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Immobilization free electrochemical biosensor for folate receptor in cancer cells based on terminal protection

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ABSTRACT

The determination of folate receptor (FR) that over expressed in vast quantity of cancerous cells frequently is significant for the clinical diagnosis and treatment of cancers. Many DNA-based electrochemical biosensors have been developed for FR detection with high selectivity and sensitivity, but most of them need complicated immobilization of DNA on the electrode surface firstly, which is tedious and therefore results in the poor reproducibility. In this study, a simple, sensitive, and selective electrochemical FR biosensor in cancer cells has been proposed, which combines the advantages of the convenient immobilization-free homogeneous indium tin oxide (ITO)-based electrochemical detection strategy and the high selectivity of the terminal protection of small molecule linked DNA. The small molecule of folic acid (FA) and an electroactive molecule of ferrocence (Fc) were tethered to 3′- and 5′-end of an arbitrary single-stranded DNA (ssDNA), respectively, forming the FA-ssDNA-Fc complex. In the absence of the target FR, the FA-ssDNA-Fc was degraded by exonuclease I (Exo I) from 3′-end and produced a free Fc, diffusing freely to the ITO electrode surface and resulting in strong electrochemical signal. When the target FR was present, the FA-ssDNA-Fc was bound to FR through specific interaction with FA anchored at the 3′-end, effectively protecting the ssDNA strand from hydrolysis by Exo I. The FR-FA-ssDNA-Fc could not diffuse easily to the negatively charged ITO electrode surface due to the electrostatic repulsion between the DNA strand and the negatively charged ITO electrode, so electrochemical signal reduced. The decreased electrochemical signal has a linear relationship with the logarithm of FR concentration in range of 10 fM to 10 nM with a detection limit of 3.8 fM (S/N=3). The proposed biosensor has been applied to detect FR in HeLa cancer cells, and the decreased electrochemical signal has a linear relationship with the logarithm of cell concentration ranging from 100–10000 cell/mL. Compared with the traditional heterogeneous electrochemical FR biosensors, the proposed biosensor owns the merits of the simplicity and high specificity, presenting the great potential application in the area of early diagnosis of cancers.

1. Introduction

Folate receptor (FR), cysteine-rich cell membrane glycoproteins, is commonly recognized as a magnetic tumor biomarker. It is highly expressed in various human malignant cancerous cells, like breast, ovarian, endometrial, brain, lung, prostate, kidneys, and so on (Henne et al., 2006; Kamaly et al., 2009; Lu et al., 2011). However, FR hardly exists in normal organs and tissues. Hence, the detection of FR can be used as a clinical diagnosis of aggressive or undifferentiated tumors at an advanced stage, which is accompanied with an increased density of FR (Li et al., 2014; He et al., 2016). Some typical methods have been extensively exploited for FR detection in the cancer diagnosis, for instance, radioimmunoassay (Antony et al., 1987), kinetic capillary electrophoresis (CE) (Petrov et al., 2005), fluorescence resonant energy transfer (FRET) (Clapp et al., 2004), surface plasmon resonance (SPR) (Cooper, 2002) *etc*. Although these methods have been identified as powerful approaches for the assays, they have some deficiencies such as tedious procedures, hazard to human health, time-consuming, sophisticated, and costly instruments.

It has been reported that FR can efficiently bind to the vitamin folic acid (FA) with high affinity (K_d~10^{-9} M) (Bharali et al., 2005),

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and its conjugates can internalize into the cell through a receptor mediated endocytosis and they have been frequently employed in bioimaging and chemotherapeutic drug delivery (Pan et al., 2013; Wang et al., 2011). The single-stranded DNA (ssDNA) could be terminally linked a small molecule, FA. When FA was bound to its protein target FR, it could be protected from the degradation by 3′-termini specific exonuclease I (Exo I) (called terminal protection) (Wu et al., 2009). The reason lies in that a steric hindrance of the bound FR macromolecules prevents Exo I from approaching and cleaving the phosphodiester bond of ssDNA from 3′-termini. Based on this principle, many sensitive and selective biosensors have been subsequently developed for FR detection (Jiang et al., 2015; Wu et al., 2011; Yang and Gao, 2014; Zhu et al., 2015). The electrochemical strategy has attracted considerable attention due to its remarkable advantages, including low cost, high sensitivity, simplicity, and rapid response (Gao et al., 2016). Many investigations have been reported on electrochemical biosensors for FR based on terminal protection of small molecule-linked DNA. For example, a novel electrochemical FR biosensor was presented via the nicking endonuclease-assisted amplification strategy (Cao et al., 2012). A highly sensitive and specific electrochemical detection of FR was developed based on hybridization chain reaction-assisted formation of copper nanoparticles (Zhao et al., 2015). A sensitive and label-free electrochemical impedance biosensor was constructed as well (Wang et al., 2014). The above-mentioned biosensors show the character of the high specificity for FR detection, however all these sensors are heterogeneous assays that probes are immobilized on the surface of electrode firstly, which is laborious and time-consuming. Furthermore, for the FA modified probe, FR interactions and the enzyme catalysis occur at the interface between the solution and the electrode surface, so the efficiencies are relative row. Moreover, the electrode modification procedures might affect the reproducibility of the biosensors. Therefore, it is desirable to develop immobilization-free based electrochemical FR biosensors in which the interaction of the probe-FR occurs in homogeneous solution and hence overcomes the drawbacks in traditional immobilization electrochemical FR biosensors.

Indium tin oxide (ITO) electrode with negatively charged surface can repel DNA because the molecular skeleton of DNA contains the negatively charged phosphate. This character has been broadly applied to develop immobilization free electrochemical biosensors for different targets (Wei et al., 2014; Xuan et al., 2013). For example, an immobilization-free and enzyme-free electrochemical nucleic acid sensing strategy was designed in light of the electrostatic repulsion between ITO electrode and the negatively charged dendrimer of DNA/PNA (Xuan et al., 2015). Given this character and the exonuclease III-assisted autocatalytic target recycling strategy, an electrochemical DNA biosensor was proposed for target DNA and protein detection (Liu et al., 2014). A label-free and enzyme-free electrochemical microRNA biosensor was presented combining this character with hybridization chain reaction (Hou et al., 2015). Our group also constructed a series of sensitive electrochemical biosensors for DNA and protein (Tan et al., 2015a, 2015b).

To the best of our knowledge, no work has been reported, which couples the high specificity of terminal protection and ITO-based immobilization-free electrochemical FR biosensor. In this study, we constructed a homogeneous immobilization-free, facile, high sensitive, and selective electrochemical biosensor for the folate receptor detection, which combines the advantages of immobilization free and the terminal protection. The proposed biosensor has high specificity and can be used to identify the over-expressed FR from the HeLa cells with high efficiency.

2. Experimental section

2.1. Reagents

Ferrocene (Fc) modified oligonucleotides (ssDNA-Fc) were synthesized by Sangon Inc. (Shanghai, China), and the arbitrary sequence was 5′-Fc **ACCTGGGGGAGTATTGCGGAGGAAGGT** -NH_2-3′. Both folate receptor (FR) and folic acid (FA) were obtained from Sigma-aldrich (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were provided by TCI (Shanghai, China). Exo I and the corresponding buffer were purchased from Thermo (Shanghai, China). Other chemicals were of analytical grade and used without further purification. Double-distilled water (Millipore, Direct-Q, 3resistance 18.2-MΩ) was used throughout the whole process. All DNA solutions were prepared in the phosphate-buffered saline (PBS, 100 mM, pH 7.4).

2.2. Preparation of folic acid linked ssDNA-Fc (FA-ssDNA-Fc)

Firstly, a ssDNA-Fc labeled with NH_2 at its 3′-end was tethered to FA via a conjugation by cross-linking agents (EDC and NHS) to form folic acid modified ssDNA-Fc (FA-ssDNA-Fc) (Wu et al., 2009). The solution was dialyzed against PBS (pH 7.4) with the 3 kD Amicon Ultra device to remove excess folic acid in process. Varying concentrations of FR was added to the FA-ssDNA-Fc solution to interact for 1 h in dark at 37 °C, and the macromolecule FR-FA-ssDNA-Fc was formed to protect the Exo I from digesting. The Exo I (100 U/mL) was added to the solution containing FA-ssDNA-Fc or FR-FA-ssDNA-Fc by incubation at 37 °C in dark for 1 h.

2.3. Electrochemical detection system

The different pulse voltammetry (DPV) was performed on a CHI 650D electrochemical analyzer (Chenhua Instruments, Shanghai, China) with a conventional three-electrode system, which consists of an ITO working, a platinum reference, and a platinum counter electrodes. The potential of the Pt reference electrode in the buffer was determined to be +0.36 V with respect to an Ag/AgCl reference electrode (Xuan et al., 2015). The ITO electrode was sonicated in a 10 g/L Alconox solution, propan-2-ol, and double-distilled water twice for 15 min in sequence before electrochemical detection, a negatively charged working electrode surface is obtained (Tan et al., 2015a,b).

2.4. Cell culture

Human cervical cancer cells (HeLa cells) were cultured in RPMI 1640 medium with 5% CO_2 atmosphere at the condition of 37 °C, which was replenished with 10% fetal bovine serum (FBS) and 100 U/mL penicillin in an incubator. The cells were incubated by trypsin-EDTA for 1 min to prevent them from detaching from the flasks. After being washed with RPMI 1640 medium several times, the HeLa cells were centrifuged at 1000 rpm for 5 min at 4 °C and resuspended in a cold CHAPS lysis buffer (0.5% CHAPS, 1 mM $MgCl_2$, 0.1 mM PMSF, 1 mM EGTA, 10% glycerol, and 10 mM Tris-HCl (pH 7.4)). Then homogeneous cell lysate (10^5 cells/mL) was obtained in ice incubating. Ultimately, different concentrations of HeLa cells were diluted by 100 mM PBS (pH 7.4) for further use.

3. Results and discussion

3.1. Principle of the proposed biosensor for FR

Scheme 1 depicts the principle of the proposed immobilization