Sensors and Actuators B 222 (2016) 198–204

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

# High-throughput, selective, and sensitive colorimetry for free microRNAs in blood via exonuclease I digestion and hemin-G-quadruplex catalysis reactions based on a "self-cleaning" functionalized microarray

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ARTICLE INFO

*Article history:*
Received 22 June 2015
Received in revised form 5 August 2015
Accepted 11 August 2015
Available online 13 August 2015

*Keywords:*
"Self-cleaning" microarray
Sample crossing contamination
Exonuclease I digestion
DNAzyme catalysis
Colorimetric assay
MicroRNA detection

ABSTRACT

A high-throughput, selective, and sensitive colorimetric method has been developed for probing free microRNAs (miRNAs) in blood based on a "self-cleaning" functionalized microarray newly fabricated. Glass substrates were first masked with a hydrophobic silane layer of hexadecyltrimethoxysilane (HDS) and then dotted with hydrophilic aminopropyltriethoxysilane (APS) embedded with nano-scaled ZnO, resulting in HDS-ZnO-APS dot microarray with highly dense ZnO-APS testing dots and depressed crossing contamination of sample droplets by the lotus-like "self-cleaning" effects of hydrophobic HDS substrate. Furthermore, ssDNA capture probes with hemin-binding sequences were covalently anchored on the amine-derivatized ZnO-APS testing dots. After miRNA target hybridization, exonuclease I was introduced to specifically digest the ssDNA probes unhybridized. Furthermore, hemin was added to form the hemin-G-quadruplex DNAzyme to achieve the ATP-enhanced catalytic amplification of visible coloration signals. Wild miRNA targets in blood could be detected in the concentration range from 0.20 pM to 1.50 nM, with the detection limit of 0.080 pM. Single-base mutation miRNAs could also be accurately identified and quantified for profiling miRNA expression pattern. Markedly different from the common microarray assays by way of sandwiched detections, such a microarray-based colorimetric method could be tailored for quantifying short-chain miRNAs of low levels in blood.

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## 1. Introduction

MicroRNAs (miRNAs) as a class of short-chain noncoding RNAs of 18–25 nucleotides that could control the expression of genes in lives [1]. The expressing levels of free miRNAs in peripheral blood have been well established to be the sensitive biomarkers for the cancer diagnostics and metastasis by way of blood [2,3]. Hence, the detection of free miRNAs has emerged as a key research field that attracts considerable attentions in recent years. Nevertheless, the quantitative profiling of free miRNAs in blood can be challenged by the short chains, low-level expressions, and nucleotide-similar sequences of miRNAs in the complicated blood background [3–6]. To date, many analysis technologies have been developed to probe free miRNAs, most known as the quantitative real-time polymerase chain reaction (qRT-PCR) [7], the Northern blotting method [8], and the electroanalysis assay [3,9]. Gracefully successive as these analysis methods could be, they might be trapped by some limitations of targeting these short-chain miRNAs. For example, the qRT-PCR provides the sensitive detection of miRNAs, however, can encounter with the low analysis selectivity or inefficiency due to the inefficient binding of the primers with such short miRNA templates [10]. Also, electroanalysis assays were conducted for miRNAs generally by way of the sandwiched detections; nevertheless, short-chain miRNA sequences could be readily unwound from

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http://dx.doi.org/10.1016/j.snb.2015.08.047
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the hybridization parts, leading to the low detection sensitivity. Remarkably, most of these classic methods might suffer from the low detection throughput, which could not satisfy the need of the analysis of multiple samples. Therefore, the development of high-throughput, selective, and sensitive candidates for the analysis of meaningful miRNAs has been an attractive but challenging issue.

Recent years have witnessed the rapid development of numerous analysis microarrays or chips serving as the multi-analysis methods with high throughput, low cost, and reduced reagents to adapt to the need of the detection flux in screening or testing multiple markers of chemical and biological importance [10–15]. However, the common microarrays with dense and arrayed testing wells or dots could encounter with a formidable challenge regarding the crossover contamination in-between the samples of complex media like blood, making it difficult to fabricate highly dense testing dots for the high-throughput analysis of multiple samples within a single experiment. Moreover, peroxidase-mimicking DNAzymes have been widely investigated due to their high catalysis, chemical stability, cost effectiveness, and easy modification [16,17]. As an example, the DNAzyme composed by the hemin-binding guanine (G)-quadruplex DNA sequences, defined as hemin-G-quadruplex DNAzymes, could present much enhanced catalysis performances comparing to hemin[16]. As a result, they have been increasingly applied for the quantitative detections of $Cu^{2+}$ ions [18], $Hg^{2+}$ ions [19], $Pb^{2+}$ ions [20], $K^{+}$ ions [21,22], $Ag^{+}$ ions [23], and DNAs [24]. For example, Dong's group reported the sensitive detection of $K^{+}$ ions by using hemin-G-quadruplex DNAzyme [21]. Willner and coworkers employed the DNAzymes as the catalytic units for the colorimetric assays of DNAs by using the rolling circle amplification process [24]. Furthermore, hemin-G-quadruplex DNAzymes have been employed to combine some signal amplification technologies using metal nanomaterials to improve the detection sensitivity [25,26].

Inspired by the hydrophobic "self-cleaning" effects of lotus leaf, in the present work, a "self-cleaning" functionalized microarray has been fabricated for the colorimetric assays of free miRNAs in blood, in combination with the selective DNA digestion of a restriction enzyme of exonuclease I (Exo I) and the ATP-enhanced catalysis of hemin-G-quadruplex DNAzyme. Glass substrates were first masked with a hydrophobic silane layer of hexadecyltrimethoxysilane (HDS) and further dotted with hydrophilic aminopropyltriethoxysilane (APS) embedded with ZnO nanoparticles (ZnO-APS), resulting the HDS-ZnO-APS dot microarray (Scheme 1A). Furthermore, DNA capture probes containing hemin-binding sequences of G-quadruplex were immobilized onto the amine-derivatized ZnO-APS testing dots of microarrays for targeting miRNAs. Then, Exo I was introduced to digest selectively the unhybridized DNA probes of single chains. Subsequently, hemin was added to bind with the DNAs probes survived by the miRNA hybridization to form the hemin-G-quadruplex DNAzyme, which could catalyze the coloration reactions of peroxidase-sensitive substrates to conduct the color changes (Scheme 1B). A colorimetric microarray analysis method was thus developed for high-throughput, selective, and sensitive detection of free miRNAs in blood, including the identification and analysis of single-base mutants for profiling miRNA expressions.

## 2. Experimental

### 2.1. Materials and instruments

Zinc acetate dehydrate, $LiOH{\cdot}H_2O$, ethanol, and toluene were purchased from Sigma–Aldrich (Beijing, China). Aminopropyltriethoxysilane (APS) and hexadecyltrimethoxysilane (HDS), ethylenediaminetetra acetic acid (EDTA), potassium chloride (KCl), tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl), rhodamine B, dimethylsulfoxide (DMSO) were obtained from Sinopharm Chemical Reagent Co. (China). Succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC), cetyltrimethyl ammoniumbromide (CTAB), and 3,3,5,5-tetramethylbenzidine (TMB) $-H_2O_2$ substrate were bought from Dibai Reagents (Shanghai, China). Blood samples were kindly provided by the local university hospital. All other reagents were of analytical grade. Deionized water (>18 MΩ × cm, RNase-free) was supplied from an Ultra-pure water system (Pall, USA). DNA exonuclease I (Exo I) containing Exo I buffer, thiolated oligonucleotides of single-stranded DNA (ssDNA) capture probes, wild miRNAs, and single-base mutant miRNAs were synthesized by Takara Biotechnology (Dalian, China), including:

DNA probe: 5′-SH-TGGGTAGGGCGGGTTGGGAAAAA CTATACAACCTACTACCTCA-3′;

Wild miRNA: 5′-UGA GGU AGU AGG UUG UAU AGU U-3′;

Single-base mutant miRNA: 5′-UGA GGU AG**A** AGG UUG UAU AGU U-3′;

Buffer solutions include the conjugation buffer (pH 7.2) containing 100 mM phosphate-buffered saline (PBS) and 150 mM NaCl; hybridization buffer (pH 7.4) consisting of 10 mM Tris–HCl,1.0 mM EDTA, 1.0 mM CTAB, and 0.50 M NaCl; DNA rinsing buffer (pH 7.4) composing of 100 mM NaCl, 10 mM Tris–HCl; Exo I buffer containing 67 mM glycine–KOH, 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol; G-quadruplex buffer consisting of 10 mM Tris–HCl, 10 mM KCl, 100 mM NaCl, 2.0 mM ATP, 0.20 μM hemin, and 0.0020% (v/v) Triton X-100.

Hydrophobicity analysis was conducted to monitor the step-by-step fabrication procedure of the microarrays using the contact-angle measurement machine (Jinhe, Jiangsu, China). Field emission scanning electron microscope (SEM, JSM-6700F, Japan) was employed to characterize the resulting surface of the ZnO-APS testing dots on microarrays. Moreover, colorimetric measurements of absorption intensities at 652 nm were performed using Infinite M 200 PRO (TECAN, Switzerland) with a home-made microplate holder adapted for the microarray tests.

### 2.2. Fabrication of DNA probe-conjugated dot microarray

The "self-cleaning" functionalized microarrays were fabricated using glass substrates (72 mm × 24 mm) that were first cleaned with fresh piranha solution ($H_2SO_4$:$H_2O_2$ = 7: 3), then washed twice in water and dried in nitrogen. Those cleaned substrates were dipped immediately into 3.0% HDS in toluene. Next, an aliquot of 1.0 μL ZnO-APS solution, which was prepared according to the procedure reported previously [27] except for using 3.0% APS, was dotted separately onto the HDS-hydrophobic substrates and further dried in vacuum to form the HDS-ZnO-APS dot microarray, as schematically shown in Scheme 1A. The change of wetability of the fabricated interfaces was characterized by the contact angle measurements. Subsequently, ssDNA capture probes containing hemin-binding sequences were conjugated to the amine-derivatized surfaces of the ZnO-APS testing dots on microarrays using SMCC cross-linker according to the instruction detailed in the reagent kit.

### 2.3. Colorimetric dot microarray analysis

Complementary wild miRNAs were spiked in blood, which was pretreated with anti-coagulant agent (i.e., heparin), and further diluted with different miRNA concentrations. The samples of miRNAs were separately added to the DNA capture probes-settled testing areas of dot microarray. The hybridization reactions were conducted at 40 °C for 30 min, and then washed twice by hybridization buffer of 50 °C to remove any non-specifically adsorbed DNA

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