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Naked eye detection of trace cancer biomarkers based on biobarcode and enzyme-assisted DNA recycling hybrid amplifications

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ABSTRACT

Naked eye-based detection has received increasing research interest due to the simplicity nature of this type of assay. However, improving the sensitivity of the naked eye detection method for the monitoring of trace amount of target molecules remains a major challenge. Herein, we describe a biobarcode and an enzyme-assisted DNA recycling hybrid amplification strategy for naked eye detection of sub-picomolar carcinoembryonic antigen (CEA), a cancer biomarker. The presence of CEA and the corresponding antibodies results in the formation of immunocomplexes and the capture of the biobarcodes in a microplate. The massive barcode DNAs released from the biobarcodes hybridize with the G-quadruplex inactive hairpin DNA probes and form catalytic nicking sites for N.BstNBI endonuclease, which cleaves the barcode DNA/hairpin partial dsDNA, releases the G-quadruplex active sequences and recycles the barcode DNA. Due to the barcode DNA recycling process, numerous G-quadruplex active sequences are generated and associate with hemin to form peroxidase mimicking enzymes, which convert colorless $ABTS^{2-}$ to green color intensified $ABTS^{\bullet-}$ to achieve naked eye detection of CEA down to 0.025 ng mL^{-1} (0.14 pM). The naked eye detection strategy reported herein can be applied also to complicated serum sample matrix, making this approach hold great promise for point-of-care diagnostic applications.

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1. Introduction

Cancer has become the number two leading causes of death in the US according to the report from National Cancer Institute. Cancer biomarkers (e.g., secreted proteins, DNA, mRNA and transcription factors) can distinguish normal or disease states (Wulfkuhle et al., 2003; Kulasingam and Diamandis, 2008; Rusling et al., 2010). The levels of cancer biomarkers can provide important information on the occurrence, existence and progression of different types of cancers (Hanash et al., 2008; Ludwig and Weinstein, 2005). Early identification and detection of these biomarkers are crucial for patient survival and successful prognosis of cancers (Giljohan and Mirkin, 2009; Wilson and Nock, 2003; Munge et al., 2011; Ferrari, 2005; Kingsmore, 2006). In this regard, intensive research focus has been directed toward developing robust analytical techniques for cancer biomarker detections.

Indeed, in the past decades, we have witnessed the demonstrations of numerous methods for the detection of low levels of cancer biomarkers by coupling effective signal amplification schemes, such as immuno-PCR (Sano et al., 1992), rolling recycle amplification (Zhao et al., 2008; Nilsson et al., (2006); Kingsmore and Patel, 2003; Demidov, 2002), biobarcode (Nam et al., 2003, 2004; Stoeva et al., 2006a, b; Thaxton et al., 2009) and functional nanomaterials (Munge et al., 2011, 2005; Wang et al., 2004; Yu et al., 2006; Mani et al., 2009; Malhotra et al., 2010), with electrical, optical or mechanical transduction techniques. Despite the advantageous high sensitivity of these approaches, these methods are reliable only in laboratory settings and are not amenable for on-site or point-of-care (POC) applications due to the requirements of highly trained personnel and complicated signal transduction means. Therefore, the development of rapid, sensitive, selective and simple alternatives for cancer biomarker detection without using advanced or complicated instruments, for example by using human naked eye, can potentially revolutionize the detection and diagnosis of cancers.

The naked eye-based detections were pioneered by Mirkin and colleagues (Mirkin et al., 1996; Storhoff et al., 1998), relying on the change of the optical properties of gold nanoparticles (AuNPs), which are strongly dependent upon the interparticle separation distance. The hybridizations between the target DNA and the DNA probes conjugated to AuNPs lead to the assembly of the AuNPs and cause a significant shift in the extinction spectrum of AuNPs, which is indicated by a visible color change from red to purple. Based on this type of target-induced assembly or disassembly of AuNPs detection mechanism, various visual sensing strategies

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have been developed to monitor different types of target molecules, including DNA (Li and Rothberg, 2004a, b; Du et al., 2006), small biomolecules (Liu and Lu, 2004, 2006; Wang et al., 2007) and metal ions (Choi et al., 2009; Darbha et al., 2008; Xu et al., 2010; He et al., 2008; Wang et al., 2008). Although these AuNP-based visual detection methods are simple, the AuNPs used in these techniques are susceptible to sensing environment (ionic strength, acidity, etc.), which may potentially lead to false positive responses (Liu and Lu, 2006; Laromaine et al., 2007). Besides, these visual detection approaches can be achieved with confidence only at high concentration of the target molecules (low sensitivity). Moreover, these strategies require the target molecules and the recognition probe molecules to be sufficiently small to guarantee a distinguishable color change upon assembly of AuNPs (Su et al., 2003). These limitations make sensitive naked eye detection of macromolecules (such as protein biomarkers) remain as a major challenge.

To explore solutions for the challenges encountered in current naked eye-based detection of low levels of protein biomarkers, we propose herein a new strategy for sensitive and visual detection of carcinoembryonic antigen (CEA, a protein cancer biomarker) based on biobarcode and enzyme-assisted DNA recycling amplifications. Our approach employs the barcode DNA as the intermediate target, which can be recycled by an endonuclease to generate massive peroxidase mimicking enzymes, known as G-quadruplex/hemin complexes. These mimicking enzymes catalyze the conversion of a colorless substrate to a green color product, which enable us to detect sub-picomolar CEA with naked eye.

2. Experimental section

2.1. Apparatus and reagents

A 2450 UV spectrophotometer (Shimadzu, Japan) was used to obtain the absorption spectra at room temperature in all experiments. A canon EOS 550D camera was used to take all the photographs.

All the DNA sequences (barcode DNA: 5′-TCATCACACTGGAAGACTC-3′; biotinylated barcode complementary DNA (c-DNA): 5′-biotin-GAGTCTTCCAGTGTGATGA-3′; G-quadruplex inactive hairpin DNA: 5′-CCCTACCCGAGTCTTCCAGTGTGATGAGGGTAGGGCGGGTTGGG-3′) were synthesized and purified by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). The nicking enzyme N. BstNBI (an endonuclease that recognizes the specific nucleotide sequence of 5′-GAGTC-3′ in a double-stranded DNA) and 10 × NEBuffer 3 (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, pH 7.9) were obtained from New England BioLabs (Ipswich, MA, USA). Hemin, 2,2-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS), H_2O_2, and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES) were purchased from Aladdin Reagents (Shanghai, China). The hemin stock solution (1 mM) was prepared in dimethylsulfoxide (DMSO) and stored at −20 °C. Streptavidin-coated magnetic microbeads (MyOne Streptavidin C1, STV-MBs) were obtained from Invitrogen Corp. (Oslo, Norway). The primary anti-CEA antibody (Ab_1), biotin-modified secondary anti-CEA antibody (biotin-Ab_2), CEA, alpha-fetoprotein (AFP), mouse IgG, vascular endothelial growth factor (VEGF) and the commercial CEA detection ELISA kit were all ordered from Biocell Co., Ltd (Zhengzhou, China). Human serum samples were provided by the 9th People's Hospital of Chongqing (Chongqing, China).

2.2. Preparation of the biobarcode conjugates

An aliquot of 200 μL STV-MBs (10 mg mL^{-1}) was washed twice with PBS buffer and incubated with PBS buffer containing biotinylated Ab_2 (2 μg mL^{-1}) for 30 min at room temperature with gentle rotation. Afterwards, 30 μL of 100 μM c-DNA was added to conjugate with Ab_2/STV-MB for 30 min at room temperature with gentle mixing. After washing the beads twice with PBS buffer and separating by an external magnet for 3 min, the beads were resuspended in PBS buffer. Finally, 30 μL of 100 μM barcode DNA was added and incubated with the c-DNA/Ab_2/STV-MBs for 30 min. The resulting biobarcode conjugates were washed with PBS buffer and stored in PBS buffer at 4 °C.

2.3. Naked eye detection of CEA by using biobarcode and enzyme-assisted barcode DNA recycling amplifications

The polystyrene microplate (NUNC, Roskilde, Denmark) was first coated with Ab_1 (10 μg mL^{-1}, 100 μL, in 0.1 M carbonate buffer, pH 9.6) at 4 °C overnight. The wells were then washed with 200 μL PBST (0.01% Tween-20) three times and blocked with 200 μL PBS–BSA (1% BSA). Then, CEA solutions (100 μL) at different concentrations or serum samples were added to the wells and incubated at 37 °C for 1 h. After washing the wells with PBST three times, the biobarcode conjugate solution was incubated with the wells for 1 h at 37 °C, followed by washing twice with PBST. Subsequently, 100 μL of nanopure water was added to the wells, and the plate was heated to 60 °C for 5 min to ensure complete release of the barcode DNAs. The barcode DNA solution was separated by an external magnet and transferred into a 1.5 mL centrifuge tube containing 10 μL of 10 μM G-quadruplex inactive hairpin sequences, 15 μL 10 × NEBuffer 3 and 30U N.BstNB I. The mixture was incubated at 55 °C for 60 min to allow recycling of the barcode DNA by the N.BstNB I enzyme (Lin et al., 2011). After the deactivation of the N.BstNB I enzyme by elevating the temperature to 90 °C, 5 μL of 1 μM hemin and 45 μL 2 × HEPES buffer (50 mM HEPES, 40 mM KCl, 400 mM NaCl, 0.1% Triton X-100 and 2% DMSO; pH 7.4) were added and incubated for 60 min at room temperature. Finally, $ABTS^{2-}$ and H_2O_2 were added to the mixture to attain final concentrations of 6 mM and 2 mM at room temperature, respectively, and photographs of the solutions were taken after 10 min of color development.

3. Results and discussion

Our naked eye sensitive cancer biomarker detection principle is illustrated in Scheme 1. The biobarcode conjugates are first prepared by co-immobilizing biotin-Ab_2 and biotin-c-DNA sequences on the streptavidin-coated magnetic microbeads (STV-MBs) through strong biotin–STV affinity interactions. This is followed by the hybridizations between the barcode DNA and the corresponding complementary strands on the STV-MBs to obtain the biobarcode conjugates (Scheme 1A). In our biobarcode design, the 1 μm MBs, instead of the common 30 nm AuNPs, are used to prepare the biobarcodes due to the following considerations. First, by using the STV-MBs, the biobarcodes can be prepared within 1.5 h, which significantly reduces the biobarcode preparation time by avoiding the lengthy and complicated aging steps required in common AuNP-based biobarcode preparation processes. Second, due to the magnetic nature of the microbeads, the biobarcodes can be easily isolated by an external magnet and the 1 μm size of the beads can increase the loading of more barcode DNA as well. For naked eye detection (Scheme 1B), the target CEA molecules are incubated with Ab_1-coated microplate, followed by the addition of the biobarcodes to form immuno-sandwich complexes in the wells of the microplate. After extensive washing to remove the non-specifically adsorbed biobarcodes, numerous barcode DNAs are released from the captured biobarcodes and transferred to a solution to hybridize with the G-quradruplex inactive hairpin DNA probes. These hybridizations

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