Analytica Chimica Acta 882 (2015) 27–31

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Sensitive detection of a serum biomarker based on peptide nucleic acid-coupled dual cycling reactions

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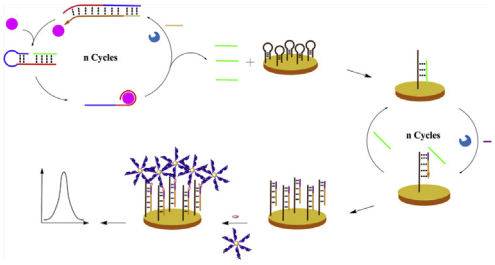
HIGHLIGHTS

- A new assay for direct serum detection of protein or hormone markers is developed.
- PNA-coupled DNA cycling reactions with dual amplification is adopted.
- The sensing system provides a detection limit of insulin as low as 1 pM.
- The assay of blood sample can discriminate GDM patients from the normal ones.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 February 2015
Received in revised form 4 May 2015
Accepted 6 May 2015
Available online 11 May 2015

Keywords:
Peptide nucleic acid
Strand displacement
Dual amplification
Insulin
Gestational diabetes mellitus

ABSTRACT

Serum level of disease markers may provide important guidance for diagnosis and prognosis. In this work, a sensitive and specific method suitable for direct serum detection of biomarkers is developed based on peptide nucleic acid (PNA)-coupled DNA cycling reactions with dual amplification. In this method, PNA released from a target-triggered homogeneous DNA cycling is employed to initiate an interface DNA cycling, and both of the cycling reactions are based on polymerase-assisted strand displacement reaction. Consequently, two PNA-coupled DNA cycling steps can take place simultaneously in one-pot, leading to greatly enhanced limit of detection and simplified operation. This method has also been successfully applied for evaluating serum insulin in pregnant women as an indicator of gestational diabetes mellitus. So the application of this method in real bio-samples may allow it to hold considerable potential in clinical practice. In addition, since there is no requirement for specific sequence of aptamer, the strategy proposed can be extended for the detection of many other protein markers and peptide-hormones in the future.

1. Introduction

Clinical diagnosis, treatment and prevention of diseases have placed great demands on the sensitive detection of biomarkers [1–3]. Therefore, a multitude of signal amplification strategies for highly sensitive measurements has been developed. Among them, DNA cycling-based approaches have arisen great scientific interest and have been intensively studied [4,5]. Traditional DNA cycling technique, such as polymerase chain reaction, is restricted to nucleotide detection, due to the thermal cycling steps employed

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http://dx.doi.org/10.1016/j.aca.2015.05.006
0003-2670/

[6,7]. Nevertheless, recent development of isothermal DNA cycling methods, such as rolling circle amplification, as well as enzyme-assisted DNA cycling, have enabled sensitive detection of a broad range of targets, including various disease marker proteins and hormones [8,9]. But the instability of DNA duplex towards temperature, salinity and enzymes in biologically complicated samples are still great concerns in designing DNA cycling-based biosensors.

Peptide nucleic acids (PNAs) are synthetic molecules consisting of peptide backbone and nucleobases, exhibiting higher affinity and sequence selectivity towards complementary DNA strand [10,11]. Meanwhile, PNAs possess improved chemical stability compared with natural nucleic acids, making PNA/DNA hybrid duplex more stable and independent of salt concentration [12]. More importantly, PNAs are not easily recognized by either nucleases or proteases [12,13], rendering them resistant to enzyme degradation in complex bio-samples detection. Owing to these properties, PNAs have been incorporated into DNA or protein detection, mainly as aptamer or initiator of the essential strand invasion and displacement steps [14,15], while the sensitivity falls short of the demand for serum detection.

In this work, as a mediator of dual enzymatic DNA cycling reactions, PNA is firstly designed to be able to be released from target-initiated in-solution DNA cycling, and the released PNA is further designed to trigger an interface DNA cycling. In the meantime, the two cycling steps are not only coupled by PNA, but also share the same mechanism of polymerase-assisted strand displacement. Therefore, the two PNA-coupled DNA cycling steps can take place simultaneously in one-pot, which may effectively enhance the limit of detection and simplify the operation of the measurements.

The proposed method has also been employed to analyze serum abundance of insulin, the model target for this study, which is known to be abnormal in diabetes mellitus [16], as well as several cancers such as pancreatic cancer, insulinoma, prostate cancer, etc. [17,18]. Particularly, serum insulin has been detected and examined in pregnant women with gestational diabetes mellitus (GDM) [19], a dangerous condition associated with a number of adverse outcomes, including excessive fetal growth, increased incidence of birth trauma and neonatal metabolic abnormalities [20,21]. In this work, the experimental results obtained by using the proposed new method can tellingly discriminate GDM from normal conditions. So it may indicate the efficiency of this method in assisting the administration of insulin-relevant pathological processes towards better patients' endings.

2. Materials and methods

2.1. Chemicals

Human recombinant insulin (>98%) was purchased from Tocris (UK). Methylene blue (MB), bovine serum albumin (BSA) and $ZrOCl_2$ were purchased from Sigma. The Klenow fragment polymerase (exo$^-$) was from New England Biolabs Inc. (USA). PNA was custom-synthesized from Shanghai Science Peptide Biological Technology Co., Ltd., with the following sequence: NH_2–CCCCCCCACC–COOH. The oligonucleotides used in this work were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). The specific DNA sequences are as follows: Hairpin DNA (5′-GGTGGTGGGGGGGGTTGGTAGGGTGTCTTCTCCCTACCAAC-H_2PO_4-3′), Primer DNA 1 (5′-GTTGGTAGGG-3′), Capture DNA (5′-SH-AAAGGTGGGGGG GTGTACCTTTGCGA-3′), Primer DNA 2 (5′-H_2PO_4-TCGCAAAGGT-3′), Signal DNA (5′-TTTCCTCTCAACTCGTA-(CH_3)6-SH-3′) and its complementary sequence (5′-TACGAGTTGAGAG-GAAA-H_2PO_4-3′). All other chemicals used were of analytical reagent grade. NEB buffer 2, used as reaction solution in the work, was from New England Biolabs Inc. (USA). 1x NEB buffer 2: 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9. All solutions were prepared with double-distilled water (specific resistance >18 MΩ/cm) purified with a Milli-Q purification system (Barnstead, USA). Human venous blood samples from GDM, normal pregnant and normal non-pregnant women were obtained from the Department of Obstetrics and gynecology, The First Affiliated Hospital of Nanjing Medical University, with the unanimous approval by the local Ethics Board. The blood samples were first centrifuged at 2500 rpm for 10 min. Then, the supernatant was collected and dialyzed. The prepared supernatant was then for the continued assay. Part of the prepared serum from normal non-pregnant women was spiked with various concentrations of insulin (2, 20, 200) pM.

2.2. Preparation of probe

Before the construction of amplification system, hairpin DNA probe was treated in a process of gradient cooling treatment to get a stable secondary structure. The process is a fully denaturation at 95 °C for 5 min followed by a gradient cooling step that drop 5°/min until cooling down to the room temperature. Then the probe was stored at 4 °C for later use. Afterwards, PNA and hairpin DNA were mixed with a concentration of 2 μM of each in the 1x NEB buffer 2. Then the mixture was incubated at 37 °C for 1 h to form hairpin DNA–PNA probe.

2.3. Gold electrode treatment and modification

First of all, a gold disk electrode (3 mm diameter) was cleaned with piranha solution (70% concentrated sulfuric acid, 30% H_2O_2) for 5 min. Then, the electrode was rinsed with double-distilled water and polished with 1 mm and 0.3 mm alumina powder in order. After that, the electrode was ultrasonicated sequentially in ethanol and water to remove residual alumina powder. At last, the electrode was immersed in nitric acid (50%) for 30 min, and electrochemically cleaned with 0.5 M H_2SO_4. The prepared clean gold electrode was incubated with 50 μL assembly solution (0.2 μM modified DNA and 10 mM TCEP in 1x NEB buffer 2) for 16 h at room temperature after being dried with nitrogen. Subsequently, the electrode was immersed in 100 μL 9-mercapto-1-nonanol (MN) solution (1 mM MN in 10 mM PBS, pH 7.4) for 3 h at room temperature. Afterward, the modified electrode was rinsed with double-distilled water to remove MN non-specifically adsorbed onto the electrode surface, followed by drying under mild nitrogen stream.

2.4. Fabrication of gold nanoparticle/DNA/methylene blue (GNP/DNA/MB) nanocomposites

Citrate-capped AuNPs with an average diameter of 13 ± 2 nm were synthesized by the citrate reduction method. In brief, trisodium citrate (5 mL, 38.8 mM) was putted rapidly to boiling solution of $HAuCl_4$ (50 mL, 1 mM) with fierce stirring. The color of the solution changed from pale yellow to deep red in a minute. The reaction was allowed to continue for 30 min under constant stirring and heat. Then, the solution was filtered with a 0.2 μm membrane filter after cooling down. The average size of the AuNPs was 13 ± 2 nm as calculated from the transmission electron microscopy (H-7650, Hitachi) images.

The above prepared AuNPs colloid was incubated with thiol-modified sequence for 16 h at room temperature. Then, the mixture was stepwise aged with 0.1 M NaCl and incubated for 24 h at room temperature. After that, centrifugation was conducted to remove redundant unmodified DNA. 1 mL of MN solution (1 mM MN in 10 mM PBS, pH 7.4) was added to the above solution for 0.5 h