Biosensors and Bioelectronics 49 (2013) 380–386

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

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

A cytometric bead assay for sensitive DNA detection based on enzyme-free signal amplification of hybridization chain reaction

Wei Ren, Hongmei Liu, Wenxia Yang, Yunlong Fan, Lang Yang, Yucong Wang, Chenghui Liu*, Zhengping Li

Key Laboratory of Medicinal Chemistry and Molecular Diagnosis, Ministry of Education; College of Chemistry and Environmental Science, Hebei University, Baoding 071002, Hebei Province, PR China

ARTICLE INFO

Article history:
Received 28 March 2013
Received in revised form
19 May 2013
Accepted 30 May 2013
Available online 7 June 2013

Keywords:
DNA analysis
Flow cytometry
Bead assay
Hybridization chain reaction
Signal amplification

ABSTRACT

A versatile flow cytometric bead assay (CBA) is developed for sensitive DNA detection by integrating the advantages of hybridization chain reaction (HCR) for enzyme-free signal amplification, flow cytometry for robust and rapid signal readout as well as magnetic beads (MBs) for facile separation. In this HCR–CBA, a biotinylated hairpin DNA (Bio-H1) is firstly immobilized on streptavidin-functionalized MBs. Upon the addition of target DNA, each target would hybridize with one Bio-H1 to open its hairpin structure and subsequently initiate a cascade of hybridization events between two species of fluorescent DNA hairpin probes (H1*/H2*) to form a nicked double helical DNA structure, resulting in amplified accumulation of numerous fluorophores on the MBs. Finally, the fluorescent MBs are directly analyzed by flow cytometry. This technique enables quantitative analysis of the HCR products anchored on the MBs as a function of target DNA concentration, and analysis of each sample can be completed within few minutes. Therefore, the HCR–CBA approach provides a practical DNA assay with greatly improved sensitivity. The detection limit of a model DNA target is 0.5 pM (3σ), which is about 3 orders of magnitude lower compared with traditional hybridization methods without HCR. Furthermore, the signal of complementary target can be clearly distinguished from that of single-base mismatched sequences, indicating the high specificity of the HCR–CBA. Moreover, this strategy is also successfully applied to the DNA analysis in complex biological samples, showing great potential in gene analysis and disease diagnosis in clinical samples.

1. Introduction

The sensitive detection of specific DNA sequences associated with pathogenic or genetic diseases has become more and more important for early clinical diagnosis and gene therapy (Huys et al., 2012). Therefore, the development of sensitive and practical DNA assays with simple and rapid procedures is highly desired (Rosi and Mirkin, 2005; Sassolas et al., 2008). Toward this goal, many signal transduction techniques such as colorimetric (Elghanian et al., 1997; Song et al., 2010), electrochemical (Li et al., 2007, 2010), fluorescent (Yang and Zhao, 2010), chemiluminescent (Freeman et al., 2011; Liu et al., 2006), light scattering (Dai et al., 2008; Du et al., 2006) and mass-based methods (Han et al., 2011, 2013) have been utilized for DNA analysis. Compared to other methods, the inherent advantages of the fluorescence detection approaches, such as high sensitivity, easy readout, low sample volume and simple operation, make them most attractive for fabricating DNA sensors (Yang and Zhao, 2010), particularly in the homogeneous assaying modes. However, a majority of the homogeneous fluorescent assays are unsuitable for the analysis of complex biological samples due to the strong light scattering and autofluorescence background in the complex matrix, which greatly limits the detection sensitivity. This is particularly challenging for nucleic acid detection in clinical samples such as serum, which is known to have a strong matrix background.

In this regard, bead assays, especially magnetic beads (MBs)-based assays, coupled with fluorescence labeling have become a fascinating choice for the detection of DNA and other biomolecules in biological samples because the beads can be easily collected, allowing abstraction of the fluorescence signal from the sample matrixes (Riahi et al., 2011; Song et al., 2011; Zhang et al., 2010). So the beads-based assays offer rapid detection and superior removal of background, which are suitable for complex samples. The general approach of bead assays has been established reasonably well so far, including binding chemistries of beads to either oligonucleotide probes or antibodies and suitable fluorescent staining method for target analytes detection. Nevertheless, there are still some drawbacks for the beads-based fluorescent

* Corresponding author. Tel./fax: +86 312 5079403.
E-mail address: liuch@hbu.edu.cn (C. Liu).

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http://dx.doi.org/10.1016/j.bios.2013.05.055

bioassays: (i) most of the reported bead assays are designed in a 1:1 binding format, namely, one target analyte can only capture one fluorescent probe on the bead surface, which greatly limit their sensitivities. In regards to DNA assays, polymerase chain reaction (PCR) is routinely used as a powerful technique for signal amplification. However, it is difficult for the PCR to produce signal reports-modified sequences directly on the beads (Niu et al., 2010). In addition, PCR is highly enzyme-dependent and usually needs demanding conditions to keep the activity of polymerase, particularly for the nucleic acid amplification on the beads surface because the enzymes may show remarkably decreased activities due to their less accessibility to the nucleic acids on the solid phase. Therefore, it is still a big challenge to find a new way to further improve the sensitivity of beads-based DNA assay; (ii) the most commonly used beads are microspheres with micron-scale diameters, so the beads-based fluorescent bioassay is performed in a suspension format rather than in a homogeneous medium. Therefore, the conventional cuvette-based fluorescence measurement on fluorometer is unfitted for the detection of fluorescence signal on the bead surface. So extra denaturalization procedures are typically needed for the beads-based DNA assay to release the fluorescent probes into solution for signal readout (Nie et al., 2009), resulting in laborious and tedious operations and thus poor reproducibility. Therefore, how to realize the fluorescence signal readout directly on the beads is still attracting great interests.

Herein, to fully address these problems, we develop a versatile MBs-based DNA assay by combining hybridization chain reaction (HCR) for enzyme-free signal amplification and flow cytometry analysis for facile fluorescence signal readout directly on the MBs (denoted as HCR–CBA approach). There are several advantages for this proposed HCR–CBA method. On one hand, unlike conventional enzyme-based amplification strategies such as PCR, in which enzyme activities are prone to be affected by the environmental media, HCR is a kinetic-controlled reaction and the amplification does not require any enzymes (Dirks and Pierce, 2004; Venkataraman et al., 2007; Yang et al., 2012). Therefore HCR is an ideal choice for DNA amplification especially in complex biosamples (Choi et al., 2010; Huang et al., 2011) and is applicable for direct amplification on the beads (Niu et al., 2010). In our design, one target DNA could initiate a cascade of hybridization events between two species of fluorescent hairpin probes to accumulate numerous fluorophores on the beads, which greatly enhanced the detection sensitivity. On the other hand, flow cytometry has offered a powerful platform for rapid measurement of the optical properties of individual microspheres at rates of up to several thousand beads per second in complex mixtures, which has been successfully applied to the detection of various biomolecules (Butterworth et al., 2011; Horejsh et al., 2005; Huang and Liu, 2010; Lin et al., 2010). Flow cytometry can give statistically quantitative counting results by measuring the fluorescence intensity of individual MBs as they pass one-by-one through a capillary under the laser excitation, so flow cytometry is particularly well-suited to the direct analysis of HCR products-enriched MBs suspension.

Therefore, by combining the distinct features of HCR for enzyme-free signal amplification, MBs for simple concentration and washing steps, and robust flow cytometry for rapid and powerful beads analysis, the proposed HCR–CBA method provides a versatile DNA assay with facile operations and greatly improved sensitivity.

2. Experimental section

2.1. Materials and reagents

All of the reagents used in this work were of analytical grade and used as purchased without further purification. Dynabeads® M-270 Streptavidin (STV) was purchased from Invitrogen Dynal AS (Oslo, Norway). All the DNA oligonucleotides (HPLC purified) used in this work were synthesized by Sangon Biotech (Shanghai, China) and the sequences adopted from the literature (Dirks and Pierce, 2004) are listed below (from 5′ to 3′, it should be noted that in the sequences of hairpin DNA probes, sticky ends are underlined and loops are italicized):

H1*: FAM-TTAACCCACGCCGAATCCTAGACT***CAAAGT**A GTCTAGGATTCGGCGTG
H2*: AGTCTAGGATTCGGCGTG***GGTTAA**CACGCCGAATCCT AGACTACTTTG-FAM
Bio-H1: Biotin-TTTTTTTAACCCACGCCGAATCCTAGACT***CAAA GT***AGTCTAGGATTCGGCGTG
Target DNA: AGTCTAGGATTCGGCGTGGGTTAA
Mis-1 DNA: AGTCTAGGATTC**A**GCGTGGGTTAA
Mis-3 DNA: AGTCTAGGA**C**TC**A**GC**A**TGGGTTAA
Del-1 DNA: AGTCTAGGATTC_GCGTGGGTTAA
Single step probe: FAM-CACGCCGAATCCTAG

2.2. Standard procedures of the HCR-CBA approach

All the reactions were carried out in a total 20 μL of SPSC buffer (0.75 M NaCl, 50 mM Na_2HPO_4, pH 7.4). Before the M-270 MBs were taken out of the stocking vial, the vial was vortexed to ensure that the beads were homogeneously dispersed. The MBs were washed three times by distilled water to remove the preservative before use. Typically, Bio-H1 (50 nM) was firstly incubated with MBs (10 μg) for 30 min to form the MBs-Bio-H1 complexes by means of the specific STV-biotin interaction, and unbound Bio-H1 were removed via magnetic separation. Then, certain amount of target DNA was added into the MBs-Bio-H1 complexes in SPSC buffer for 1 h at room temperature with shaking. Afterwards, the MBs were magnetically separated and then incubated with 500 nM H1* and 500 nM H2* respectively in SPSC buffer with shaking for 2 h at room temperature to conduct the HCR reaction. Finally, each sample was directly diluted to 500 μL with SPSC buffer and immediately subject to flow cytometry analysis. For cytometric analysis, standard high-speed running conditions with ultrapure water-based sheath fluids were used and 10,000 events were collected for each sample. Fluorescence signals of the HCR products-anchored MBs were detected by FL1 (FITC/FAM) channel at 530/30 nm using a FACSCalibur flow cytometer (BD Biosciences, San Jose, USA) with 488 nm laser excitation, and the flow cytometry data were acquired using CellQuest Pro software (BD Bioscience). The mean fluorescence intensities (MFI) of the fluorescent histograms were used for the quantitative analysis of target DNA.

After being separated from the matrix and washed with SPSC buffer with the help of a magnet, fluorescence imaging test of the HCR products-loaded MBs was also performed on a FV-1000 laser-scanning fluorescence confocal microscope (Olympus) by using a 488 nm laser and the fluorescence emissions were collected in the range of 500–600 nm.

3. Results and discussion

3.1. Design principle of the HCR–CBA method for DNA detection

The principle of the proposed HCR–CBA is illustrated in Fig. 1. Dynabeads M-270 are uniform (2.8 μm), superparamagnetic and STV-functionalized beads with hydrophilic surface. According to the sequence of the proof-of-concept DNA target, two species of