Original article

Sensitive detection of microRNAs using polyadenine-mediated fluorescent spherical nucleic acids and a microfluidic electrokinetic signal amplification chip

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1. Introduction

Cancer mortality has significantly increased in the recent years and approximately 10 million deaths have been reported worldwide each year [1]. An early cancer diagnosis system is critical for patient survival. Biomarker-based cancer diagnostic tests can significantly improve early detection and subsequent treatment strategies [2]. MicroRNAs (miRNAs) are a class of small endogenous RNA molecules of approximately 20–24 nucleotides that play important roles as post-transcriptional regulators of gene expression [3]. Recently, aberrant miRNA expression was found to be implicated in the development of several cancers, including colorectal [4], lung [5], breast [6], and prostate [7] cancers. Thus, miRNAs are potentially promising biomarkers of cancer that can be used in the early diagnostics and monitoring of various tumors.

Considering the clinical relevance of miRNAs, several analytical methods have been developed. Northern blotting robustly detects miRNAs; however, this technique is restricted by the low sensitivity, labor intensiveness, and long duration [8]. Microarrays, reverse transcription quantitative polymerase chain reaction, and RNA sequencing are widely used because of their excellent analytical performances [9]. However, these methods generally involve tedious sample processing methods and expensive equipment or lack sensitivity. miRNAs are typically present in trace amounts and are present in varying concentrations in...
different biological fluids. Moreover, they are implicated in cancer development. Therefore, a cost-effective, sensitive, and rapid assay for measuring miRNA levels in different biological samples is warranted.

In recent decades, gold nanoparticle (AuNP)-based fluorescent spherical nucleic acid (FSNA) probes have garnered considerable attention in biosensor research due to their high sensitivity and selectivity, large surface area, and low toxicity [10–12]. Although the Au-thiol connection strategy has been certified to be useful in the fabrication of FSNA, it is limited by the total number and conformation of surface-tethered DNA molecules. These factors affect hybridization and specific DNA-Au binding [13–15]. Previous studies have reported that polyadenine (polyA) sequences have a high affinity for AuNP surfaces [16–18]. Pei et al. [14] proposed a salt-aging strategy for preparing FSNA with modification-free, diblock DNA oligonucleotides. In addition, they demonstrated that polyA sequences provide anchoring stability and can form an upright conformation, which facilitates DNA hybridization through the elimination of nonspecific binding. However, this salt-aging method requires approximately 3 days; moreover, the salt concentrations must be controlled to avoid AuNP aggregation. A recent study reported a novel FSNA freezing-based labeling strategy. This method involves a single step, does not include salt-aging or thiols, and is less time-consuming and more cost-effective than the salt-aging method [19]. Because of these characteristics, the freezing-based labeling strategy has shown significant potential for diverse biosensor targeting, including miRNAs.

Microfluidic chips are miniaturized, portable, and cost-effective entities that are widely used to detect disease-related biomarkers [20]. For example, Yang et al. [21] reported an exosome capture and relevant RNA detection method for non-small cell lung cancer diagnostics based on cationic lipoplex NPs in a microfluidic device. Gao et al. [22] developed a surface-enhanced Raman spectroscopy-assisted pump-free microfluidic immunoassay for prostate cancer screening, which showed excellent specificity and sensitivity. In recent years, with increasing development in nanoscience, micro-nanofluidic systems combined with different nanostructures have become topical. These novel nanofluidic devices can pre-concentrate samples more efficiently than pure microfluidic devices because of their ion concentration polarization (ICP) abilities [23,24]. Microfluidic chips with ICP-based electrokinetic signal amplification (ESA) have been extensively studied for diverse charged biomolecules and were found to display high sensitivity, indicating that this is a promising method of detection of trace analytes [25,26]. Moreover, multi-channels can be easily fabricated on one chip and can simultaneously and efficiently determine multiple targets.

Considering these advantages, we fabricated a biosensor system that detects miRNAs in a sensitive and specific manner by combining FSNA biosensor and ESA chips (FSNA-ESA) technology. This method can sensitively detect miRNAs in serum and urine matrix within 30 min. Additionally, the simultaneous detections of multibiomarkers improved diagnostic accuracy, increased detection efficiency, and reduced costs [27]. To this end, we further developed a method for the detection of multiple miRNAs (miRNA-21, miRNA-141, and miRNA-373) that are highly expressed in prostate cancer [7] in a specimen based on a three parallel channel (TPC) microfluidic chip.

2. Experimental

2.1. Materials

All oligonucleotides (Table S1 and the Supplementary data) and RNase inhibitors were supplied by Sangon Biotech Co., Ltd. (Shanghai, China). HAuCl4 and sodium citrate were obtained from National Pharmaceutical Group Corporation (Beijing, China). Phosphate buffered saline (PBS; 1× PBS, pH 7.4) was supplied by Guangzhou Alexan Biotech Co., Ltd. (Guangzhou, China). Sodium chloride and platinum wire electrodes were purchased from Tianjin Fuchen Chemical Reagents Factory (Tianjin, China) and Xiya Chemical Technology Co., Ltd. (Linyi, China), respectively. Syldrg 184 silicone elastomer polydimethylsiloxane (PDMS) and 20% (m/m) Nafion resin were provided by Dow Corning (Midland, MI, USA) and Sigma-Aldrich (Shanghai, China), respectively. Microfluidic chips were prepared using glass as a substrate, PDMS and curing agent were mixed (10:1, m/m) as a cover plate. All chemicals were of analytical grade and prepared in ultrapure water (18.2 MΩ cm). Centrifuge tubes and pipette heads were autoclaved before use.

Direct current (DC) power (MP3001D, Maisheng, Dongguan, China) was used to supply DC voltages for microfluidic ESA. Fluorescence was recorded using an inverted fluorescence microscope (Leica DMIL LED, OSRAM GmbH, Wetzlar, Germany) equipped with a charge-coupled device camera (Leica DFC 360 FX) and images were quantified by free-software ImageJ. AuNPs and FSNA were characterized using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), a Malvern Zetasizer Nano ZS instrument (Malvern, Melvin, UK), and transmission electron microscopy (TEM) (Hitachi, Ltd., Tokyo, Japan).

2.2. AuNP and FSNA synthesis

As previously reported, 13-nm AuNPs were synthesized using the sodium citrate reduction method [28]. Briefly, 1.7 mL of 1% HAuCl4 and 48.3 mL of ultrapure water were added to a three-necked flask and boiled in an oil bath with stirring. Subsequently, 5 mL of 38.8 mM sodium citrate was rapidly added to the boiling solution. When the solution changed color from yellow to deep red (within approximately 15 min), the heating was stopped and the solution cooled to room temperature (25 °C) with stirring.

FSNAs were synthesized by the freezing-based labeling method. First, 2 μL (100 μM) of capture DNA sequence with polyA tails (polyA-DNA) was added to 100 μL of AuNP solution. This mixture was incubated at −20 °C for 2 h and then thawed. Second, 104 μL of buffer A (1 x saline sodium citrate (SSC) pH 7.4 containing 150 mM NaCl and 17 mM phosphate) and 2 μL (100 μM) of reporter-DNA were added to the thawed solution and incubated for 35 min to form FSNAs. To remove free reporter-DNA, FSNAs were washed four times with buffer B (0.5 x SSC pH 7.4 containing 75 mM NaCl and 8.5 mM phosphate) by centrifugation at 13,000 r/min at 4 °C for 15 min. Finally, FSNAs were dispersed in buffer B and stored at 4 °C in the dark.

AuNP and FSNAs preparation for TEM was performed as follows: FSNAs and AuNP solutions were diluted five times in ultrapure water, and 20 μL of aliquot was added to a TEM copper grid and air-dried for 5 min. TEM images were acquired on a Hitachi TEM at an operating voltage of 200 kV. AuNP and FSNAs characterization presented in Fig. S1 and the Supplementary data [29–31].

2.3. Microchip fabrication

The silicon mold substrate of the PDMS microchip was fabricated using standard soft lithography techniques. The ESA microchip contained one Nafion line and one PDMS chip with two parallel microchannels (width = 200 μm and height = 45 μm). A Nafion line was made using micro-flow patterning [32]. Briefly, 1.2 μL of Nafion resin was loaded into a PDMS mold, which had one 45-μm thick and 400-μm wide straight microchannel. Nafion resin was patterned on a glass substrate and followed the shape of the PDMS mold due to capillary forces. The PDMS mold was removed...
and the Nafion line on the substrate solidified by heating at 95 °C for 3 min. Finally, this Nafion-patterned glass slide was irreversibly bonded to a PDMS chip after oxygen plasma treatment, and an H-shaped PDMS chip generated. The Nafion membrane was perpendicular to parallel microchannels, and reservoirs were constructed using 10-μL pipet tips inserted into reservoirs of the PDMS chip.

2.4. Sample preparation

To prepare samples for miRNA-21 detection, 20 μL of FSNAsolution and 180 μL of different miRNA-21 concentrations were incubated in a 200-μL reaction system for 30 min at room temperature. After this, 6-carboxyfluorescein (6-FAM)-labeled reporter-DNA in the solution was generated by centrifugation at 13,000 r/min for 15 min. The biological samples contained 1% serum or 1% urine. Samples for three-target detection were prepared as follows: three FSNA probes were designed according to miRNA-21, miRNA-141, and miRNA-375 sequences, and three targets were simultaneously detected in the TPC biochip.

3. Results and discussion

3.1. Principles of the FSNA-ESA system

An overview of the method principle is shown in Fig. 1. First, FSNA were constructed using the freezing method based on intrinsic affinity between polyA-DNA sequences and the Au surface [16,19]. Second, 6-FAM-labeled reporter-DNA hybridized with the recognition polyA-DNA block, resulting in the fluorescence quenching of reporter-DNA via fluorescence resonance energy transfer [33]. Reporter DNA was replaced by a more efficient hybridization process between miRNAs and polyA-DNA in the presence of target miRNAs; thus, reporter-DNA bridization process between miRNAs and polyA-DNA in the presence of target miRNAs; thus, reporter-DNA fluorescence was restored. Finally, in an electric field, free reporter-DNAs accumulated on the front of the Nafion membrane due to Nafion ion selectivity [34]. In essence, reporter-DNAs were efficiently stacked by ESA on the biochip. Reporter-DNA fluorescence intensity was proportional to the miRNA concentration in biological samples and was used to quantitate the miRNA.

![Fig. 1. Schematic illustration of the fluorescent spherical nucleic acids (FSNA)-electrokinetic signal amplification (ESA) system for miRNA detection. GND: ground; AuNP: gold nanoparticle; PolyA: polyadenine; miRNA: microRNA.](image)

3.2. Optimization of polyA-DNA

The loading density of polyA-DNAs on AuNP surfaces could significantly affect FSNAsensitivity. To facilitate rapid and sensitive miRNA determination, polyA tail lengths of 10, 20, 30, and 40 nucleotides (nt) were investigated. PolyA 10-DNA showed failed labeling and was unable to protect AuNPs from aggregation (Fig. 2A). This might be due to a tendency to form secondary structures in recognition sequences, thus shielding A bases [19]. With increasing polyA lengths, the fluorescence intensity (F0) was enhanced until polyA length reached 30 nt. However, fluorescent signal intensity decreased when polyA length increased to 40 nt. This was possibly due to fluorescence recovery in FSNA probes which were affected by DNA-capped AuNP density and related to polyA length. Thus, shorter polyA tails produced a higher surface density and steric hindrance, which led to inefficient hybridization. Moreover, longer polyA tails had a lower surface polyA-DNA density; therefore, reporter-DNA levels on AuNP surfaces were smaller [33] and generated lower fluorescence intensity. The ideal balance between surface density and reporter-DNA levels was achieved when polyA tail length was 30 nt; therefore, polyA 30 was selected for further studies.

Additionally, the fluorescence intensity enhanced with the increasing molar ratio between polyA-DNA and AuNP from 50:1 to 200:1, and reached a plateau when the molar ratio further increased (Fig. 2B). This was because polyA 30-DNA/AuNPs (200:1) provided enough sites to hybridize target miRNA-21; therefore, the 200:1 molar ratio was selected for further studies.

3.3. Optimizing buffer conditions

Buffer concentration is a key factor determining enrichment efficiency. In this study, PBS concentrations from 0.025× to 1× were investigated. As shown in Fig. 3A, fluorescence intensity increased when the PBS concentration was increased from 0.025× to 0.1×, but decreased when the concentration was greater than 0.1×. This result showed that the length of the depletion region (shown by the green arrow in Fig. 3A) decreased with the increased PBS concentration. Only a small number of cations in low concentration of PBS were pumped into the microchannel by electroosmotic flow (EOF). Negatively charged molecules had to move further toward the anodic reservoir and maintain electro-neutrality at the depletion boundary. However, a mass of cations were contained in higher concentration PBS solution, so anionic analytes had to move slightly toward the anode. On one hand, the shorter depletion region formed a stronger electric field gradient distribution, which benefited the compression of fluorescence bands. On the other hand, higher ionic strength could destroy the electrical neutrality of the depletion zone and induce sample leakage, which was not conducive to reporter-DNA accumulation [26]. Therefore, 0.1× PBS was selected for further studies.

CH3CN is commonly used as an additive for on-line sample pre-concentration, which enables the rapid accumulation of reporter-DNAs via transient pseudo-isotachophoresis [35]. We investigated CH3CN concentrations which ranged from 0% to 8% (V/V) in PBS. The fluorescence intensity of different CH3CN concentrations after 30 min of ESA increased with the increased CH3CN concentration of up to 3% (V/V), but then decreased at higher CH3CN concentrations (Fig. 3B). This phenomenon was explained by the changing distribution of the electric field in the microchannel caused by CH3CN, thereby improving the enrichment efficiency of ESA. However, excessive CH3CN generated an EOF mismatch between sample and running buffer and led to low detection sensitivity. Therefore, 3% (V/V) CH3CN was finally selected.
3.4. Effects of Naﬁon on membrane dimensions

As a highly negatively charged nanoporous material, Naﬁon maintains its cation permselectivity. The ICP effect induced by Naﬁon is one of the most efﬁcient ways to pre-concentrate low abundant molecules [34]. We hypothesized that the stacking efﬁciency of the ESA system was up to the area of the Naﬁon membrane (deﬁned by width and depth). To identify how Naﬁon membrane dimensions affected sensitivity, different membrane widths and depths were investigated. Ion depletion zone length and ﬂuorescence intensity increased by increasing membrane width and depth. However, ﬂuorescence intensity decreased dramatically when membrane width and depth were greater than 400 and 45 μm, respectively (Figs. 3C and D). We hypothesized that when Naﬁon membrane width and depth were decreased, microchannel conductivity was more dominant than Naﬁon and formed a shorter depletion zone, resulting in sample leakage [36]. A larger Naﬁon membrane generated a fast vortical motion and a longer depletion zone which reduced the enrichment efﬁciency. Considering this, a Naﬁon membrane with width and depth dimensions of 400 and 45 μm, respectively, was ﬁnally chosen.

3.5. ESA system speciﬁcity for miRNA-21 detection

Speciﬁcity is vital for the real application of the FSNA-ESA system. FSNA-ESA selectivity for miRNA-21 was tested under different conditions, including blank control, 10 nM non-complementary sequences (miRNA-141 and miRNA-375), 10 nM random RNA, 10 nM single-base mismatch (mis-1), and 1 nM miRNA-21. Only miRNA-21 induced a considerable increase in ﬂuorescence intensity, whereas the ﬂuorescence intensity of mis-1 was much lower than of miRNA-21 (Fig. 4A). These results indicate that our system is signiﬁcantly speciﬁc for miRNA-21, which is primarily attributed to molecular hybridization.

3.6. Method validation

Under optimal conditions, linearity was measured by assessing the relationship between ﬂuorescence intensity (F−F0) and the natural logarithm of miRNA-21 concentrations in the range of 1×10^1-5×10^4 pM. Fluorescence enrichment bands and the miRNA-21 calibration curve are shown (Fig. 4B). Good linearity (r = 0.9947) and repeatability (relative standard deviation values were 3.1% and 4.0% for intraday (n = 6) and inter-day analyses (n = 6), respectively) were obtained for miRNA-21. The limit of detection (LOD) (S/N = 3) was 1.3 pM, which was calculated using the equation: LOD = 3 × SD/k (SD = standard deviation of the blank determination (n = 11) and k = slope of the linear regression curve). Our results and data from previous studies were also compared (Table 1).
and indicated relatively better sensitivity and a lower LOD for our method. Furthermore, to explore the applicability of our system, miRNA-21 levels in human serum and a urine matrix were detected. As shown in Table 2, miRNA-21 recoveries in 1% human serum and 1% urine samples were 104.0%–113.3% and 104.9%–108.0%, respectively, and were acceptable [40]. These results suggested our method worked well with complex matrices and showed great promise for early clinical diagnostics.

### 3.7. TPC biochip for the simultaneous detection of three miRNAs

As shown in Fig. 5A, the TPC chip comprised three parallel channels (red lines) for online sample enrichment, and the two channels (blue lines) at the side for buffers. Further, miRNA-21, miRNA-141, and miRNA-375 were selected to verify TPC biochip feasibility. As shown in Figs. 5B and C, miRNA fluorescence intensities ($F$–$F_0$) were enhanced in the TPC biochip in 30 min ESA. Based on these results, our biochip FSNA-ESA system showed excellent potential of simultaneously detecting multiple disease-related miRNAs in clinical samples.

### 4. Conclusions

We developed a programmable, highly efficient, and homogeneous miRNA detection system based on polyA-mediated FSNA and an ESA microchip. We demonstrated higher sensitivity compared with previous FSNA sensors. The system LOD was 1.3 pM. Additionally, non-specific adhesion effects were eliminated by simply modulating polyA tail length which favored molecular hybridization. Significantly, our new method is universal, so different miRNAs or DNAs can be detected by altering polyA-DNA recognition sequences. Furthermore, a TPC biochip was used to simultaneously detect three different miRNAs (miRNA-21, miRNA-141, and miRNA-375) related to prostate cancer, and was confirmed as an attractive method of improving sample throughput. Considering these advantages, our method showed great potential as a routine tool for miRNA analysis in disease diagnostics and clinical settings.

### CRediT author statement

Jun Xu: Methodology, Validation, Software, Data curation, Investigation, Writing - Original draft preparation; Qing Tang: Methodology, Validation; Runhui Zhang: Validation; Haoyi Chen: Software; Bee Luan Khoo, Xinguo Zhang and Yue Chen: Writing -
Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2022.05.009.

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