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A novel sandwich assay with molecular beacon as report probe for nucleic acids detection on one-dimensional microfluidic beads array

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Abstract

A novel sandwich assay with molecular beacons as report probes has been developed and integrated into one-dimensional microfluidic beads array (1-D chip) to pursue a label-free and elution-free detection of DNA/mRNA targets. In contrast with the immobilized molecular beacons, this sandwich assay can offer lower fluorescence background and correspondingly higher sensitivity. Furthermore, this sandwich assay on 1-D chip operating in conjunction with molecular beacon technique allows multiple targets detection without the need of laborious and time-consuming elution, which makes the experiment process simple, easy to handle, and reproducible results. In the experiment, the synthesized DNA targets with different concentrations were detected with a detection limit of ∼0.05 nM. Moreover, the mRNA expression changes in A549 cells before and after anticancer drug 5-flouorouracil treatments were detected and the results were validated by the conventional RT-PCR method.

Keywords: Molecular beacon; Sandwich assay; Microfluidic; One-dimensional beads array

1. Introduction

The development of simple and efficient methods or platforms for the rapid detection of nucleic acids is very important for the early and precise diagnosis of various genetic diseases [1–3]. Recently, the areas of multianalyte assays and sensors have become the focus of active research aiming for the use of smaller sample volumes, shorter assay times, simpler assay protocols, reduced cost per test, and larger information acquisition [4–6]. Biochip, as one of the most promising tools for the nucleic acids detection, has shown its predominance for its micromation, high efficiency and facile operation. And on-chip nucleic acids detection always offers efficient hybridization and high sensitivity for the high local probes concentration resulting from the high density of immobilized probes [7–9].

Recently, we have developed a novel one-dimensional beads array (1-D chip), which combines the advantages of beads array (encoding and high throughput capabilities) with the rapid binding kinetics of homogeneous assays, and the liquid handling functions of microfluidics [10]. As demonstrated previously, the newly fashioned 1-D chip has been successfully used for proteins expression study. To further extend its applications for the nucleic acids detection is obviously a promising work, which represents an important step in the direction of making this system serve as a universal chemical and biological detection platform.

Simple platforms need simple and sensitive detection methods to fully exhibit their powerful capabilities. Traditionally, the nucleic acids detection on chip always require samples labeling or elution process, which is laborious and time-comsuming [11–14]. Recently, the development of molecular beacons (MBs) has offered researchers an elution-free and label-free method for the detection of nucleic acids with excellent specificity and sensitivity. The MBs have been extensively used in homogeneous hybiridization assays in vitro and in vivo [15–19]. However, the immobilized MBs used for on-chip detection of nucleic acids often have relatively high fluorescence background and the sensitivity is not satisfied [20–23]. The nucleic acids samples need to be amplified by PCR, which undoubtedly has increased the complication of the assay and limited its applications on chip. Clearly, the development of new high-sensitivity and simple methods suitable for rapid and direct (i.e., without amplification)

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detection of nucleic acids on chip has represented an important challenge.

In this paper, we describe a novel sandwich assay with MBs as report probes. An extended application of 1-D chip is demonstrated for the rapid and direct nucleic acids detection based on this novel sandwich assay. The superfluous MB report probes in this sandwich assay exist in a homogeneous solution rather than immobilized directly on the solid surface, which allow them have low fluorescence background and relatively high sensitivity. Moreover, the using of MBs has made the sandwich assay no need of the laborious and time-consuming elution process. This design integrates the advantages of both MBs [15–19] and the conventional sandwich array [13,14], which offers a label-free, elution-free, sensitive and good reproducible detection of nucleic acids. In the experiment, multiple synthesized cDNA samples (same sequence with a special fraction of human gene c-myc mRNA) were detected simultaneously in a single micro-channel based on the 1-D chip. The achieved detection limit is ∼0.05 nM. Furthermore, we have examined the total mRNA samples extracted from the A549 cells and the anticancer drug 5-flouorouracil (5-FU) treated A549 cells to detect the human gene c-myc mRNA expression changes. The results were validated by the conventional method RT-PCR [24,25].

2. Experimental

2.1. Chemicals and reagents

The biotinylated capture probes, molecular beacons, biotinylated molecular beacons, oligonucleotide target, primers for PCR, and other oligonucleotides used in this study were synthesized and purified by Takara Corporation (Dalian, China) and are summarized in Table 1. The capture probe and MB are specially designed to detect the mRNA of human gene c-myc. And the specificity of these sequences is confirmed by BLAST in Gene Bank.

PDMS prepolymer and curing agent were obtained from Dow Corning Corporation Midland, MI, U.S.A.; RNase inhibitor, AMV reverse transcriptase, DNA Taq polymerase and dNTPs mixture were obtained from Takara Dalian Corporation. Porous silica microbeads (Ø 50 μm), polymethobromide (PB), biotinylated BSA, streptavidin and DEPC were purchased from Sigma. Dextran sulphate (DS) was purchased from Tianjin H&Y Bio. Co. Ltd. Trizol reagent kit was provided by Invitrogen Corporation. The lung cancer cells are cultured and treated with 5-FU in our lab.

TM buffer (10 mM Tris–HCl buffer containing 100 mM Mg^{2+}), DEPC treated ultrapure water was dealt with sterilization then used in all experiments reported in this paper. All the vessels in the experiment concerning with the mRNA were treated with 0.1% DEPC 2 h first, then dealt with sterilization.

2.2. Modification of microbeads

DNA functionalization was performed as follows: surface of microbeads was first activated with Na_2CO_3 (2.0 M) for 30 min. The activated beads were then suspended in 250 μL (1 μM) biotinylated BSA solution for about 24 h in a low temperature shaker (4 °C, >300 rpm) to binding the biotinylated BSA by physical absorption. Streptavidin (1 μM) was added to form a bridge for the following bindings of biotinylated capture probes (1 μM). Both streptavidin and biotinylated capture probes were linked to the surface successfully within 20 min. Washing with ultrapure water thoroughly was performed between each step. The modified microbeads were stored at 4 °C. A condition of the temperature −20 °C is suggested for long-term storage.

The biotinylated molecular beacons were modified on the microbeads with the same method.

2.3. Fabrication of 1-D chip

The fabrication of 1-D chip refers to our previous work [10]. Briefly, a PDMS replica was molded on a silica stencil-plate which was designed by us and fabricated by Research Institute of Micro/Nano Science and Technology, Shanghai Jiao Tong University. Then the PDMS replica was sealed with a top quartz glass slide. To reduce the non-specific absorption the regents or samples, the inner walls of the channel were treated by dynamic coating with 5% PB and 3% DS [26]. The microbeads modified with biotin-MBs were put into the microchambers by using the vacuum tweezers (Nikon Narishige, model: NT88NEN, Nikon Corp., Japan) under the microscope (Leica DM IRB, Leica Corp., Germany).

Table 1
Names and sequences (5′-3′ orientation) of the oligonucleotides used in this study

Name	Sequence
Capture probe	GAGACGTGGCACCTCTTGAGAAAAAAAAAA-biotin
MB	TAMRA-CCAGTCGCTGCGTAGTTGTGCTGATGGACTGG-DABCYL
Immobilized MB	FAM-CCAGTCGCTGCGTAGTTGTGCTGATGGAC(biotin-dT)GG-DABCYL
Target	CTCAAGAGGTGCCACGTCTCCACACATCAGCACAACTACGCAGC
Primer 1	AGCCCACTGGTCCTCAAGA
Primer 2	GTTCGCCTCTTGACATTCTCC
Primer 3	TCCTGGAGAAGAGCTACTA
Primer 4	GTACTTGCGCTCAGGAGGAG

The MB was labeled 5′ TAMRA and 3′ DABCYL. And the immobilized MB was labeled 5′ FAM and 3′ DABCYL. Moreover, a biotin molecule was labeled on the T base in the middle of stem. The capture probe was labeled 3′ biotin. The Primer 1 and Primer 2 are the forward and reverse primers for c-myc, respectively, and the Primer 3 and Primer 4 are the forward and reverse primers for β-actin, respectively.

2.4. The mRNA samples preparation and RT-PCR

Total RNA was isolated from A549 cells and 5-FU-A549 cells (A549 cells treated with 5FU for 18 h) by using Trizol kit according to the manufacturer's instructions, and then quantified by a Spectrophotometer (DU800, BECKMAN COULTER). Same amount (1 μg) of two RNA samples was reverse transcribed in two 20 μL tubes, respectively, with 50 pM oligo(dT) primer, 1.0 mM each nucleotide, 5 U AMV RTase, 30 U RNase inhibitor, 4 μL 5× reverse transcriptase buffer, at 42 °C for 1 h, and the enzymes were then denatured by heating for 2 min at 94 °C. PCR was performed in 50 μL volumes of 5 μL 10× PCR buffer, 200 μM each nucleotide, 0.4 μM each primer (see Table 1), 2.5 U of Taq DNA polymerase and 1.5 μL RT product by a thermocycler (GeneAmp. PCR system 2700, Applied Biosystems) with the following cycles: 25 cycles of 50 s 94 °C, 50 s 55 °C, 1 min 72 °C. Five microlitre of each PCR product was electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

2.5. General process of the experiment

The chip channel was introduced with the TM buffer in advance, then 2 μL cDNA/mRNA samples were introduced into the inlet of the 1-D chip, and the samples flowed through the DNA functional beads under gravity. After 30 min hybridization at 37 °C, the 20 nM MBs were introduced to substitute for the samples. The MBs would hybridize with the cDNA/mRNA samples that captured by the immobilized capture probes and fluorescence signals were obtained by the Cool CCD system in invert fluorescence microscopy. The fluorescence intensity was scaled by general software "Image J".

3. Results and discussion

3.1. Design of the 1-D chip and the probes

The central component of the newly fashioned 1-D chip is an extremely versatile polymer platform with many cabinets along a single microchannel. Each of the microfabricated chambers holds a single bio-functional bead, with interconnecting channels for liquid flow [10]. While samples flow through the microchannel, targets can be recognized and captured by the corresponding bio-functional microbeads (the scheme is shown in Fig. 1a). 1-D chip integrates the technique of both beads array and microfluidic system, which can offer advantages of little sample consumption, low potential of contaminations, easy operation, are cheap and reusable. Moreover, such a design makes it potentially possible to pursue high-throughput nuclei acids detections in a single channel.

The immobilized MBs can offer a label-free and elution-free detection of nucleic acids on chip, which makes the assay simple and easy to operate. But they often have relatively high fluorescence background and the sensitivity is not satisfied with direct (i.e., without amplification) detection of nucleic acids. To solve this problem, in this work, the MB has been integrated with the conventional sandwich assay. The scheme of the novel sandwich structure is shown in Fig. 1b. Such a new sandwich structure comprises three parts of oligonuclotides sequences as biotinylated capture probes, targets and MBs report probe. The target is a long oligonucleotide fraction, which contains the complementary sequences to the capture probe and the MB. After the hybridization of the targets with the capture probes, the MBs were added to replace the superfluous targets and hybridized with the targets captured on the microbeads. Then the MBs forms a rigid probe-target duplex, the fluorophore and quencher are forced to separate and fluorescence is restored, while the superfluous MBs maintains the hairpin structure under a condition of homogeneous solution in the chip channel.

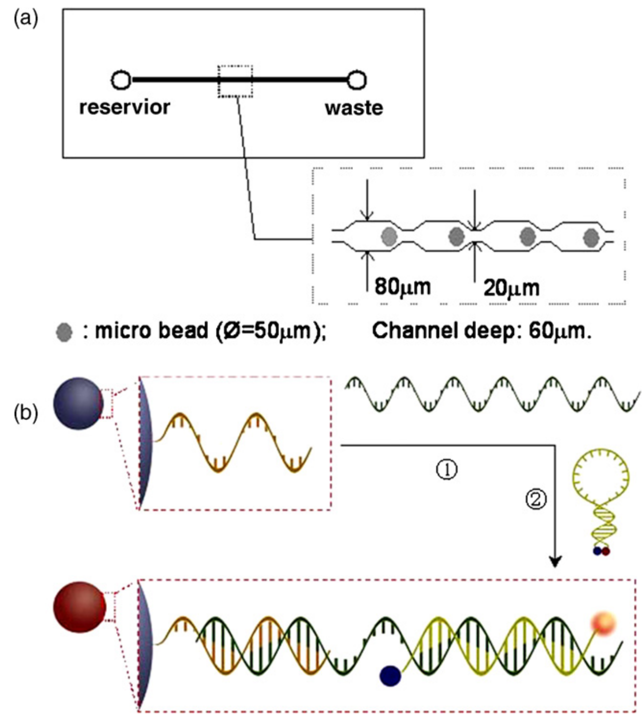

Fig. 1. Scheme of the 1-D Chip and the novel sandwich assay with MBs as the report probes. The capture probes were modified on the surface of the microbeads through a linkage of biotin–streptavidin.

3.2. Contrast of the MB in sandwich assay with the MB immobilized on beads surface

To obtain a contrast results between the MB in sandwich assay and the MB immobilized on the micro-beads surface, we have design and synthesized another immobilized MB, which has the same sequence with the MB used in the sandwich assay. And each MB has fluorescence enhancement over 20-fold after hybridization with target cDNA in homogenous solution. The immobilized MB is labeled 5′ FAM and 3′ DABCYL, respectively. And a biotin is labeled on the T base in the middle of stem for immobilization (see Table 1). The biotinylated MBs were immobilized on the microbeads surface to detect 5 nM target cDNA on 1-D chip. At the same time, we also detected the 5 nM target cDNA on 1-D chip by the novel sandwich assay with the MBs as report probes. The results are shown in Fig. 2. To obtain a clear fluorescence signal relative to the background,

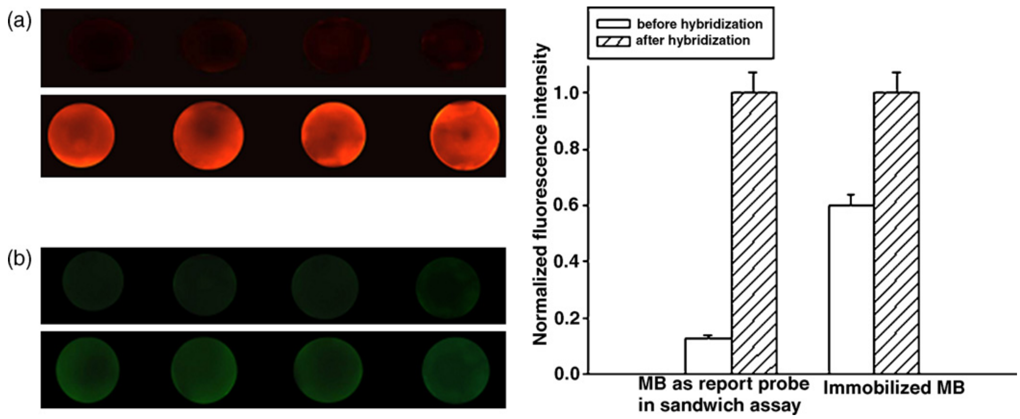

Fig. 2. Images and normalized fluorescence intensities of the MBs as report probes in sandwich assay and the immobilized MBs. (a) Images of the MBs as report probe in sandwich assay before and after hybridization with its target cDNA. (b) Images of the immobilized MBs before and after hybridization with its target cDNA. And the right column plot is the normalized fluorescence intensity of the images.

the two assays with different method have adopt different CCD exposure time. However, it is obviously that the novel sandwich assay with MB as report probe has relatively low fluorescence background and correspondingly high sensitivity. Refer to our previous work that is based on the immobilized MB detection of nucleic acids on 1-D chip, the sensitivity of this novel sandwich assay with MB as report probe on 1-D chip has been improved at least 10-fold.

3.3. The cDNA target detection on chip

Based on our 1-D chip, to evaluate detection sensitivity, dynamic range, and reproducibility, we prepared a four-fold redundant microbeads array (that is, four identical beads are used to measure each target) to detect the synthesized cDNA (the same sequence with a special mRNA fraction of human gene c-myc). Fig. 3 shows the fluorescence images of the beads array at various cDNA concentrations, together with five-fold redundancy beads for comparison (20 nM random sequence was added instead of complementary target sequence). Satisfactory reproducibility was obtained. And on the basis of a signal-to-noise ratio of 3, the achieved detection limit is ~0.05 nM with a saturation concentration of 10 nM. It is important to note that the total volume of the micro-channel is <0.1 μL and the samples for each assay we used is <2 μL. that is to say we can detect ~100 amol nucleic acids molecules in a total volume of 2 μL by using our 1-D chip and the novel sandwich assay.

3.4. The detection of mRNA sample on chip and validation by RT-PCR

This sandwich assay with the MB as report probe has provided a simple and sensitive method for oligonucleotides detection on chip. Here, on the basis of the calibration studies, we have further determined the mRNA expression change of human gene c-myc before and after anticancer drug 5-FU treatment. C-myc is one of the famous tumor-associated genes, and its expression plays important roles in cell growth, differentiation, and malignant transformation. The total mRNA samples (3 $\mu g\ \mu L^{-1}$) were extracted from A549 cells and 5-FU treated (18 h) A549 cells, respectively. The results are shown in Fig. 4. Through contrasting Fig. 4a with b, it is obvious that c-myc mRNA is significantly downregulated in A549 cells after 5-FU treatment for 18 h. To validate the results obtained by the arrays, electrophoretic images of the c-myc RT-PCR products are illustrated in Fig. 4c with gene β-actin as invariant

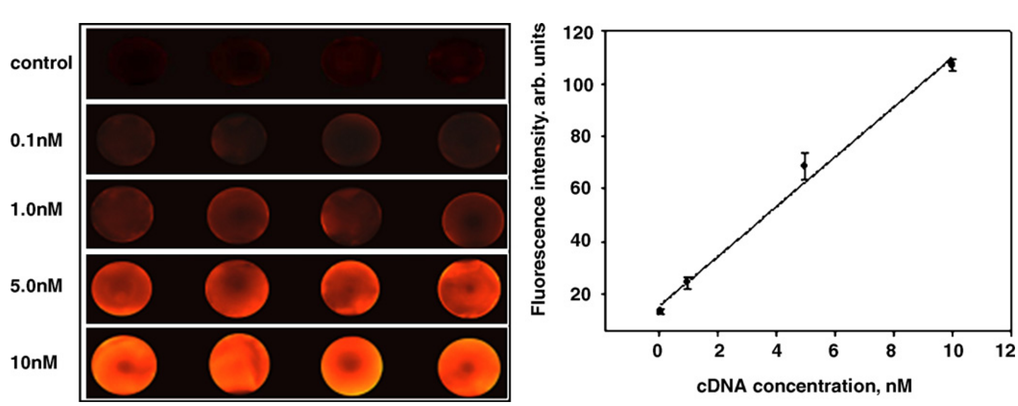

Fig. 3. Detection of c-myc cDNA targets with different concentrations using 1-D chip. Left: the fluorescence images of four-fold redundant beads array for c-myc cDNA, together with four-fold redundant beads for control (random sequence). Right: the fluorescence enhancement is increased linearly with increasing target cDNA concentration in 0.05–10 nM range.

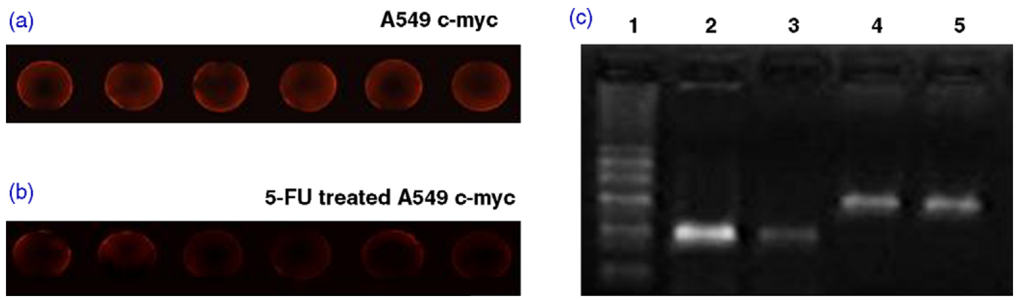

Fig. 4. Detection of mRNA samples using 1-D chip (a) and (b) are images six-fold redundant beads array detecting A549 c-myc and 5FU treated A549 c-myc mRNA, respectively. (c) Electrophoretic image of RT-PCR. Lane 1 is DNA maker. Lanes 2 and 3 are the c-myc RT-PCR products of A549 and 5-FU treated A549, respectively. And lanes 4 and 5 are the β-actin RT-PCR products of A549 and 5-FU treated A549, respectively, as invariant expression reference.

expression reference, which shows an agreement to the array results.

4. Conclusion

We have extended the applications of the 1-D chip for directly nucleic acids detection by using a novel sandwich assay with molecular beacon as report probe. As a demonstration, the cDNA samples with different concentration and the mRNA samples extracted from cells are detected. Such a sandwich design offers a simple, sensitive, elution-free and label-free method for the rapid and direct nucleic acids detection on 1-D chip.

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