

Supporting Information

A Portable Point-of-care Testing System to Diagnose Lung Cancer through the Detection of Exosomal miRNA in Urine and Saliva.

Ping Zhou,^a Fei Lu,^a Jianbo Wang,^b Kaiye Wang,^a Bo Liu,^a Na Li*,^a and Bo Tang*,^a

1. Materials and Reagents

The miRNA-205 and epithelial cell adhesion molecule (EpCAM) aptamers were purchased from Sangon Biotech Co., Ltd. Lateral flow test strip Milenia Genline Hybridetect 01 was purchased from Milenia Biotec. The DNA Reporters labeled with Biotin and FAM were purchased from Takara Biotechnology (Dalian) Co., Ltd. Duplex-specific nuclease was purchased from Evrogen JSC (Shanghai, China). RNase Inhibitor was purchased from Beijing Solarbio Science & Technology Co., Ltd. Tetraethyl orthosilicate (TEOS) was purchased from Alfa Aesar Chemical Ltd. (Tianjin, China), and (3-aminopropyl)-triethoxysilane (APTES) was purchased from Heowns Biochemical Technology Co., Ltd. (Tianjin, China). The experimental water used was Mill-Q secondary ultrapure water (18.2 MΩ•cm). The chemical reagents used in the experiment were analytical grade and without purification. A549 and Beas-2b cells were obtained from the ATCC and maintained as described previously.

2. Methods

2.1 The synthesis of Fe₃O₄@SiO₂ nanoparticles

Ferric chloride (1.3 g, 8 mmol) and trisodium citrate (0.2 g, 0.68 mmol) were dissolved in 20 ml of ethylene glycol, stirring at room temperature to dissolve the ferric chloride. Then, anhydrous sodium acetate (1.2 g) was added to the reaction system, and continued stirring for 30 min. Subsequently, the solution was transferred into a Teflon-lined stainless steel autoclave, and heated to 200 °C for 10 h.^{S1} Fe₃O₄ was obtained by magnetic separation from the reaction system, and was washed three times with absolute ethanol and water. To coat the Fe₃O₄ with SiO₂ layer (Fe₃O₄@SiO₂), the Fe₃O₄ (20 mg) was dispersed in absolute ethanol solution (40 mL), then ammonium hydroxide (800 μL) and TEOS (400 μL) were added into the solution, followed by stirring with 12 h.^{S2} Fe₃O₄@SiO₂ nanoparticles was obtained by magnetic separation and washed 3 times with water and ethanol.

2.2 The synthesis of Fe₃O₄@SiO₂-aptamer nanoparticles

The surface of Fe₃O₄@SiO₂ was functionalized with amino-group by APTES. Firstly, the Fe₃O₄@SiO₂ (10 mg) were dispersed in a mixed solution of water (8 mL) and absolute ethanol (20 mL), after stirring at room temperature for 600 rpm, 5 min. Then, the ammonium hydroxide and APTES were added in the reaction solution, respectively. After 12 h, Fe₃O₄@SiO₂-NH₂ was generated successfully and obtained by magnetic separation, and then washed 3 times with water and ethanol. Secondly, the aptamer DNA of EpCAM with carboxyl was modified on the surface of Fe₃O₄@SiO₂-NH₂ by the dehydration condensation reaction between amino and carboxyl. The aptamer DNA solution (100 μM, 5 μL) was added in PBS buffer (pH=7.4) containing EDC (0.1 mmol) and NHS (0.2 mmol). After reacted at room temperature for 0.5 h, the carboxyl group on aptamer DNA was activated. And then, 1 mg Fe₃O₄@SiO₂-NH₂ in PBS buffer was added into the

solution.^{S3} Mixed the reaction overnight, the resulting Fe₃O₄@SiO₂-aptamer NPs (FSAs) were obtained by magnetic separation and washed twice with water. Finally, the FSA nanoparticles were redispersed in PBS buffer.

The supernatant of the above reaction and the aptamer DNA solution containing EDC and NHS (total DNA, 100 μ M, 5 μ L) were purified by Amicon-3K 5 times with PBS solution. Next, the concentrations of two DNA solution was measured by nanodrop. The aptamer loading on the Fe₃O₄@SiO₂-NH₂ NPs (L_{aptamer}) was calculated following formula:

$$L_{\text{aptamer}} = m_{\text{total}} * (C_{\text{supernatant}} / C_{\text{total}}).$$
 Among them, m_{total} is the quality of total aptamer DNA. $C_{\text{supernatant}}$ is the concentration of supernatant. And C_{total} is the concentration of total aptamer DNA. After calculating, the aptamer loading on the magnetic nanoparticles were 2.6 μ g / mg.

2.3 The detection of miRNA in vitro

The sequence of miRNA-205 was displayed in table S1. Some reagents were prepared for the next reaction. The miRNA-205 and Reporter DNA were firstly dissolved in RNase-free water. 10 X DSN buffer was taken out from -20 °C refrigerator for melting. The total volume of DSN reaction reagents is 20 μ L, containing 10 X DSN buffer (2 μ L), duplex-specific nuclease (0.1 U), Reporter DNA (10 μ M, 2 μ L), DNase Inhibiter (20 Unites), miRNA-205 (2 μ L) and sterile water (11.5 μ L). The reaction took place in an RNase-free micro tube, incubated at 37 °C for 30-60 min. For terminating the reaction, the 2 X DSN stop solution was added to the tube, mixed and stayed at room temperature for 5 min. Subsequently, HybriDetect 1 lateral flow strips (Milenia) were used for reading out the fluorescein isothiocyanate (FITC) and Biotin labeled DNA Reporter. The DSN reaction solution (20 μ L) was added to HybriDetect 1 assay buffer (100 μ L, Milenia) and ran on HybriDetect 1 lateral flow strips (Milenia). All experiments were repeated at least three times.

2.4 Cell control

Non-small cell Lung cancer cells (A549) and human normal lung epithelial cells (Beas-2b) were used in this work. Two cell lines were cultured in dulbecco's modified eagle medium (DMEM) with 10 % fatal bovine serum (FBS) and 1% Penicillin-Streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Anaerobic culture conditions were 5% CO₂, 1 % O₂ and 94% N₂, at 37 °C.

2.5 Exosomes extraction from A549 and Beas-2b cells

When the cell confluency reached about 80 %, the media was removed. And then the cell was treated with the new DMEM media without FBS containing 0.6 % insulin-transferrin-selenium (ITS-G). Then the cells continued to culture in a humidified incubator at 37 °C for 48 h. Exosomes were obtained from the supernatant by gradient centrifugation. Firstly, the supernatant media was collected and centrifuged (Eppendorf, Centrifuge 5424 R) at 500 g, 4 °C for 5 min to remove the cellular debris, then sequentially centrifuged at 2000 g for 10 min and at 10000 g for 30 min to removing other debris. Finally, exosomes precipitation was obtained by ultra-high speed centrifugation (Beckman, U.S.A., Optima XPN-100 Ultracentrifuge) at 120, 000 g for 1 h. Exosomes were washed with PBS, followed by ultra-high speed centrifugation at 120, 000 g for 1 h.

2.6 The detection of exosomal miRNA

Exosomes were obtained by centrifugation method or based on the FSAs. The protocol of exosome extraction by gradient centrifugation was same as above. For the other experiment, firstly, FSAs was dissolved in PBS buffer (1 mg mL⁻¹) and mixed with exosome solution in microtube. Notably, the exosome solution includes the culture solution of A549 cells or Beas-2b cells. And

then put the microcentrifuge tubes in a DNA mixer to rotate and mix the solution for 0.5 h at room temperature. After that the exosomes were binding with FSAs, and the FSAs were obtained by magnetic separation. Next, the precipitation was treated with TRIzol Reagent at 4 °C for 10 min to obtain the lysate of exosome. Notably, the TRIzol Reagent is not the RNA extraction kit, and it have be used in this work for breaking the exosomes. Subsequently, the exosome lysate (2 µL) was added into DSN reaction system which mentioned at earlier. For the remaining steps, please referring to the detection of miRNA in vitro in the above. All experiments were repeated at least three times.

2.7 The detection of exosomal miRNA in urine or saliva

The urine or saliva samples came from healthy people or lung cancer patients. At first, 400 µL urine or 100 µL saliva was incubated with FSAs solution (1 mg, 0.5 mg mL⁻¹) at 37 °C for 1 h. Then the FSAs-exo was separated from the solution by magnetism. And the precipitation was dissolved in TRIzol buffer at 4 °C for 10 min. Subsequently, 2 µL buffer was added to DNS-based detection solution, and the mixture was incubated at 37 °C for 0.5 h. This reaction system was stopped by 2X EDTA buffer. Finally, 20 µL reaction solution was mixed with 100 µL HybriDetect 1 assay buffer (Milenia) and ran on HybriDetect 1 lateral flow strips (Milenia). The total assay time of the exosomal miRNA detection is 40 min after further optimization of reaction conditions, which includes 10 min exosomes capture, 10 min exosomes lysis, 15 min DSN reaction and 5 min strip running. All experiments were repeated at least three times.

2.8 The quantification of detection results by Image J

The test line and control line in the strip are quantified with Image J, respectively. We treated the mean intensities of test and control line as 100 %, and calculated the percentage of test line in two lines. The experiments were repeated at least three times, and calculated the error bars.

2.9 The extraction of miRNA

Total miRNA was extracted from 10 mL urine based on miRNeasy extraction Micro Kit (TianGen, DP501). According to the protocol of the manufacturer, miRNA was achieved. The concentration and the quality of the RNA were analyzed by nanodrop.

2.10 The generation of cDNA

The cDNA is synthesized by program containing poly A tailing modification and reverse transcription reaction using miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN, KR211). According to the manufacturer's protocol, 7 µL total miRNA as the template was added to the reaction mixture. The mixture was reacted under the program at 42 °C for 60 min and then 95 °C for 3 min. Polymerase chain reaction (PCR) instrument was purchased from Bioer Technology Co., Ltd. (Hangzhou, China, LineGene 9620). The concentration and the quality of the cDNA were analyzed by nanodrop.

2.11 quantitative Real time polymerase chain reaction (qRT-PCR)

qRT-PCR assay was performed with miRcute Plus miRNA qPCR Kit (SYBR Green, TIANGEN, FP411). According to the manufacturer's protocol, 2 µL cDNA as the template was added to the SYBR Green mix. The specific PCR primers of miRNA-205 and RNU-6 were purchased by TIANGEN BIOTECH (Beijing) CO., LTD. Data was normalized with RNU-6 and relative expression of miRNA-205 was determined by the $2^{-\Delta\Delta C_t}$ method. All experiments were repeated at least three times.

2.12 Western blotting

Exosome samples were resolved by SDS-PAGE (10%) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% fat free dry milk and incubated

with anti-CD9 and anti-CD81 (two antibodies were purchased from Santa) antibodies overnight at 4 °C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent. The blots were analyzed using a Amersham Imager 600 (GE AI600, America). All experiments were repeated at least three times.

2.13 The calculation of limit of detection^{S4-S5}

The calibration graph of DSN and strip based detection could be fitted into the following regression equation: $y=4.48252 \cdot C_{\text{miRNA}}-2.07147$; $R^2=0.961$. The limit of detection (LOD) was calculated via the interception point between the standard curve and background + 3σ level line. The LOD was calculated to be 7.76 pM. All experiments were repeated at least three times.

2.14. The Stability experiments of lung cancer diagnostic kit (LCDK)

The FSAs, DSN reaction mixture and lateral strips were stored at 4 °C for 1, 3, 7, 14, 30 and 180 days before using, respectively. The exosome samples were the supernatant solution after A549 cells treated with FBS-free DMEM containing 0.6 % insulin-transferrin-selenium (ITS-G) for 48h (2 mL / T25). Then test the samples under the above detection protocol. All experiments were repeated at least three times.

3. Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shandong Normal University and written informed consents were signed by all patients.

Table S1 RNA and DNA sequences employed in this work.^{S6-S7}

Sequence (5'-3')
Reporter : FITC-AGACT CCGGT GGAAT GAAGGA -Biotin
miRNA-205: UCCUU CAUUC CACCG GAGUCU
Aptamer-EpCAM: HOOC-CAC TAC AGA GGT TGC GTC TGT CCC ACG TTG TCA TGG GGG GTT GGC CTG

Table S2. The formula of DSN reaction system.

Reagent	Concentration	Volumes in 20 uL total volumes
10X DSN buffer	10X	2 uL
Duplex-Specific Nuclease	0.5 U/ uL	2 uL
Reporter	10 uM	2 uL
RNase Inhibiter	40 U/ uL	0.5 uL

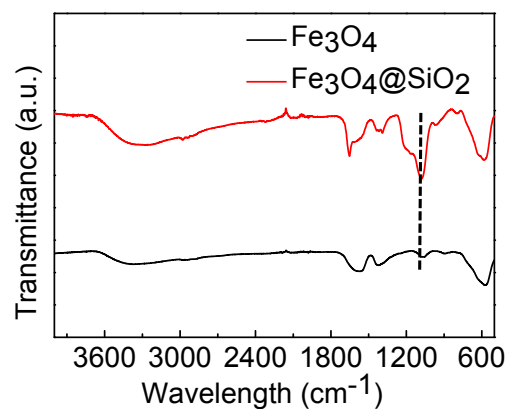


Figure S1. The FT-IR spectra of Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{SiO}_2$.

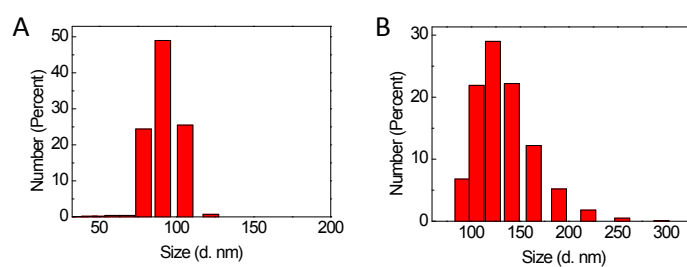


Figure S2. The DLS images of A549-exo (A) and Beas-2b-exo (B).

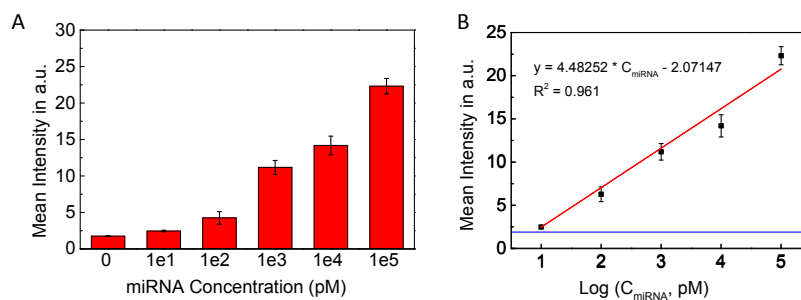


Figure S3. A) Quantitation of bands intensity from detection in Figure 3B. B) The linear fitted graph of DSN and strip based detection performance. ($y = 4.48252 * C_{\text{miRNA}} - 2.07147$, $R^2 = 0.961$).

The blue line shows the background + 3σ level which intercepts with the linear fit at $x = 0.89$, corresponding to $C_{\text{miRNA}} = 7.76 \text{ pM}$.

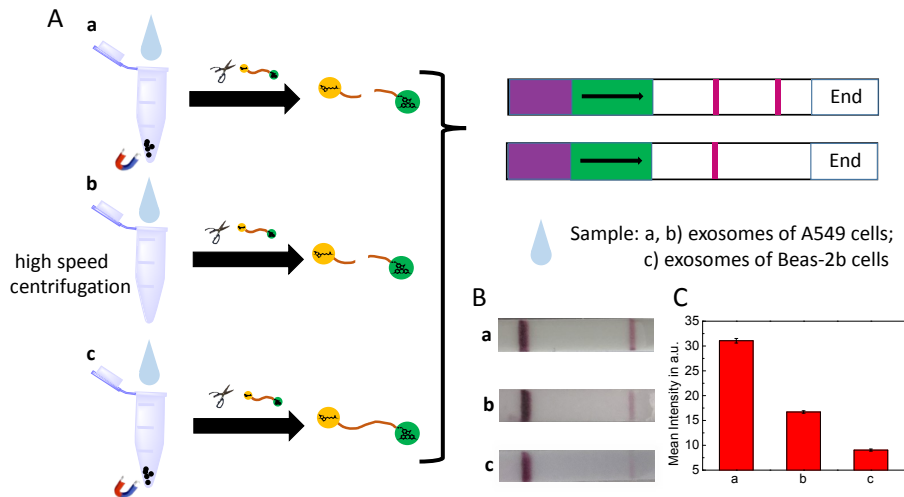


Figure S4. A). The schematics of exosomal miRNA detection for different samples through DSN-Strips system. B). The detection results from (A). C). Quantitation of band intensity from detection in (B).

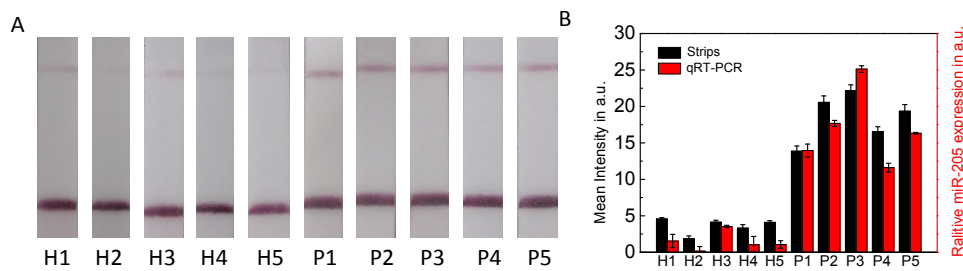


Figure S5. A) The detection of exosomal miRNA, using 10 saliva samples from 5 lung cancer patients (P1-P5) and 5 healthy people (H1-H5). B) Quantitation of band intensity from detection in A (black), H1-H5 as the control groups in qRT-PCR assay (red), and the error bars of the qPCR measurement represent \pm SEM of n = 3 measurements.

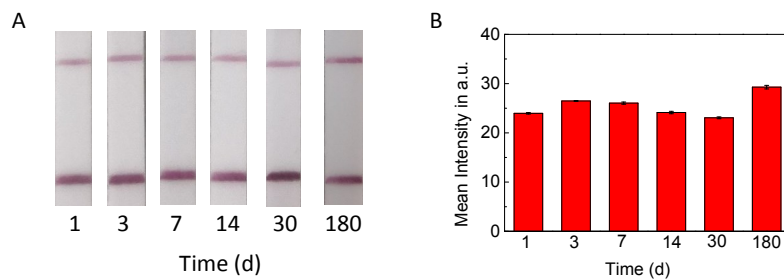


Figure S6. A) The detection of exosomal miRNA by kit after stored at 4 °C for 1, 3, 7, 14, 30 and 180 day. B) Quantitation of band intensity from detection in A.

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