (molecular mass ~68 kDa), and dimethyl sulfoxide (DMSO) were acquired from the Sigma-Aldrich Corporation. A 10 mM phosphate buffer solution (PBS, pH 7.4) was used as the rinsing solution and SNA diluent. A $[Fe(CN)_6]^{3-/4-}$ solution containing 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, and 0.1 M KCl (as the supporting electrolyte) was prepared as a redox probe for the measuring system. Doubly distilled water was used in all experiments. All reagents were analytical grade and used as received, without further purification.

2.2. Apparatus and measurements

The morphologies of the BSA-incorporated Ag nanoflowers were investigated using transmission electron microscopy (TEM; JEOL 2011) and field-emission scanning electron microscopy (FESEM; Philips XL30, the Netherlands), at accelerating voltages of 200 and 10 kV, respectively. EIS was performed, using an Autolab PGSTST 30 analyzer (Metrohm Autolab BV, Switzerland), in 0.1 M KCl solution containing 10 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) as the redox probe, at an open-circuit potential. The impedance spectra were recorded in the frequency range 10^{-1}–10^5 Hz, with a signal amplitude of 5 mV. Cyclic voltammetry (CV) was performed using a CHI 1030 electrochemical workstation with a conventional three-electrode system. A modified electrode was used as the working electrode, with a platinum wire as the auxiliary electrode and a saturated calomel electrode as the reference. Zeta potential was measured in Zeta potential analyzer (Malvern Instruments ZS 90). MTT assays were performed using an enzyme-labeled microplate reader (SUNOSTIK SPR-960).

2.3. Cell culture and collection

Human colon cancer DLD-1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. The DLD-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 (Matsumoto et al., 2005). The DLD-1 cells were trypsinized for 2–3 d in 0.25% trypsin solution, collected from the culture medium by centrifugation at 1000 rpm for 5 min, and then washed twice with 1 × PBS (pH7.4). The sediment was resuspended in the 1 × PBS to obtain a 0.5 mL homogeneous cell suspension. Cell suspensions of different concentrations were prepared from this stock. The cell numbers were determined using a Petroff–Hausser counter.

2.4. Fabrication of BSA-incorporated Ag nanoflowers/GA-modified electrochemical cytosensor

The method for synthesizing the sensing film (BSA-incorporated Ag nanoflowers) is key to our research. Briefly, BSA (5 mg mL^{-1}, 10 mL) and $AgNO_3$ (10 mM, 10 mL) aqueous solutions were mixed in a 50 mL beaker. The mixture was stirred at room temperature for 10 min, and then the solution was placed in a water-bath at 55 °C for 5 min. Ascorbic acid (50 mg) was then rapidly added to the solution. The mixture was kept at 55 °C for