Sensors and Actuators B 248 (2017) 187–194

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

A novel label-free homogeneous electrochemical immunosensor based on proximity hybridization-triggered isothermal exponential amplification induced G-quadruplex formation

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ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form 24 March 2017
Accepted 29 March 2017
Available online 31 March 2017

Keywords:
Label-free
Hemin
Homogeneous
Proximity hybridization

ABSTRACT

A label-free homogeneous electrochemical immunosensor was developed for sensitive and selective detection of carcino-embryonic antigen (CEA) based on proximity hybridization triggered isothermal exponential amplification induced G-quadruplex formation. The presence of CEA promoted the formation of a proximate complex via the proximity hybridization of the DNA strands labelled to affinity ligands, which unfolded the molecular beacon, the stem part of molecular beacon as a primer hybridize with the template to initiate the isothermal exponential amplification process. Thus, with the electrochemical indicator hemin selectively intercalated into the multiple G-quadruplexes, a significant electrochemical signal drop is observed, which is dependent on the concentration of the target CEA. Thus, using this "signal-off" mode, a simple, label-free homogeneous electrochemical strategy for sensitive CEA assay with a detection limit of 3.4 fg/mL is readily realized. Furthermore, this method also exhibits additional advantages of simplicity and low cost, since both expensive labeling and sophisticated probe immobilization processes are avoided. Its high sensitivity, acceptable accuracy, and satisfactory versatility of analytes led to various applications in bioanalysis.

1. Introduction

An immunoassay is considered as a powerful tool for disease diagnosis [1–7], environmental monitoring [8,9], and food safety [10,11]. Recently, electrochemical immunosensors have gained increasing attention and are considered to be one of the most promising methods in the quantitative detection of proteins because of their specific advantages, such as low cost, excellent detection limits, fast response, and easy handling [12–15]. Various electrochemical immunoassays based on labeling technologies with electroactive substances, enzymes, and nanomaterials, have been developed to amplify the tracing signal [16–19]. These electrochemical methods generally exhibit high detection sensitivity and high signal to noise ratio. However, the aforementioned methods are based on the heterogeneous assay that needs the immobilization of recognition element on the electrode [20,21]. Thus, the development of immobilization-free electrochemical strategies is highly desirable.

Homogeneous electrochemical biosensor has drawn an increasing number of attentions in recent years, because the hybridization between DNA and the recognition by the enzyme occur in the homogeneous solution without the immobilization of any biorecognition probe [22–26]. The signal outputs of these homogeneous electrochemical biosensors are recorded via detecting the diffusion current of DNA labeled by electrochemical active compounds (methylene blue or ferrocene) in the solution [27–29]. Although these homogeneous electrochemical strategies enabled low detection limit, they still require modification of an electroactive substance at its terminal [30,31]. In order to avoid the unnecessary labeling process, it is desirable to design a convenient and efficient signal output electrochemical system for label-free homogeneous detection.

In this paper, we construct a label-free homogeneous electrochemical immunoassay based on proximity hybridization-

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http://dx.doi.org/10.1016/j.snb.2017.03.152
0925-4005/

triggered isothermal exponential amplification (IEA) for producing many G-quadruplex/hemin complexes. Hemin consists of an iron (III) ion held in a heterocyclic ring, known as a porphyrin [32]. The hemin use as an electron transfer medium based on the reversible redox of Fe (III)/Fe(II) of hemin [33,34]. Unlike the most commonly used redox mediator, hemin was not covalently modified at the end of the oligonucleotide but could formate of G-quadruplex/hemin complex. For example: Pu group have used hemin/G-quadruplex concatamers as trace labels for sensitive detection of microRNAs [35].

In this paper, using CEA as a model tumor marker, the target CEA-induced proximity hybridization to trigger isothermal exponential amplification for sensitive electrochemical homogeneous immunoassay was developed (Scheme 1). The presence of target CEA triggered the formation of sandwich proximity immunocomplex, which unfolded the molecular beacon (MB). Subsequently, this separated stem DNA as a signal primer to hybridize with the template-DNA (T-DNA) and initiates IEA. The regenerated primer-template can continue to produce oligonucleotide G-quadruplex in a manner of linear amplification. The production of reporter oligonucleotide acts as a scaffold for the synthesis of G-quadruplex/hemin. The intercalated hemin molecules are further prevented from reaching the electrode surface because of the electrostatic repulsion between the negatively charged ITO electrode surface and the G-quadruplex. Thus, with fewer free hemin molecules present in solution, a significant electrochemical current drop is detected. Using this "signal-off" mode, facile and sensitive homogeneous electrochemical detection of CEA is readily realized.

2. Experimental section

2.1. Reagents

Nt·BstNBI endonuclease and DNA polymerase were obtained from New England Biolabs. Deoxyribonucleotides (dNTPs) were obtained from Fermentas Biotechnology Co. Ltd (Canada). Prostate-specific antigen (PSA), immunoglobulin G (IgG), platelet derived growth factor (PDGF), CA125, tris (2-carboxyethyl) phosphane hydro-chloride (TCEP), glucose (Glu), uric acid (UA), and dopamine (DA), were obtained from Sigma-Aldrich Chem. Co. Water was purified with a Milli-Q purification system (Branstead, USA) and used throughout the work. All chemicals used in this work were of analytical grade. The buffers used in the study were HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) for target binding. The washing buffer was PBS (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.1 M NaCl, pH 7.5). DNA oligonucleotides used in this work were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China).

DNA1: 5′-SH-TTTTTTTTTTTTTTTTGTGAGGGA**ACGGTCCTTG**-3′

DNA2: 3′-SH-TTTTTTTTTTTTTTTTCACTCCCT-**GCCAGATT-AGGTAAC**-5′

DNA3: 5′-ATTGCACCTGACGACGGTCAG-3′

G-quadruplex sequences: 5′-GGGTTAGGGTTAGGGTTAGGG-3′

Molecular beacon (MB): 3′-GTTACCTAATCTGGCTGCCAGGAAC GTACAGATTAGGTAAC-5′

Fluorophore-linker-MB:3′-FAM-GTTACCTAATCTGGCTGCCAGGAAC GTACAGATTAGGTAAC-BHQ-5′

Template DNA (T-DNA):3′-GTTACCTAATCTGTACCTCAGTT-CTCCCAA

CCCGCCCTACCCAA-5′

2.2. Apparatus

Differential pulse voltammetric (DPV) measurements were performed using a CHI 660E electrochemical analyzer (Shanghai, China). A three-electrode system was employed, with an indium tin oxide (ITO) electrode as the working electrode, an Ag/AgCl as the reference electrode, and a platinum wire as the auxiliary electrode. The ITO electrode was prepared as follows: first, ITO electrode was sequentially sonicated in an Alconox solution (8 g of Alconox per liter of water), acetone, and ultrapure water for 15 min each. Then, the ITO electrode was immersed into 1 mM NaOH solution for 5 h at room temperature and sonicated in ultrapure water for 15 min. After these cleaning procedures, a negatively charged working electrode surface was obtained. All the fluorescence measurements were performed on a Hitachi F-7000 spectrofluorimeter (Hitachi, Japan). The excitation wavelength was 418 nm, and the spectra are recorded between 570 and 650 nm. The fluorescence emission intensity was measured at 592 nm. Circular dichroism (CD) spectra were obtained on a JASCO J-815 spectrometer (Tokyo, Japan) at room temperature. CD spectra were recorded between 220 and 350 nm with a 10 mm path length quartz cuvette. The scan rate was set at 100 nm/min with a response time of 1 s and a bandwidth of 0.5 nm. The spectra were averaged over 3 scans. Zeta-potential analysis was performed on a Zetasizer (Nano-Z, Malvern, UK).

2.3. Preparation of DNA-labeled antibody

The DNA labeled antibodies (Ab-DNA) were synthesized according to the previous work [36]. Briefly, anti-CEA antibody (2 mg mL^{-1}) was first reacted with a 20-fold molar excess of sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) in PBS (55 mM phosphate, pH 7.4, 150 mM NaCl, 20 mM EDTA) for 2 h at room temperature. In parallel, 3 μL of 100 μM DNA1 or DNA2 was reduced with 4 μL of 100 mM DTT in PBS for 1 h at 37 °C. Both products were purified by ultrafiltration (10 000 MW cutoff membrane, Millipore), and the buffer was changed to PBE (55 mM phosphate, pH 7.4, 150 mM NaCl, 5 mM EDTA). After the products were mixed to incubate overnight at 4 °C and the unreacted DNA was removed by ultrafiltration (100,000 MW cutoff membrane, Millipore), the Ab-DNA was obtained.

2.4. Measurement procedure

The isothermal amplification reaction was carried out in 25 μL of total reaction solution consisting of 10 μL of 1 μM Ab1-DNA1 and Ab2-DNA2, 5 μL of various concentrations of CEA, 5 μL of 1 μM MB, 5 μL of 1 μM T-DNA, 5U DNA polymerase, 3.0 mM dNTP and 6U nicking enzyme. The one-pot reaction system was incubated at 37 °C for 50 min in a mixed buffer of NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) and CutSmart Buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 mg/mL BSA, pH 7.9). The reaction solution and 5 μL hemin (12 μM) were dropped onto the ITO for electrochemical detection, which could adequately cover the electrode working area. DPV from 50 to 750 mV (vs Ag/AgCl) with pulse amplitude of 50 mV and a pulse width of 200 ms was recorded in the above-mentioned reaction mixtures.

3. Results and discussion

3.1. Feasibility of the electrochemical immunoassay

The proof-of-concept experiments were carried out to investigate the feasibility of the proposed strategy for CEA assay. To verify that proximity hybridization could open MB, hybridization tests using free DNA strands in solution were performed. A fluorophore and a quencher were modified at both ends of the MB. As shown in Fig. 1, by itself or in the absence of target CEA, a weak fluorescence emission was observed (curve a) since the fluorescence of FAM was quenched by the black hole quencher (BHQ). In addition, for the