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Design strategy for a novel electrochemically active–inactive switching molecular beacon based on Hemin for SNPs and insulin detection directly in homogenous solution

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ABSTRACT

Herein, a novel and convenient electrochemically active–inactive switching molecular beacon based on hemin (Hs-MB) has been designed for easy discrimination of single nucleotide polymorphisms (SNPs) and sensitive detection of insulin. The electrochemically active changing capability of Hs-MB is based on two identical hemin groups labeled at both ends of MB sequence in dimer or monomer forms depending on the conformation of MB which is in stem-loop structure or line shaped structure. The Hs-MB assay permits discrimination of SNPs and the highly sensitive and specific detection of insulin with detection limit successively as low as 0.5 pmol/L. Even at a very low target concentration, the Hs-MB assay also shows a good specificity in the presence of other potentially interfering components. The experimental results also show that Hs-MB can also be used for the accurate and rapid monitoring of insulin secretion by glucose-stimulated from MIN6 cells at different time periods, demonstrating that Hs-MB has potential in monitoring of biomarker variation in vivo.

1. Introduction

Ultrasensitive analysis of biomarkers in complex samples is of great importance for biological studies and clinical diagnosis. Insulin, a peptide hormone produced by β-cells of the pancreatic islets, has important effects on physiological glycometabolism due to its role in the absorption of glucose from blood for its conversion into glycogen or fat, or into both in the case of the liver. It is associated with diabetes and other related diseases [1,2]. Thus, sensitive and selective detection of insulin [3,4] is of profound value in the diagnosis of infection and a multitude of other disease states, as well as in assessments of general physiological trauma [5].

Conventional immunoassays are sensitive and still standard technologies nowadays, but they generally require complicated operation steps, expensive instruments, and qualified personnel. Recently, several innovative methods, including fluorescent technique, such as fluorescent molecular beacon (F-MB), quantum-dots (QDs) and oxidized carbon nanoparticles (OCNPs) based aptasensors has been developed with great advantages as well as limitations [6,7] and offered new opportunities for improved performance in protein biomarker detection [8–10]. However, the applications of these methods were limited by facile photo-bleaching of F-MB, the toxicity of QDs and false positive results (FPR) caused by the weak interaction between OCNPs and DNA.

Compared to optical transduction mechanisms, Plaxco and his coworkers focused on the electrochemical molecular beacons (E-MBs) for the electrochemical detection with high sensitivity and selectivity, fast and portable, simple instrumentation and low-cost [11,12]. As is well known, such E-MBs are usually designed to be immobilized onto the electrode surface [13,14], which may, somehow, cause lower available hybridization efficiency and higher background noise, possibly arising from spatial hindrance effects.

We have designed a new type of electrochemically active–inactive switching molecular beacon (EAIS-MB), such as CAs-MB [15,16]. Similar as F-MB, the CAs-MB was non-fixed on the electrode surface and just free in the solution, and displays electrochemically active–inactive behavior between its hairpin and extended structures. It can direct detection of the target in homogeneous solution and deliver satisfactory results.

Hemin, the iron complexes of porphyrins, forms the active sites of a variety of vitally important enzyme systems. Compared with carminic acid, the nonemer of hemin is higher electrochemical activity and it can be held together in formation of π–π dimers or μ-oxo dimers (a single-

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oxygen bridging the metal atoms) depending on the conditions [17–19]. Therefore, hemin is chosen as the candidate for the stratagem another EAIS-MB, which is Hs-MB.

Hence, the aim of this paper was to design a sensitive, convenient, and robust electrochemical Hs-MB strategy for the detection of bioactive molecules to meet the increasingly rigorous requirements of medical research and clinical diagnosis. Therefore, taking advantage of the Hs-MB probe and the amplification efficiency of the cascade signal amplification strategy, the proposed method provides an ultrasensitive platform for insulin detection.

2. Materials and methods

2.1. Instrumentation

Electrochemical measurements were performed with a CHI 660E electrochemistry workstation (Chenhua Instruments, China) in three electrode systems, where a platinum wire (φ = 0.5 mm) as counter electrode, Ag/AgCl (3 mol/L KCl) electrode as reference electrode and a bare glassy carbon (GC) electrode (3 mm diameter) as working electrode at ambient temperature (22 ± 2 °C). In cyclic voltammetry (CV) mode, scan range was from − 0.6–0.0 V and scan rate was 50 mV/s. In differential pulse voltammetry (DPV) mode, pulse width was 50 ms, pulse amplitude was 50 mV, pulse period was 200 ms, and the scan range was from 0.3 V to − 0.8 V. The electrolyte solution was 0.1 mol/L phosphate buffer solution (pH 7.4) containing 0.1 mol/L NaCl. A Waters HPLC system model 2487 was equipped with a dual-wavelength UV detector and a Waters Symmetry C18 (150 mm × 4.6 mm) column (Milford, USA). UV–vis absorption spectra were recorded on a Cary Varian 50 Probe.

2.2. Materials

All oligonucleotides were synthesized and HPLC-purified by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The oligonucleotide sequences are listed in Table S1. Exo III was purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Hemin, insulin, immunoglobulin G (IgG), lysozyme, and thrombin were purchased from Sigma-Aldrich. N-hydroxysuccinimide (NHS), N,N-diisopropylethylamine (DIPEA), dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), hydrogenperoxide (30%), 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), glucose, and 2-mercaptoethanol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The mouse insulinoma pancreatic β-cell line, MIN6 cells, was purchased from Shanghai Meixuan Biotechnology Co., Ltd. Fetal bovine serum, penicillin, and streptomycin were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK). All other chemicals were obtained from Shanghai Chemical Reagents (Shanghai) and used without further purification. Milli-Q water (resistance > 18 MΩ cm) was used in all experiments.

2.3. Fabrication of Hs-MB

Hs-MB was prepared by the reaction between hemin active esters and hairpin-shaped oligonucleotides. Briefly, hemin active ester was synthesized by slowly mixing hemin (97.8 mg), HATU (85.6 mg), NHS (17.26 mg), and DIPEA (74 μL) in anhydrous DMF (10 mL) under pure nitrogen in a sealed 25 mL flask. After 30 min of magnetic stirring in the dark at room temperature, a ten-fold volume of diethyl ether was poured into the reaction mixture, and the black precipitate was collected by centrifugation. After rinsing twice with diethyl ether, the precipitates were dried by N_2 flow and stored at − 20 °C. The product was characterized as hemin$(NHS)_2$ by MALDI mass measurements with a major peak at *m/z* 811.15 (The calculated weight of M·H+ (M = $C_{42}H_{38}FeN_6O_8$) is 811.15 g/mol; Fig. S1).

The synthetic procedure for Hs-MB was similar to that for hemin $(NHS)_2$. Briefly, excess hemin active ester, 5 OD MB sequence, and DIPEA (20 μL) were added into a solution of 50% DMF and 50% water (pH 8.5). The reaction was allowed to proceed in the dark with gentle stirring for one hour at room temperature. 0.1 vol of 3 mol/L NaCl (95% ethanol as the solvent) and 2.5 volumes of ice-cold absolute ethanol were added into the reaction mixture, and the mixture was kept at − 20 °C for 30 min to allow the product to precipitate. The brown-black precipitate was collected by centrifugation for 15 min at 12,000 rpm and then redissolved in water for HPLC analysis (A: 0.1 mol/L aqueous triethylammonium acetate; B: acetonitrile; linear gradient; Fig. S2). Further purification was performed with a Sephadex LH-20 exclusion/reversed-phase column to desalt and remove unbound hemin. Finally, the pure Hs-MB solution was freeze-dried and stored at − 20 °C. MALDI mass measurements of Hs-MB showed a measured MW of 10,403.5 g/mol (calculated MW: 10,403.2 g/mol; Fig. S3).

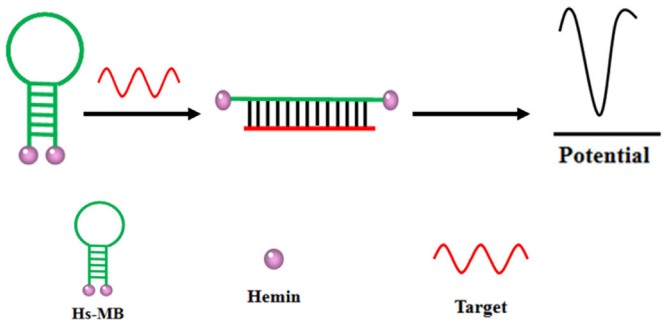
2.4. Continuous sampling monitoring of insulin secretion by glucose-stimulated MIN6 cells at different time periods

MIN6 cells were maintained in Dulbecco's modified Eagle's medium containing 5 mmol/L glucose with 11% (v/v) heat-inactivated fetal bovine serum, 0.1 mmol/L of 2-mercaptoethanol, 100 units/mL of penicillin, and 100 μg/mL of streptomycin in a 5% CO_2 atmosphere at 37 °C [20]. For continuous sampling monitoring of insulin secretion from glucose-stimulated MIN6 cells at different time periods, different final concentrations of glucose were added (5, 10, 15, 25 mmol/L) to the MIN6 cells (1×10^6 cells/well). Every two minutes, 200 μL of liquid was removed from the medium as the sample used for the next insulin detection. The detailed procedure for insulin detection was as follows. First, the aptamer-binding reaction was performed by mixing 10 μL of H1-H2 duplex (18 nmol/L) and 50 μL of various concentrations of insulin samples followed by incubation at 37 °C for 30 min. Then, 20 μL of H3 (90 nmol/L) and 5 μL of Exo III (60 U/μL) were added into the above solution with continuous incubation at 37 °C for 1 h. The mixture was then heated to 70 °C for 10 min and cooled to room temperature. Then, 180 nmol/L of Hs-MB and 100 μmol/L of $ZnCl_2$ were added and the mixture was incubated at 37 °C for 1 h. Finally, the electrochemistry measurements were performed in HEPES buffer solution (pH 7.4) with volumes of 500 μL.

3. Results and discussion

3.1. Characteristics of Hs-MB

The operating principle of Hs-MB is shown in Scheme 1. In the absence of the target sequence, the MB possesses a stem-loop conformation with the two identical hemin groups in close proximity to become dimeric forms, leading to suppression of the electrochemical signal. Upon the addition of the target DNA, the loop domain hybridizes with it, forcing the Hs-MB to unfold and leading to physical separation



Scheme 1. Schematic illustration of Hs-MB hybridized with a target gene segment.