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# 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

Shuying Li[a], Rui Li[a], Minmin Dong[a], Liyan Zhang[a], Yao Jiang[a], Lijun Chen[b], Wei Qi[a], Hua Wang[a,c,*]

[a] Shandong Province Key Laboratory of Life-Organic Analysis, College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu City, Shandong Province 273165, PR China
[b] Hospital of University, Qufu Normal University, Qufu City, Shandong Province 273165, PR China
[c] Jining Functional Materials and Surface Treatment Technology R & D Center, Southern Shandong Academy of Engineering Technology, Jining City, Shandong Province 272000, PR China

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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.



## 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

* Corresponding author at: Shandong Province Key Laboratory of Life-Organic Analysis, College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu City, Shandong Province 273165, PR China.
*E-mail address:* huawangqfnu@126.com (H. Wang).

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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.

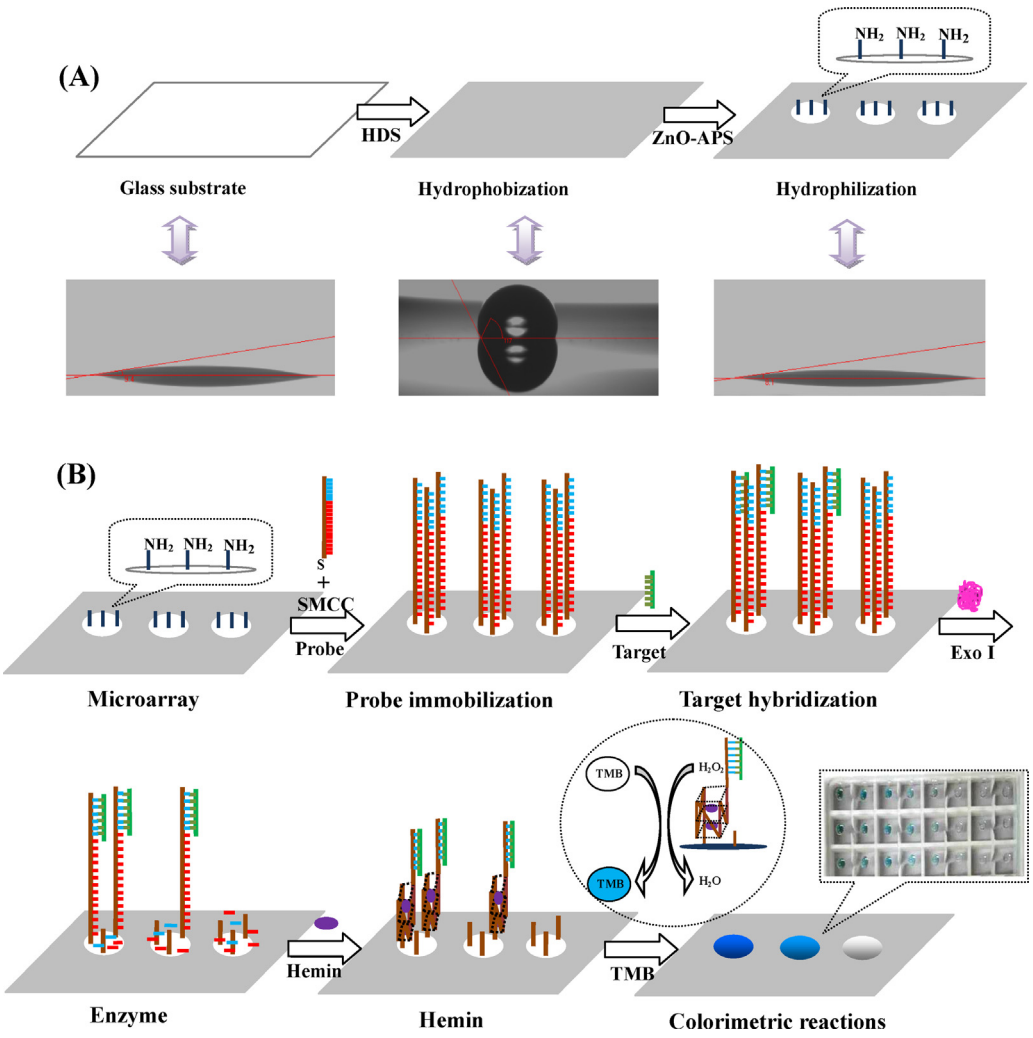
Hydrophobicity analysis was conducted to monitor the step-by-step fabrication procedure of the microarrays using the contact-angle measurement machine (Jinhe, Jiangsu, China). Field emission scanning electron microscope (SEM, JSM-6700F, Japan) was employed to characterize the resulting surface of the 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




**Scheme 1.** (**A**) Schematic illustration of the fabrication process of the "self-cleaning" functionalized microarray by the HDS hydrophobization of glass substrate and the ZnO-APS hydrophilization of testing areas, of which the changes of interface wetabilities were characterized by the contact angle measurements; (**B**) Schematic illustration of the principle and procedure of the microarray-based colorimetric assays for miRNAs, including the immobilization of DNA probes containing hemin-binding sequence, hybridization of miRNA target, Exo I digestion of unhybridized ssDNA probes, hemin binding, and catalytic TMB-$H_2O_2$ reactions.

probes or mismatched miRNA targets. Then, an aliquot of Exo I (0.50 U/mL) was introduced to be incubated at 37 °C for 30 min. After that, the microarray was flushed twice with Exo I reaction buffer and PBS, respectively. Furthermore, an aliquot of the G-quadruplex buffer containing 2.0 mM ATP and 0.20 μM hemin was separately dropped onto each of the testing dots to be further incubated at 25 °C for 1.0 h to form the hemin-G-quadruplex DNAzyme, followed by being washed twice with the G-quadruplex buffer. Subsequently, an aliquot of 5.0 uL of TMB-$H_2O_2$ substrate was dropped onto each of the testing dot areas of microarrays to be incubated for 20 min for the absorbance measurements at 652 nm using the home-made microplate reader. In addition, according to the procedure above, the experiments of mutant-level identifications were conducted for the single-base mutant miRNAs with different concentrations spiked in the wild miRNA samples at the fixed total miRNAs of 0.50 nM.

## 3. Result and discussion

### 3.1. Microarray-based colorimetric detection procedure

The "self-cleaning" functionalized microarrays were fabricated by the procedure schematically illustrated in Scheme 1A. Glass substrates were first masked with a hydrophobic HDS layer to obtain the lotus-like "self-cleaning" effects against the crossover contamination of sample droplets. Hydrophilic ZnO-embedded APS was then dotted on the hydrophobic HDS substrate to create the amine-derivatized ZnO-APS testing areas forming the HDS-ZnO-APS dot microarrays for covalently anchoring DNA capture probes. Notably, ZnO nanoparticles herein were employed to work with APS to achieve the improved hydrophilicity and amine-functionalized interface of testing dots for anchoring DNA probes, so as to facilitate the highly sensitive analysis for low-level miRNAs afterwards. The main principle and procedure of the high-throughput dot microarray assays for miRNAs are schematically illustrated in Scheme 1B, mainly including the DNA probe immobilization, miRNA target hybridization, Exo I digestion, hemin binding, and TMB-$H_2O_2$ reactions catalyzed by the hemin-G-quadruplex DNAzyme. As depicted in Scheme 1B, miRNA targets were captured by the immobilized ssDNA probes onto the dot microarray thus forming double-stranded DNAs (dsDNAs). A selective digestion was then conducted for the unhybridized ssDNA probes by using Exo I. Subsequently, hemin was introduced to bind with the DNA probes survived by the miRNA target hybridization, leading to hemin-G-quadruplex DNAzyme that would catalyze the coloration reactions of TMB-$H_2O_2$ substrates

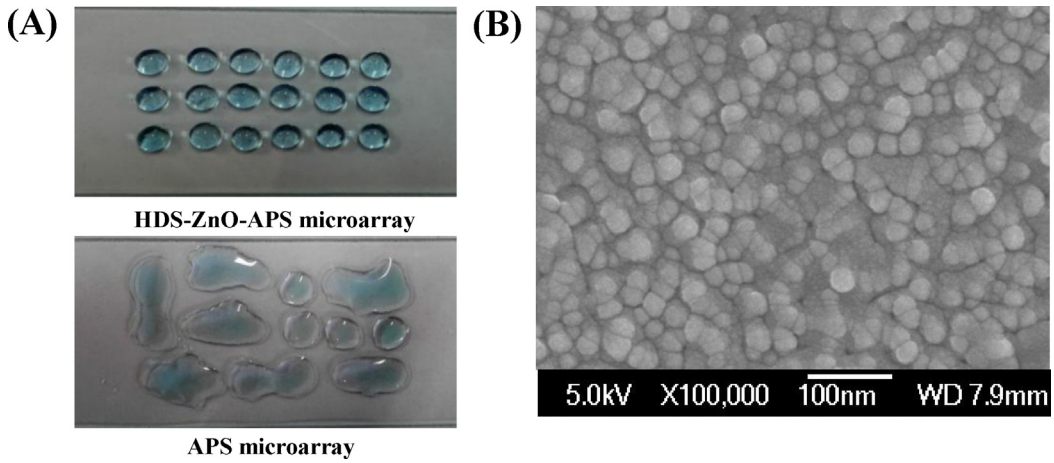



**Fig. 1.** (**A**) Comparison of the capacities against the crossing contamination of sample droplets on the HDS-ZnO-APS microarray and the common APS-derivatized one under the same fabrication conditions, with the amplified photographs of the TMB-$H_2O_2$ reaction products of miRNA colorimetry. (**B**) Representative SEM image of the resulting surface of ZnO-APS nanocomposites of the testing dot areas on microarrays.

to conduct the color changes. The resulting various colorations could be correlated to the targeting miRNA concentrations for the high-throughput microarray analysis. Moreover, the performance against the sample crossing contamination of the HDS-ZnO-APS microarray newly fabricated was investigated by the colorimetric TMB-$H_2O_2$ reactions, comparing to the common APS-derivatized microarray (Fig. 1A). It was found that highly dense and independent testing dots were constructed on the developed microarray as demonstrated by the reaction product drops. Importantly, the crossing contamination of sample droplets could be apparently depressed due to the "self-cleaning" effects of hydrophobic HDS interfaces in-between the testing dots. In contrast, the APS-derivatized microarray might present the serious sample crossover contamination or interference resulting in the disorderly expanded testing dot areas. Furthermore, the resulting ZnO-APS testing dots on the microarrays were characterized by SEM imaging (Fig. 1B). Accordingly, a uniform distribution of numerous spheric ZnO-APS nanocomposites with a size of about

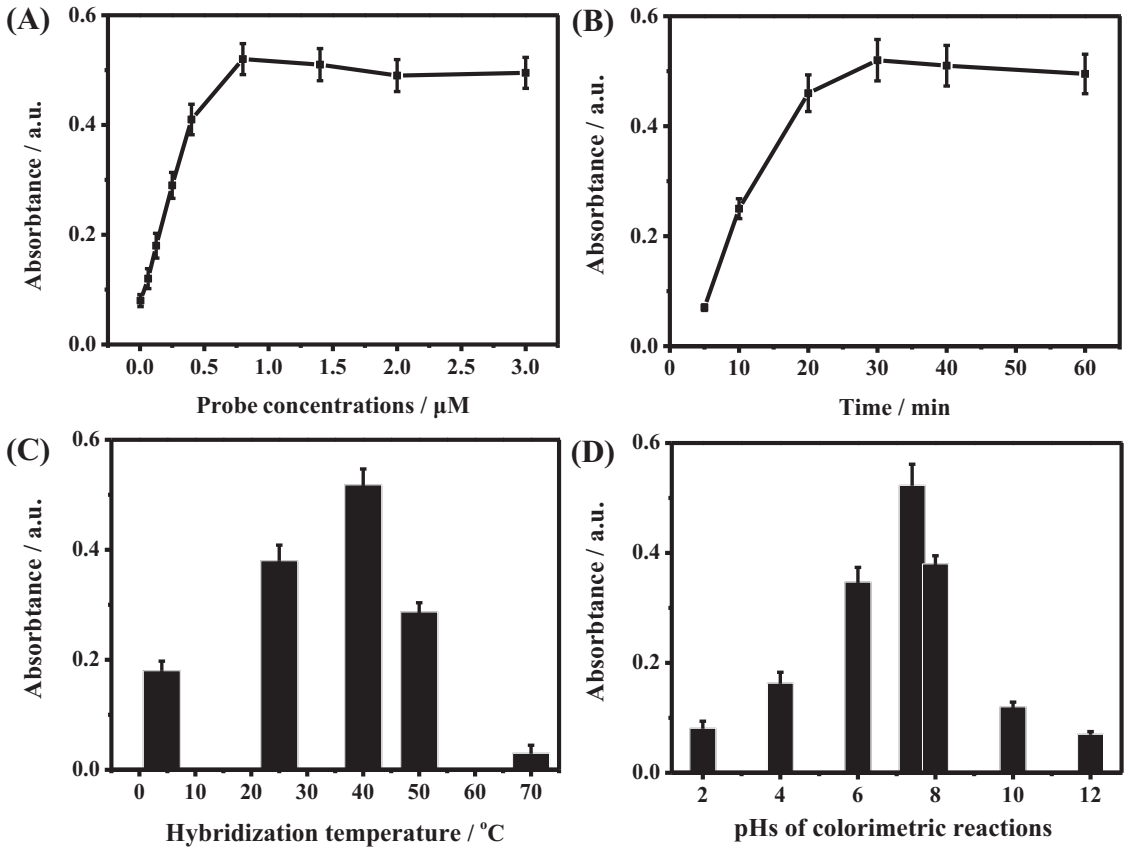



**Fig. 2.** Optimization of the main conditions for the microarray-based colorimetric assays of miRNAs including (**A**) DNA capture probe concentrations, (**B**) hybridization time, (**C**) hybridization temperature, and (**D**) pH values of colorimetric reactions. The UV absorbance values of TMB-$H_2O_2$ reaction products were recorded at 652 nm.

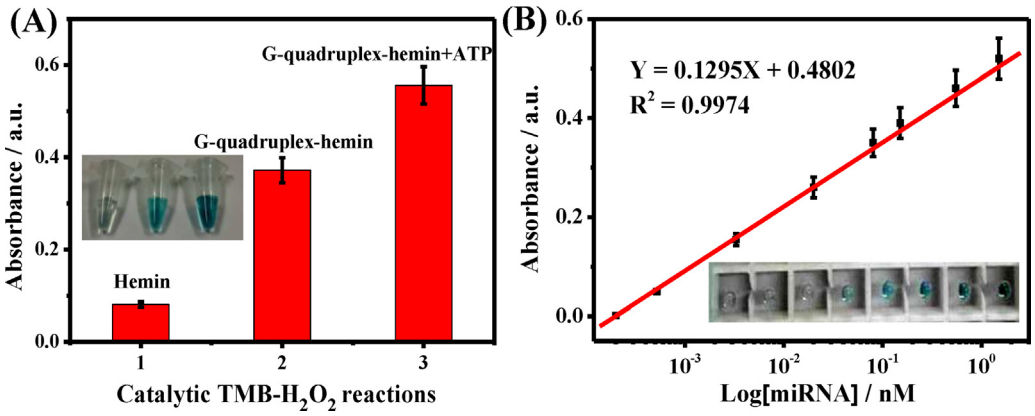



**Fig. 3.** (**A**) Comparable results of TMB-$H_2O_2$ reactions catalyzed separately by hemin and G-quedruplex-hemin DNAzyme in the presence and absence of ATP (2.0 mM) using 1.0 nM G-quadruplex and 0.20 μM hemin, with the corresponding photographs of the reaction products of TMB-$H_2O_2$ substrates (Fig. 3A, insert); (**B**) the calibration curve describing the relationship between the absorbance changes and targeting miRNAs spiked in blood with different concentrations, with the corresponding photographs of colorimetric products on the developed microarray (Fig. 3B, insert). The UV absorbance values of TMB-$H_2O_2$ reaction products were recorded at 652 nm.

30.0 nm was observed, indicating that a large testing area could be thus expected for the ZnO-APS dots created on the microarrays.

### 3.2. Optimization of the main detection conditions

The concentrations of DNA capture probes were first optimized by measuring the colorimetric responses to miRNAs. As is shown in Fig. 2A, the colorimetric responses could increase with the increasing DNA probe concentrations till 0.80 μM. Interestingly, too high concentrations of DNA probes might trigger the decreased signals, presumably due to that too high density of DNA capture probes on the ZnO-APS testing areas might negatively influence the formation of the G-quadruplex structures for catalyzing the coloration reactions. Therefore, 0.80 μM of DNA capture probes was selected for all colorimetric assays. Moreover, the hybridization time was explored (Fig. 2B). Obviously, the coloration responses increased as the hybridization time increased, and tended to be steady after 30 min, which is thus chosen for the hybridization reactions. Furthermore, experimental investigations on the hybridization temperature were carried out, with the data manifested in Fig. 2C. As shown in Fig. 2C, the coloration responses could depend on the hybridization temperature, indicating that 40 °C is sufficient for the hybridization. Of note, the hybridization signals could decay when the temperature was higher than 54 °C of dsDNA melting temperature. Additionally, the pH-dependent coloration reactions were studied (Fig. 2D). It was found that the coloration responses could peak at pH 7.4, which is thereby selected as the optimal one in the experiments.

### 3.3. Colorimetric analysis of wild miRNA samples

Under the optimized experimental conditions, the microarray-based colorimetric method was employed to detect the complementary wild miRNA samples (Fig. 3). Here, ATP was introduced into the coloration reactions to improve the catalysis performance of hemin-G-quadruplex DNAzyme. As is shown in Fig. 3A, the catalytic TMB-$H_2O_2$ coloration reactions could be apparently enhanced in the presence of ATP, as also demonstrated in the corresponding photographs (Fig. 3A, inset). A deeper color was observed for the reactant substrate catalyzed by hemin-G-quadruplex in the presence of ATP. Here, ATP and hemin could simultaneously anchor at the different binding sites of the oligonucleotides of the DNA probes so that the binding affinity between DNA probes and hemin could increase, thus leading to the increased catalytic activity of hemin-G-quadruplex DNAzyme, as also mentioned elsewhere [28]. Fig. 3B manifests the coloration responses to free wild miRNA targets with different concentrations in blood, with the photograph of the corresponding reaction products (Fig. 3B, inset). A linear relationship was obtained for the current responses versus miRNA concentrations ranging from 0.20 pM to 1.50 nM ($R^2$ = 0.9974) with the detection limit of 0.080 pM estimated by the 3$\sigma$ rule, which was much lower than those of other colorimetric miRNA detections

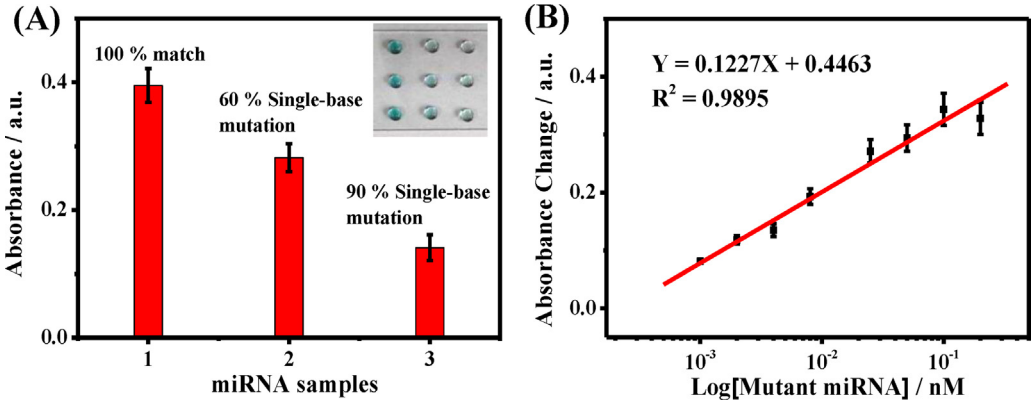



**Fig. 4.** (**A**) Comparable identification of miRNAs with different mutant percentages in the complementary miRNA samples at the fixed total miRNAs of 0.15 nM, with the corresponding photographs of the microarray of three replicated experiments (Fig. 4A, insert); (**B**) the calibration curve for quantifying mutant miRNAs spiked in blood with different concentrations in the fixed total miRNAs of 0.50 nM. All of the absorbance changes were recorded for the colorimetric TMB-$H_2O_2$ reactions on the developed microarray. The UV absorbance values of TMB-$H_2O_2$ reaction products were recorded at 652 nm.

reported elsewhere [29,30]. Here, the highly selective and sensitive detections of miRNAs might separately result from the special digestion of Exo I for unhybridized ssDNA probes and the ATP-enhanced catalysis activities of hemin-G-quadruplex DNAzyme for TMB-$H_2O_2$ reactions aforementioned.

Moreover, the developed microarray-based colorimetric method was applied to identify the single-base mutant levels of miRNAs through the great difference of melting temperature between the non-complementary and complementary hybridized products (Fig. 4). Fig. 4A manifests the comparable identification of single-base mutant miRNAs with different mutant percentages in miRNA samples, with the photograph of the corresponding colorimetric results (Fig. 4A, inset). A big response difference was observed, showing the mutant identification ability of the developed microarray-based colorimetric method. Furthermore, the mutant levels in the miRNA samples were quantified based on the mutation-induced changes in the coloration reactions (Fig. 4B). Accordingly, a linear relationship was achieved for the changes of product absorbances versus the concentrations of single-base mutant miRNAs ranging from 1.0 pM to 200.0 pM ($R^2 = 0.9895$). Therefore, the developed microarray-based colorimetric method could determine the free miRNAs in blood with high selectivity and sensitivity, together with the abilities of identification and quantification of the single-base mutant levels of miRNAs for profiling the gene expression pattern.

## 4. Conclusion

A functionalized microarray was successfully fabricated with the "self-cleaning" hydrophobic substrate, highly dense testing dots, and depressed crossing contamination of sample droplets. A microarray-based colorimetric method was thus developed for probing low-level free miRNAs in blood by combining the specific Exo I digestion of unhybridized ssDNA probes and ATP-enhanced catalysis of hemin-G-quadruplex DNAzyme. Single-base mutation levels of miRNAs could also be accurately identified and quantified for profiling gene expression pattern. Remarkably, this special detection format can be suitably tailored for quantifying the short-sequence miRNAs, which might circumvent the unwinding risk of hybridized miRNAs commonly encountered in the sandwiched assays elsewhere. The so developed microarray-based colorimetric strategy for miRNA detections is high-throughput, selective, and sensitive, holding great promise of wide applications in the clinical laboratory for the diagnosis of cancer and the warning of cancer metastasis.

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## Biographies

**Shuying Li** received her bachelor degree in Chemical Engineering and Technology from the QuFu Normal University, China, in 2013, and now is a postgraduate student in Analytical Chemistry at the same university. Her research interests include Biosensors and Medical Detectors R&D.

**Rui Li** received her bachelor degree in Chemical Education from the QuFu Normal University, China, in 2013, and now is a postgraduate student in Analytical Chemistry at the same university. Her research interests include Biosensors and Medical Detectors R&D.

**Minmin Dong** received her bachelor degree in Chemistry Application from the QuFu Normal University China, in 2013, and now is a postgraduate student in Analytical Chemistry at the same university. Her research interests include Biosensors and Medical Detectors R&D.

**Liyan Zhang** received her bachelor degree in Chemistry Education from the QuFu Normal University in China, in 2014, and now is a postgraduate student in Analytical Chemistry at the same university. Her research interests include Biosensors and Medical Detectors R&D.

**Yao Jiang** received her bachelor degree in Chemical Engineering and Technology from the QuFu Normal University, China, in 2014, and now is a postgraduate student in Analytical Chemistry at the College of Chemistry and Chemical Engineering in the same university. Her research interests mainly include Biosensors and Medical Detectors R&D.

**Lijun Chen** received her bachelor degree of Clinical Medicine from Hunan University of Chinese Medicine, China, in 1991, and now is working at the Affiliated Hospital of Qufu Normal University as Attending Physician. Her research interests mainly involve Clinical Detection and Diagnosis.

**Wei Qi** received her Ph.D. from Chinese Academy of Sciences, China, in 2009, and now is working at Qufu Normal University as an associate professor in Chemistry. Her research interests mainly include Colloid and Interface Chemistry.

**Hua Wang** received his Ph.D. from Hunan University, China, in 2004, and now is working at Qufu Normal University as a professor in Chemistry. His research interests mainly include Chemo/Biosensors, Advanced Functional Materials, and Organic Synthesis.