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ARTICLE

In Situ Multiplex Detection of Serum Exosomal MicroRNAs Using All-in-One Biosensor for Breast Cancer Diagnosis

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Herein, a simple all-in-one biosensor based on DNA three-way junction has been constructed for in situ simultaneous detection of multiple miRNAs by competitive strand displacement. In our design, three oligonucleotides (Y1, Y2 and Y3) of Y-type scaffold were extended at their 5' ends by introducing three single-stranded recognition sequences with quenchers (BHQ1, BHQ2 and BHQ2), respectively. Subsequently, three reporter sequences labeled with different fluorophores (FAM, Cy3 and Cy5) were bound to corresponding recognition sequences to form the multicolour DNA biosensor that gives a self-quenched fluorescence. The biosensor can effectively enter into exosomes and then hybridize to the complementary miRNAs targets to form longer duplexes and release the reporter sequences, thus activating the readable fluorescence signals for simultaneously detecting multiple miRNAs in exosomes. As a proof of principle, miR-21, miR-27a and miR-375 were chosen as model targets because of their high expressions in breast cancer cells (MCF-7). Fluorescence signals of MCF-7 exosomes after being treated with the biosensor exhibited the positive correlations to their concentrations and the limits of detection were determined to be 0.116 μg/mL, 0.125 μg/mL and 0.287 μg/mL exosomes by detecting three exosomal miRNAs (miR-21, miR-27a and miR-375), respectively. In contrast, there were no obvious correlations between fluorescence intensities and control MCF-10A exosome concentrations. Importantly, by testing multiple exosomal miRNAs using the biosensor in clinical serum samples, breast cancer patients can be effectively differentiated from healthy donors. Consequently, the developed biosensor demonstrates the high potential as a routine bioassay for multiplex quantification of exosomal miRNAs in clinical diagnosis.

Introduction

Exosomes have gained world-wide attention in recent years due to the pathological significance and potential clinical diagnosis value.[1-5] As nanosized extracellular vesicles (30-150 nm in diameter), exosomes are secreted by various cells and circulate in most of the body fluids, such as blood, urine, saliva, breast milk, cerebrospinal fluid, etc.[6-8] Currently, many reports evidenced that exosomes play an important role in mediating intercellular communications because they carry abundant important biomolecules (e.g., nucleic acids, proteins, growth factors, etc.) that originated from parental cells and convey information to recipient cells during circulation.[9-12] Therefore, exosome-associated biomolecules may be ideal candidate biomarkers for early diagnosis of diseases, such as cancers.[13-15]

MicroRNAs (miRNAs) are a class of non-coding RNAs, approximately 22 nucleotides in length, which involve in a wide range of biological processes including cell proliferation, differentiation, immunological response and tumorigenesis.[16,17] Recent studies have shown that many miRNAs that are highly correlated to the development and malignant progression of cancers were found in cancer exosomes.[18,19] It is noteworthy that the distinctive phospholipid bilayer structure of exosomes can protect their miRNAs contents from RNase digestion and other environmental disturbance.[20] Therefore, exosomal miRNAs can act as the non-invasive biomarkers and have the potential for early diagnosis and prognostic monitoring of cancers.[21,22]

At present, quite a few detection methods for exosomal miRNAs have been constructed, such as real-time quantitative (RT-PCR)-based strategy,[23] a ratiometric fluorescent probe,[24] (hybridization chain reaction) HCR- and DNAzyme-based sensing method reported in our previous work.[25] However, the majority of these approaches are limited to extracting total RNAs from exosomes by multi-step operations including exosomes lysis, RNA extraction, cDNA synthesis and RT-PCR analysis, which make RNA easy to degrade and reduce the extraction amount, thereby affecting the reproducibility of results. Meanwhile, these procedures are time-consuming, laborious, especially technically challenging in extracting low

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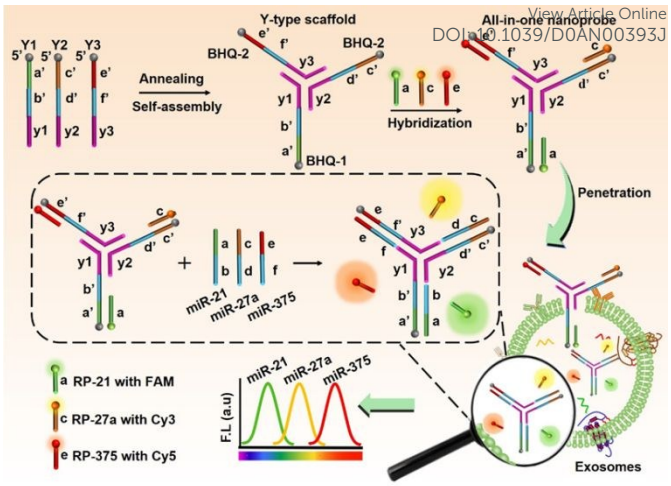
expression levels of exosomal miRNAs from microsamples Thus, a simple and in situ method seems to be a better candidate for direct multiplex detection of exosomal miRNAs. However, most of these approaches for exosomal miRNAs quantification mainly focused on the analysis of a single biomarker, which may yield false-positive results and cannot be sufficient to guide the diagnosis of disease in many cases.[26,27] Although Rhee' group[28] had reported a simple multiplexed method for in situ detection of exosomal microRNAs, the proposed assay was performed with three individually independent fluorescent dye-labeled molecular beacons, which may cannot guarantee the delivery of three molecular beacons into exosomes in an equal amount, resulting in notable signal deviation and failing to accurately quantify the contents of exosomal miRNAs. The integration of multiple recognition sequences into a probe (all-in-one) can ensure the equal amount delivery of three different sensing probes into exosomes and are not easily susceptible to the interference in the lumen of exosome, thus accurately reflecting the differential expression levels of multiple target miRNAs in nanosized exosomes. However, the all-in-one strategy has not employed for in situ multiplex detection of exosomal miRNAs. Herein, we constructed a multicolour all-in-one biosensor based on DNA three-way junction for in situ simultaneous quantification the contents of the multiple miRNAs in the whole exosomes (**Scheme 1**). As a proof of principle, three exosomal miRNAs including miR-21, miR-27a and miR-375 were used as model targets due to their high expression levels in breast cancer cells.[28] In our design, the biosensor contains three backbone oligonucleotides (Y1, Y2 and Y3) of Y-type scaffold, which were extended at their 5' ends by introducing three single-stranded recognition sequences for targeting different miRNA (miR-21, miR-27a and miR-375), and further modified with different quenchers (BHQ1, BHQ2 and BHQ2) at the most end. The other three dye-terminated oligonucleotides (RP-21, RP-27a and RP-375) with different fluorophores (FAM, Cy3 and Cy5) served as reporter sequences, which were identical to the partial sequences of the corresponding targets. These three reporter sequences (RP-21, RP-27a and RP-375) were firmly bound to the extended recognition sequences of Y1, Y2 and Y3, respectively, which



Scheme 1. Schematic representation of in situ multiplex detection of serum exosomal microRNAs using all-in-one biosensor for breast cancer diagnosis.

yielded multicolour all-in-one biosensor in the absence of target exosomal miRNAs and thus produced the self-quenched fluorescence. When entering exosomes, the recognition sequences quickly hybridized to the complementary targets miRNAs to form longer duplexes with thermodynamically favorable configuration and liberate the reporter sequences, thereby activating the readable fluorescence signals. By calculating the fluorescence signal, the developed biosensor can simultaneously quantify the multiple miRNAs contents in exosomes. This present study indicates that the multicolour biosensor possesses considerable potential as a multiplex sensing platform and additionally expands the wide applications for more exosome-associated biomarkers in clinical diagnosis.

Experimental

Chemical reagents

Membrane permeabilization reagent Streptolysin O (SLO) and Dulbecco's phosphate-buffered saline (D-PBS) were purchased from Sigma−Aldrich. Tris(2-carboxyethyl)phosphine (TCEP), BCA protein assay, Dulbecco's modified Eagle's medium (DMEM), minimal essential growth medium (MEGM), fetal bovine serum (FBS), penicillin/streptomycin, cholera toxin and 0.22 μm syringe-filters were purchased from Thermo Scientific HyClone (MA, U.S.A.). Anti-human-CD63 and Anti-human-Alix was obtained from Liaoning Rengen Biosciences Co. Ltd. The ExoQuick-TC™ reagent was purchased from System Biosciences. Four kinds of cells (MCF-7, MCF-10A, L02 and NIH/3T3) were obtained from the Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Serum samples from three breast cancer patients and two healthy donors were provided by Hunan Province Tumor Hospital (Changsha 410013, Hunan, China). All the chemicals were at least of analytical reagent grade unless otherwise specified. All buffers were prepared with ultra-pure MilliQ water (18.2 MΩ cm). The reaction buffer was 50 mM Tris-HCl containing 8 mM $MgSO_4$ (pH 7.5). All oligonucleotides were synthesized by Sangon Co. Ltd. (Shanghai, China). DNA sequences used in this article were listed in **Table S1**.

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Preparation of biosensor and PAGE analysis

The construction of all-in-one biosensor based on DNA three-way junction was present as follows: Firstly, stoichiometric amounts of three oligonucleotides Y1, Y2, and Y3 at their 5' ends modified with quenchers (BHQ1, BHQ2 and BHQ2) were mixed to form Y-type scaffold. The mixtures then heated to 95 °C for 5 min and slowly cooled down to room temperature (25 °C) for at least 2 h. Subsequently, stoichiometric amounts of three reporter sequences with different fluorophores (FAM, Cy3 and Cy5) and Y-type scaffold were incubated for 30 min at 37 °C to form all-in-one biosensor.

Polyacrylamide gel electrophoresis (PAGE) was carried out to characterize the assembly process of all-in-one biosensor and studied the performance of strand displacement reactions. In brief, 12 μL of different loading samples containing 8 μL of reaction products, 2 μL of 6× loading buffer and 2 μL of 1× SYBR Gold were separated by 15% PAGE. PAGE was run in 1× TBE buffer at 80 V voltage for 90 min at 4 °C and finally imaged by Azure Biosystems C600 Multifunction Imaging System (Azure Biosystems, U.S.A.). Signals of FAM, Cy3 and Cy5 were excited by three-color RGB light including Cy2 (460 nm), Cy3 (526 nm) and Cy5 (628 nm), respectively. The concentrations of all loading samples were 1 μM.

Cell culture and exosome isolation

MCF-7 cells (human breast cancer cells), L02 cells (normal human hepatocyte cancer cell) and NIH/3T3 cells (mouse embryonic cells) were cultured in DMEM media containing 10% exosomes-depleted FBS and 1% penicillin and streptomycin. MCF-10A cells (normal mammary epithelial cells) were cultured in MEGM containing 10% exosomes-depleted FBS and cholera toxin. All cells were incubated in a humidified atmosphere of 5 wt %/vol CO_2 at 37 °C. After 48 h of incubation, exosomes were collected from cell culture supernatant and isolated by ExoQuick-TC™. According to the manufacturer's recommendations, isolated procedures were as follows: the supernatant was first centrifuged at 3000 g for 15 min to remove cells and cell debris. Then, the supernatant was incubated with ExoQuick-TC™ overnight at 4 °C. The next day, the mixture was centrifuged at 1500 g for 30 min, and the isolated exosomes were resuspended in 100 μL of DPBS and stored at -80 °C for further use.

For the production of exosomes-free FBS, the commercialized FBS was centrifuged at 120,000 g for 12 h at 4 °C by a micro ultracentrifuge refrigerated centrifuge (Cs150FNX Hitachi, Japan). The collected supernatants were then filtered using a 0.22 μm syringe-filter and stored at 4 °C for further use.

For the investigation of the stability of the biosensor in serum, serum sample was first ultracentrifuged at 100,000 g for 2.5 h at 4 °C to remove the exosomes. The synthesized all-in-one biosensor were then added in the prepared 60% serum sample. Next, three different fluorescence signals were measured respectively. The detection procedure in serum samples was the same as that in Tris-HCl buffer and cell culture medium.

Characterization and quantification of exosomes

The morphology of isolated exosomes was characterized by using transmission electron microscopy (TEM). In brief, isolated exosome was fixed with 2% paraformaldehyde for 1 min. After that, the mixture was loaded on formvar carbon-coated copper grids for 10 min, negatively stained with 2% phosphotungstic acid for 5 min and then washed twice with DPBS. Subsequently, the prepared sample was dried completely at room temperature and imaged by a Tecnai G2 20 STwin (FEI, Czech Republic). Furthermore, the hydrodynamic diameter was measured by Zetasizer 3000HS (Malvern Instruments, Worcesterhire, UK). Next, the concentrations of isolated exosomes were determined by BCA protein assay. Besides, the contents of three exosomal miRNAs derived from MCF-7 and MCF-10A were determined by quantitative real time polymerase chain reaction (qRT-PCR, Sangon Co. Ltd., Shanghai, China).

Multiplex detection of synthetic miRNAs in buffer solution

150 nM biosensor was incubated with various concentrations of miRNA targets (miR-21, miR-27a, miR-375) and their control sequences, respectively, in 100 μL of 50 mM Tris-HCl buffer (pH 7.5, 8 mM $MgSO_4$). After 5 min of incubation at 37 °C, the fluorescence intensities were evaluated by Hitachi F-7000 fluorescence spectrometer at corresponding excitation wavelengths. The fluorescence intensity of FAM was measured with the excitation wavelength at 488 nm and emission wavelength at 520 nm. The fluorescence intensity of Cy3 was measured with the excitation wavelength at 530 nm and emission wavelength at 570 nm. The fluorescence intensity of Cy5 was measured with the excitation wavelength at 600 nm and emission wavelength at 670 nm. The signal-to-background ratio (SBR) was regarded as the indicator of hybridization reactions ($SBR=F/F_0$, where F is the fluorescence emission intensity in the presence of target, and F_0 is the background fluorescence signal). Besides, taking the detection of miR-21 as an example, biosensor and control biosensor without the recognition sequence for miR-21 were also made to study the specificity of the developed method.

To investigate the stability of the proposed biosensor, we chose cell culture medium and FBS serum (60%) to simulate the complex physiological environments. 100 nM of biosensor was incubated in Tris-HCl buffer, cell culture medium and FBS se-rum (60%), respectively. After 2 h of incubation at 37 °C, the fluorescence intensities were evaluated by Hitachi F-7000 fluorescence spectrometer at corresponding excitation wavelengths.

Multiplex detection of miRNAs in exosomes

To enhance the delivery of biosensor, exosomes were treated with 0.8 U/mL SLO by reversible permeabilization method, which was activated by adding 5 mM TCEP to 10 U/mL SLO for 1 h at 37 °C. Then, 100 nM biosensor was added into 100 μL of solutions containing various concentrations of SLO-treated exosomes derived from MCF-7 and MCF-10A cells, respectively, and then incubated for 30 min at 37 °C. Subsequently, the expression contents of three miRNA targets were determined by measuring the fluorescence intensities at corresponding excitation wavelengths. Besides, to investigate the selectivity of biosensor, SLO-treated exosomes derived from different types of cells (MCF-7, MCF-10A, L02, NIH/3T3) were respectively incubated with 100 nM biosensor for in situ multiplex detection of miRNAs. As a control, the biosensor

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without recognition sequence for miR-21 was also delivered into MCF-7 exosomes for miR-21 detection.

To demonstrate the multiplex detection of exosomal miRNAs in clinical samples, plasma samples from two healthy donors and three breast cancer patients were collected from Hunan Cancer Hospital and the Affiliated Cancer Hospital of Xiangya School of Medicine. Before experiments, the plasma exosomes were respectively isolated by the commercially available ExoQuick-TC™ reagent. Then, 100 nM biosensor was added into SLO-treated exosomes derived from five clinical samples and incubated for 30 min at 37 °C. The differential expressions of three miRNAs were investigated by testing the fluorescence intensities.

Ethics statement

Clinical samples from two healthy donors and breast cancer patients were collected from Hunan Cancer Hospital and the Affiliated Cancer Hospital of Xiangya School of Medicine. All experiments were performed in accordance with the Guidelines of Clinical Sample Management Rules of Hunan Cancer Hospital, which were reviewed and approved by the Ethics Committee at Hunan Cancer Hospital. Informed consents were received from the blood donors of this project.

Results and discussion

Construction and characterization of all-in-one biosensor

To investigate the feasibility for miRNAs detection, three different competitive strand displacement reactions were first proved by gel electrophoresis (**Fig 1A**). In the absence of the miR-21 target, reporter sequence RP-21 with FAM (lane 1) was bound to Y1 to form a duplex-DNA structure (RP-21-Y1) with lower migration speed (lane 2). After adding miR-21, a distinct band of RP-21 (blue, bottom in lane 3) and a new hybridization product of Y1 and miR-21 (top in lane 3) were observed, indicating the excellent recognition for miR-21. Similarly, two bright bands of RP-27a (green, bottom in lane 7) and RP-375 (red, bottom in lane 11) were also clearly seen upon addition of miR-27a and miR-375. These results demonstrated that three miRNA-targets could be successfully detected by competitive strand displacement. Subsequently, the potential ability of the developed biosensor platform for simultaneous detection of three miRNAs was further investigated by electrophoresis (**Fig 1B**). Bright bands migrated slower by continuously additional DNA strands (from lane 1 to 4), suggesting that three reporter sequences can successfully assemble onto a Y-type scaffold to form all-in-one biosensor (lane 4). In the presence of three miRNA, three different color bands were simultaneously observed in the bottom of lane 8, which were consistent with the phenomenon from lane 5 to lane 7, evidencing that the simultaneous detection of three miRNA targets can be achieved by the biosensor. The structure of all-in-one biosensor was characterized by atomic force microscopy (AFM) (**Fig. S1**).

Subsequently, we excluded the mutual interference of three

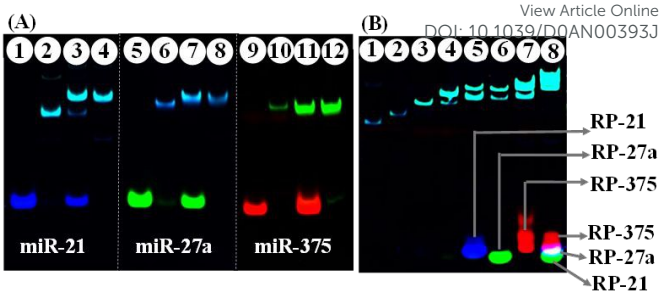

Figure 1. PAGE analysis of single (A) and multiplex (B) detection method for miRNAs by competitive strand displacement reactions. (A) Single target detection. lane 1: RP-21, lane 2: RP-21 + Y1, lane 3: RP-21 + Y1 + miR-21, lane 4: Y1 + miR-21, lane 5: RP-27a, lane 6: RP-27a + Y2, lane 7: RP-27a + Y2 + miR-27a, lane 8: Y2 + miR-27a; lane 9: RP-375, lane 10: RP-375 + Y3, lane 11: RP-375 + Y3 + miR-375, lane 12: Y3 + miR-375. (B) Multiple target detection. lane 1: Y1, lane 2: Y2, lane 3: Y3, lane 4: Y1 + Y2 + Y3 + RP-21 + RP-27a + RP-375 (biosensor), lane 5: Biosensor + miR-21, lane 6: Biosensor + miR-27a, lane 7: Biosensor + miR-375, lane 8: Biosensor + miR-21 + miR-27a + miR-375.

different kinds of fluorescence signals (Fig. S2). The fluorescence intensities of FAM, Cy3, and Cy5 were significantly increased after adding three corresponding miRNA-targets. Moreover, it was noteworthy that the fluorescence signal of one dye in the presence of the specific target was comparable to that in the presence of three targets, suggesting that the existence of one target had little impact on the fluorescence signal of others. Hence, the simultaneous detection of multiple miRNAs can be realized by the proposed biosensor. The kinetics studies were then performed by monitoring the fluorescence signals over different time points (**Fig. S3**). The results displayed that fluorescence intensities restored quickly within 100 s upon additional synthetic miRNA targets and remained stable afterward. To achieve a complete response, we chose 5 min as incubation time in the subsequent experiments. In addition, the fluorescence intensities increased in a concentration-dependent manner, which suggested that the biosensor could simultaneously recognize three specific targets (**Fig. S4**). The limits of detection (LOD) were determined to be 462 pM for miR-21 (**Fig 2A**), 301 pM for miR-27a (**Fig 2C**), and 154 pM for miR-375 (**Fig 2E**), respectively. (LOD=3σ/S, σ=standard deviation of the blank probe values, S=slope of the calibration curve). Selectivity is an important factor for simultaneous detection of multiple targets in exosomes. We then used miR-200b, miR-141 and let-7d as controls. Fluorescence intensities of biosensor incubated with miR-21 (**Fig 2B**), miR-27a (**Fig 2D**), miR-375 (**Fig 2F**) increased remarkedly, implying that the biosensor was high enough to discriminate

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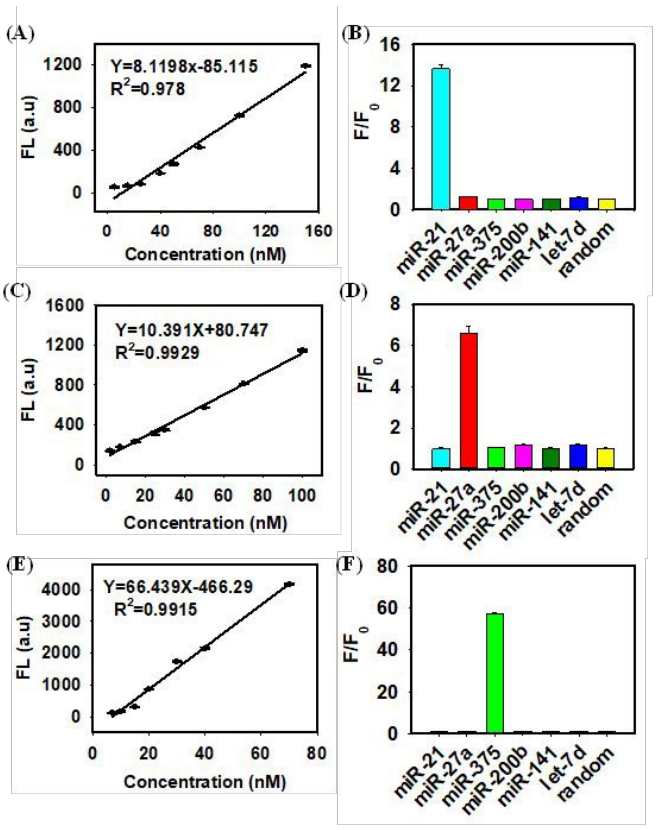

Figure 2. The linear relationship between fluorescent signals of biosensor and synthetic miR-21 (A), miR-27a (C) and miR-375 (E) concentrations ranging from 5 to 140 nM, from 2 to 100 nM and from 7 to 70 nM, respectively. Selectivity of biosensor for miR-21 (B), miR-27a (D) and miR-375 (F), respectively. All values are mean ± SD (n=3).

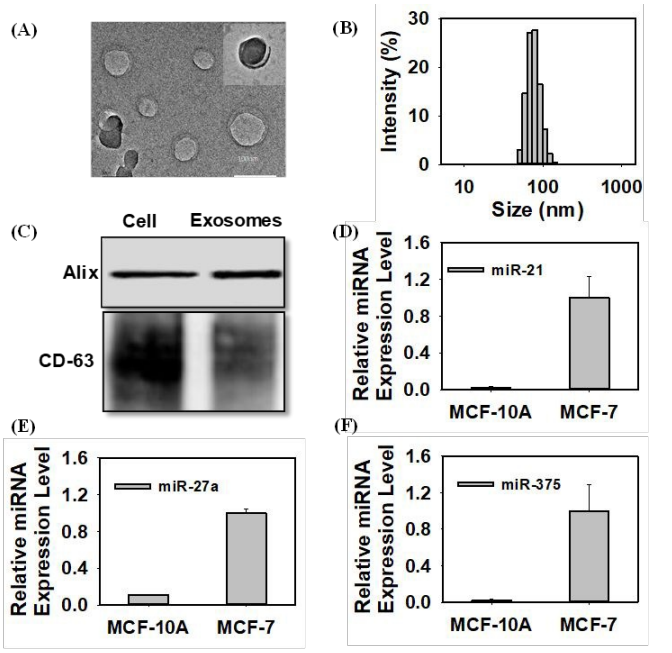

Figure 3 TEM image of MCF-7 exosomes (A). Inset: the membrane structure of exosome. DLS measuring size distribution of MCF-7 exosomes (B). Western blot image of CD-63 and Alix from cell lysates and corresponding exosomes (C). Relative levels of exosomal miR-21 (D), miR-27a (E) and miR-375 (F) derived from MCF-7 and MCF-10A cells by real-time qPCR using U6 RNA level as an internal control for data normalization. All values are mean ± SD (n=3).

miRNAs targets from controls. In addition to the above selectivity tests, a control biosensor without recognition sequence (e.g. miR-21) was also employed to further study the specificity performance of biosensor (**Fig. S5A**). The fluorescence intensity generated by biosensor was much higher than that of control biosensor. Moreover, we investigated the applicability of biosensor in the presence of nontargets miRNA (**Fig. S6**). Even if the presence of 20-fold concentration of three nontarget miRNAs, there is almost no effect on the fluorescence intensities of three target miRNAs, indicating the good applicability of the developed biosensor. Based on the above results, the multiplex biosensor has good sensitivity, selectivity and applicability for target miRNAs, implying the potential application for exosomal miRNAs detection.

Isolation and characterization of exosomes

To obtain the exosomes, MCF-7 cells were cultured with exosome-free FBS for two days. After that, the collected supernatant including exosomes were isolated using ExoQuick kit or ultracentrifugation. TEM image (**Fig 3A**) showed the round structures and phospholipid bilayer membrane structure of isolated exosomes. The size of isolated exosomes was determined to be approximately 80 nm by dynamic light scattering (DLS), which was consistent with the previously reported literatures (**Fig 3B**).[29,30] Besides, the exosome markers were further identified by western blotting (WB) against two of exosomes-enriched proteins, CD-63 and Alix, which expressed on the membrane and in the lumen of exosomes, respectively.[31,32] The distinct bands of the abundant CD-63 and Alix in MCF-7 exosomes validated the efficiency of the exosome isolation (**Fig 3C**). These results evidenced that those obtained vesicles were typical exosomes and these isolated exosomes can be directly used in the following experiment. The non-tumorigenic epithelial cells (MCF-10A) were widely used as a control cells and treated with the above-mentioned procedures. To analyse the expression levels of three different miRNAs in MCF-7 and MCF-10A exosomes, we extracted exosomal miRNAs and then measured the relative amounts of miR-21, miR-27a and miR-375, respectively, by using the real-time PCR analysis. The higher amounts of miR-21, miR-27a and miR-375 were existed in MCF-7 exosomes than that of MCF-10A exosomes (**Fig 3D-F**), proving that three cancer-related exosomal miRNAs would serve as potential biomarkers for clinical diagnosis.

To ensure the complete reaction between biosensor and exosomal miRNAs, 30 min of incubation time was used in the subsequent exosomes tests based on the kinetics results in solution. According to the previous reported literature, SLO[33,34] has the merits of simple, non-toxic, and rapid delivery when compared with other delivery strategies (e.g. microinjection and electroporation), which can be used as ideal reversible membrane permeabilization tool to enhance the delivery of biosensor into exosomes. Therefore, we optimized the concentrations of SLO to obtain higher fluorescence recoveries. The optimal concentration of SLO was 0.8 U/mL and was used for the following experiments (**Fig. S7A**). In addition, we investigated the enhanced delivery of biosensor into exosomes by using SLO. A lower concentration of

exosomes treated with SLO displayed a higher fluorescence signal compared with twice concentrations of exosomes without SLO, which indicated that SLO can effectively facilitate the delivery of biosensor into exosomes, thus improving the sensitivity and specificity of the proposed approach (**Fig. S7B**). It was also surmised that the exosome membrane was damaged after SLO treatment, thus liberating intra-exosomal miRNA into the solution. The liberated intra-exosomal miRNAs in solution could hybridize with biosensor and generate strong fluorescence signals. Thus, we carried out experiments to rule out this possibility (**Fig. S8**).

In situ and multiplex analysis of exosomal miRNAs

We carefully evaluated the differential expression levels of three miRNAs in MCF-7 and MCF-10A exosomes. As shown in **Fig 4A, C and E**, the fluorescence intensities increased in a dose-dependent manner by adding MCF-7 exosomes over various concentrations ranging from 0 to 240 μg/mL. Whereas, no significant changes of fluorescence intensities were observed with increasing concentrations of MCF-10A exosomes, indicating that MCF-10A exosomes express lower levels of three miRNAs than of MCF-7 exosomes. In addition, fluorescence signals of MCF-7 exosomes incubated with the biosensor exhibited the positive correlations to their concentrations with good linear correlations (R^2 = 0.99). By detecting three exosomal miRNAs (miR-21, miR-27a and miR-375), the LOD were determined to be 0.116 μg/mL (**Fig 4B**), 0.125 μg/mL **(Fig 4D**) and 0.287 μg/mL (**Fig 4F**) exosomes, respectively. These results were correlated very well with real-time PCR analysis in **Fig 3D-F**, indicating that the biosensor can

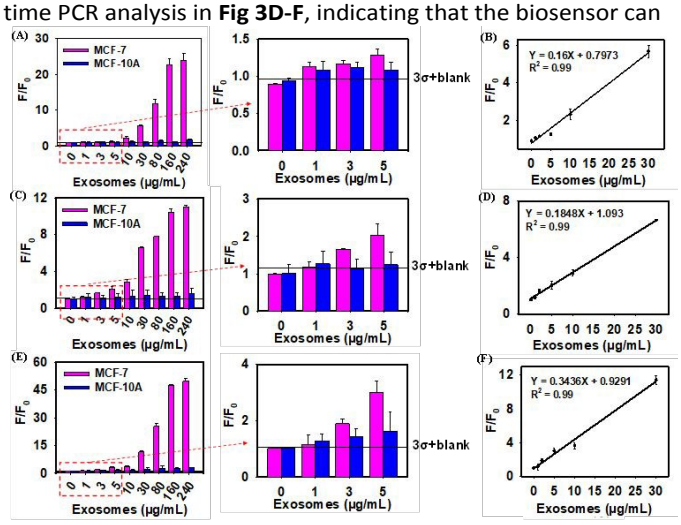

Figure 4 Multiplex detection of miR-21 (A), miR-27a (C) and miR-375 (E) in MCF-7 and MCF-10A exosomes over various concentrations ranging from 0 to 240 μg/mL. Linear dynamic range of fluorescence intensity of exosomal miR-21 (B), miR-27a (D) and miR-375 (F) with exosomes concentrations, respectively. All values are mean ± SD (n=3).

effectively enter into exosomes and quantitatively respond to multiple miRNAs with increasing concentrations of MCF-7 exosomes.

Except for MCF-10A exosomes mentioned-above, we also chose L02 and NIH/3T3 exosomes, as controls, to further demonstrate the selectivity of the proposed assay toward other types of exosomes. **Fig 5A-C** displayed that the fluorescence intensities of three miRNA targets in MCF-7 exosomes were much higher than that in MCF-10A, NIH/3T3 and L02 exosomes. Besides, there is almost no effect on detecting three MCF-7 exosomal miRNA even when mixed with MCF-10A exosomes at higher concentrations. Similar to the detection result of control biosensor in solution (**Fig. S5A**), a negligible fluorescence signal of exosomal miR-21 was observed by control biosensor when compared with biosensor (**Fig. S5B**). These results further proved that MCF-7 exosomes expressed higher levels of miRNA-21, miR-NA-27a and miR-375 than MCF-10A exosomes, confirming that biosensor has a better capability of simultaneously quantifying the differential expression levels of exosomal miRNAs and discriminating cancer exosomes from normal exosomes with high specificity and sensitivity. In addition, the relative standard deviations (RSD) of reproducibility (n=4) was 0.96%, when the exosome concentration was 2 μg/mL, suggesting the outstanding reproducibility of the proposed biosensor. Moreover, no significant difference of fluorescence intensities was examined when the biosensor incubated in the complex environment, such as cell culture medium and FBS serum, demonstrating the good stability of the DNA nanosensor (**Fig. S9**). The comparison of currently available detection method for exosomal miRNA were list in **Table S2**. At present, the commonly used detection approaches have some shortcomings including single target detection, long time-consumption and complicated operation. Though its sensitivity, in this work, is not as good as some electrochemical, surface-enhanced Raman scattering (SERS)

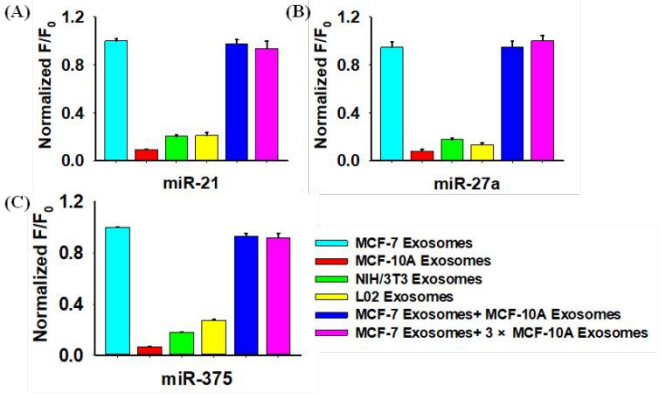

Figure 5 Selectivity studies of the multiplex detection method for three exosomal miRNAs. Detection of miR-21 (A), miR-27a (B) and miR-375 (C) derived from MCF-7, MCF-10A, NIH/3T3, L02 and mixtures of MCF and MCF-10A exosomes. Exosomes used in this study were in the equal concentrations (85 μg/mL).

and signal amplification strategy-based methods, our developed method with a good reproducibility and stability is simple, cost-effective, easy-operation, and a shorter time duration.

Multiplex determination of serum exosomal miRNAs from clinical patients

To demonstrate the clinical application of multiplexed detection method for miRNAs, the serum exosomes were isolated and collected from three breast cancer patients (P1, P2, P3) and two healthy individuals (H1, H2). The fluorescence

signals of equal amounts of exosomes derived from five clinical samples were measured, respectively, after being incubated with the biosensor. As displayed in **Fig 6A-C**, the fluorescence signals of three serum exosomal miRNA generated from the breast cancer patients were significantly higher than that from the healthy donors, demonstrating that the higher contents of three miRNAs in breast cancer patients than that in the healthy individuals. All these results obtained from five clinical samples were also displayed in **Fig 6D**, further implying that the proposed method can simultaneously and quantitatively detect different expression levels of three exosomal miRNAs from clinical samples. Therefore, the developed biosensor is more readily applicable to clinical research and can also be a potential tool for early cancer diagnosis.

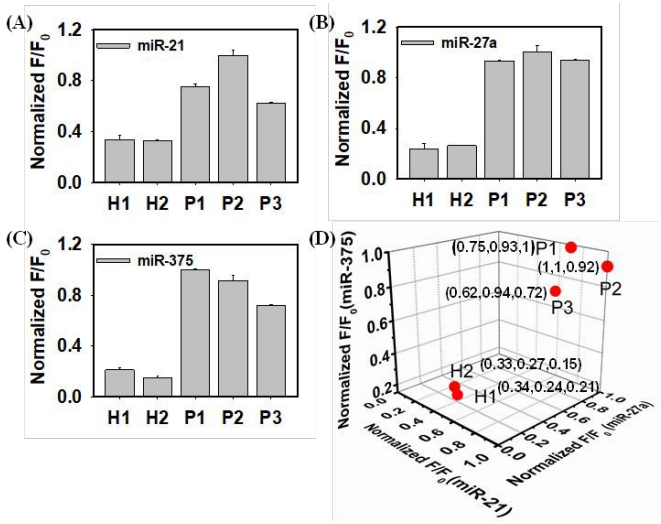

Figure 6 Multiplex detection of serum exosomal miR-21 (A), miR-27a (B) and miR-375 (C) collected from three breast cancer patients and two healthy individuals. (D) Sum of the results of panels (A-C).

Conclusions

In summary, we have developed a simple all-in-one biosensor based on DNA three-way junction for in situ simultaneous detection of three cancer-related exosomal miRNAs by the strand displacement reaction. With the introduction of all-in-one conception, the biosensor is based on the integration of multiple recognition sequences, which can ensure the equal amount delivery of three different sensing probes into exosomes and decrease the signal interference, thereby improving the accuracy for multiplex detection of exosomal miRNAs. The rationally designed biosensor can effectively enter into exosomes and specifically bind with target exosomal miRNAs to generate fluorescence signals for in situ quantification the differential expressions of multiple miRNAs in circulating exosomes. Moreover, the developed biosensor can successfully overcome many drawbacks of the PCR-based technology and offer many advantages, such as high accuracy, easy operation, simple design probe, low cost and non-invasive. Using miR-21, miR-27a and miR-375 as model targets, the proposed biosensor for miRNAs can discriminate MCF-7 exosomes from control exosomes even at higher concentrations. More importantly, with the ability to directly quantify the multiple exosomal miRNAs, breast cancer patients can be effectively differentiated from healthy donors. Therefore, the present approach can open the door to providing more precise information for prognostic and treatment on cancer patients in early- and late-stage, and offer the possibility for the non-invasive early diagnosis of cancer with lower miRNAs expressions when combined with nucleic acid signal amplification technology

Conflicts of interest

There are no conflicts to declare.

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Graphical abstract

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