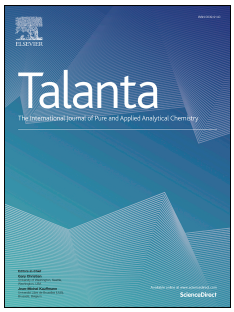

Structure-switching aptamer triggering hybridization displacement reaction for label-free detection of exosomes

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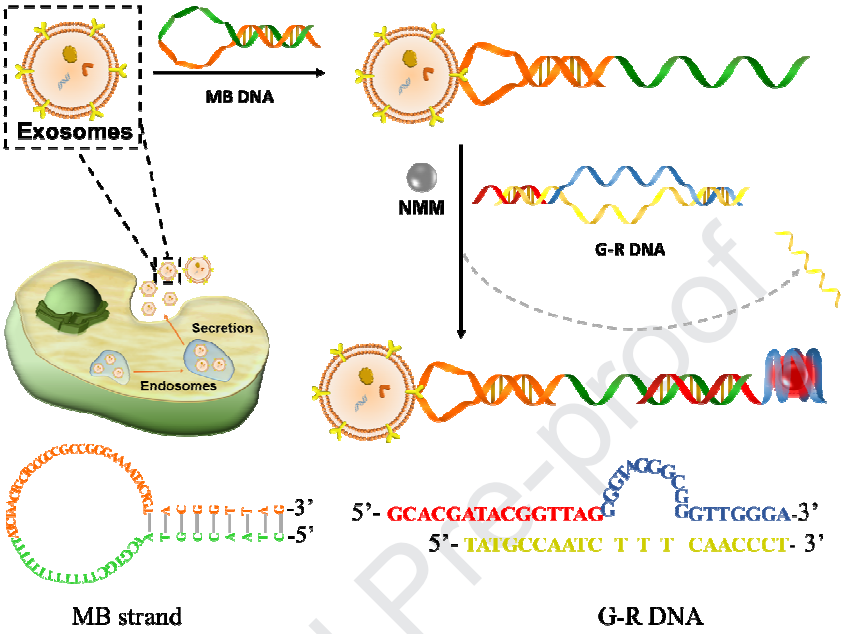
Structure-Switching Aptamer Triggering Hybridization Displacement Reaction for Label-free Detection of Exosomes



A novel label-free and activatable aptamer probe was reported for detection of exosomes in human blood.

Structure-Switching Aptamer Triggering Hybridization Displacement Reaction for Label-free Detection of Exosomes

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Abstract

Exosomes play important roles in intercellular communications, tumor migration and invasion. However, the specific detection of cancer exosomes remains as a big challenge due to its low concentration in biofluids. Therefore, the sensitive and selective detection of cancer cells-derived exosomes has attracted growing attention owing to their potential in diagnostic and prognostic applications. Activatable strategies have received great attention for the detection of low abundant analytes due to their high sensitivity. Herein, based on molecular recognition between DNA aptamer and exosome surface biomarker (protein tyrosine kinase-7), a novel activatable and label-free strategy was designed for highly sensitive and specific sensing of exosomes. In this work, the target exosomes trigger strand replacement reaction to form G-quadruplex, which result in an obvious fluorescence enhancement of N-methylmesoporphyrin IX due to the bonding between G-quadruplex and N-methylmesoporphyrin IX. Under the optimum experimental conditions, the linear range for exosomes was measured to be 5.0×10^5-5.0×10^7 particles/μL and the detection limit (LOD) was calculated to be 3.4×10^5 particles/μL (3σ). This assay possesses high specificity to distinguish exosomes derived from different cell lines, and has successfully been validated in patient and healthy plasma samples. Furthermore, the probe can effectively detect the exosomes in 30% fetal bovine serum, indicating that the biological matrix has a negligible effect on this method. This developed label-free, convenient and highly sensitive biosensor will offer a great opportunity for exosomes quantification in biological study and clinical application.

Keywords: Exosomes; aptamer; label-free; fluorescent sensor; quantitative detection.

1. Introduction

Exosomes, referring to extracellular vesicles secreted from almost all types of cells with a size range from 30 to 150 nm, play a pivotal role in intercellular communication and cancer metastasis [1-3]. Since exosomes are present in the circulation system and typically carry tons of biomolecules derived from parent cells, including lipids, proteins and nucleic acids, they have been regarded as noninvasive biomarkers for cancer diagnosis and prognostic assessment [4, 5]. Therefore, the development of exosomes biosensors is of great significance in understanding the biological functions of exosomes and for disease diagnosis.

In recent years, various analytical methods have been constructed to detect analytical sample [6-8]. Among them, several strategies have been developed for detecting and profiling exosomes, such as nanoparticle tracking analysis (NTA) [9], colorimetric assay [10, 11], electrochemical detection [12], flow cytometry [13], microfluidics [14, 15], enzyme-linked immunosorbent assays (ELISA) [16], fluorescent sensors [17, 18] and others [19, 20]. Though these methods can detect and distinguish different types of exosomes, there are still some problems that restrict their further applications [21, 22]. For example, NTA only can be used to provide an estimated number of exosomes within narrow ranges at a high concentration level (1×10^{7}-10^{9} particles/mL). Moreover, lipoprotein particles, apoptotic body and large protein aggregates may interfere with exosomes assay [23]. In addition, most of the above-mentioned assays are based on protein recognition ligands, suffering from expensive cost, complex experimental procedure and poor stability. Thus, the development of facile method to perform rapid marker screening and sensitive exosomes detection is still highly desired.

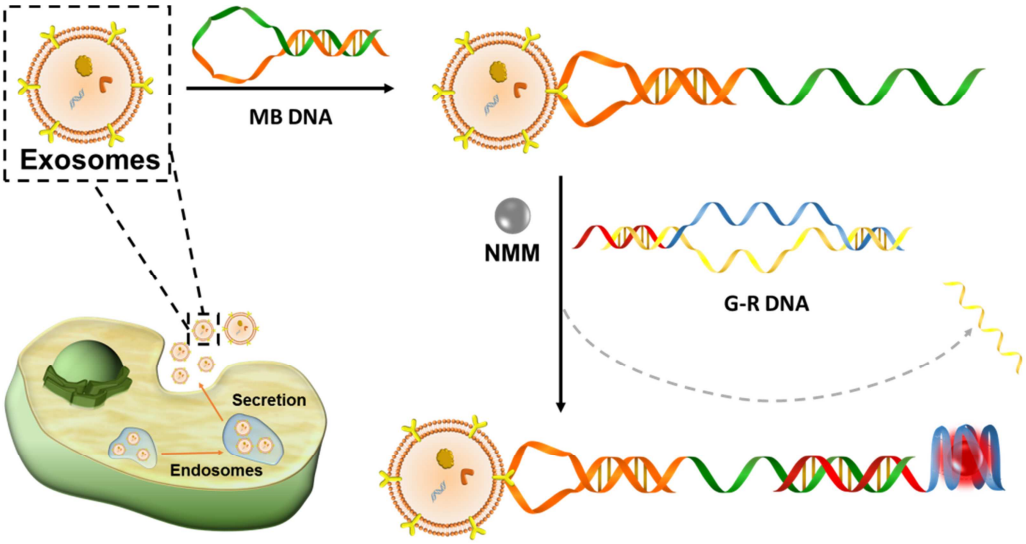
Aptamers, which are generated by a technique called systematic evolution of ligands by exponential enrichment (SELEX) [24, 25], exhibit high binding affinity and specificity toward targets [26, 27]. Compared with antibodies, aptamers provide many indisputable and convincing advantages, such as easy synthesis and chemical modification, high stability and good reproducibility. Due to the similarity between parent cells and exosomes, cell aptamer-based sensing strategy can be a useful tool for exosomes assay [28, 29]. Using different measuring techniques, several of aptamer-based assays for quantitation and profiling of exosomes have been reported [19, 30, 31]. In

these projects, it can be found that the aptamer molecules labeled with fluorescent dyes either at the ends or at the active site are generally applied to provide a signal. However, the binding capability of the aptamer is most likely to be affected by the extra modification with dyes. Moreover, it is laborious and costly. Thus it is highly demanded to design a label-free, cost-effective and activated strategy for quantitative detection of exosomes.

In order to improve the sensitivity of analytical methods, various strategies have undergone remarkable growth during recent years. For example, by employing the polymer polypyrrole as the fluorescence quencher, Ramanavicius et al. reported some immunosensors for targets detection [32, 33]. Activatable probes also have emerged as an active area of research due to their high sensitivity. These kind of probes only generate signal under specific target stimulation, resulting in effectively reducing the background signal and improving the sensitivity. Based on structure-switching aptamer triggered hybridization chain reaction, several activatable approaches have been constructed to detect cancer cells [34]. Therefore, label-free and activatable probes can be skillfully designed for high sensitivity detection of exosomes.

In this work, based on molecular recognition triggered structure-switching aptamer, we proposed a novel label-free and activatable platform for sensing of exosomes, as illustrated in Scheme 1. In this design, an aptamer sgc8, which can specifically bind with the tyrosine kinase-7 (PTK7) on the surface of exosomes [28, 35, 36], was chosen as the model. The hairpin DNA strand (hereafter named MB), contained a target recognition domain (orange line) and a trigger domain (green line) for initiation of strand displacement reaction. We chose N-methylmesoporphyrin IX (NMM) as signal reporter which can insert G-quadruplex structure and produced a strong fluorescence enhancement. In the absence of target, the G-rich DNA chain was blocked in the double strand (hereafter named G-R DNA) and it was greatly hindered to fold into G-quadruplex structure, thus leading to a low background signal. However, in the presence of target exosomes, sgc8 bounded with the PTK7 and underwent structure switching of MB, following by the explosion of the trigger domain. Then the trigger domain initiated the strand displacement reaction to expose the G-rich domain and then form a G-quadruplex structure (dark green), leading to a significantly fluorescence signal. Therefore, a linear relationship was established for exosomes detection with the increase in fluorescence intensity. The strategy exhibited a high sensitivity to exosomes secreted from CCRF-CEM cells with a detection limit of 3.4×10^5 exosomes/μL. It also showed a high selectivity

toward target exosomes compared to other types of exosomes. Moreover, this method was also successfully applied to detect and distinguish exosomes in patients from healthy donor plasma samples. Therefore, this label-free and activatable fluorescence aptamer strategy will provide a useful platform for detecting and profiling exosomes and possess great promise for cancer early diagnosis.



Scheme 1. Schematic diagram of the biosensor for quantification of exosomes by activatable and label-free design.

2. Experiment section

2.1. Reagents and instruments

The details of the instruments were provided in Supporting Information. All oligonucleotides used in this work, Fetal bovine serum (FBS), RPMI-1640 medium, Dulbecco's modified eagle medium (DMEM) and phosphate buffer solution (PBS) were obtained from Sangon Biotech Co., Ltd (Shanghai, China). The sequences of oligonucleotides involved are listed in Table S1, and the predicted secondary structure simulation diagram of the MB DNA and G-R DNA were provided in Fig.S1. Ultrafiltration membranes with the pore diameter of 0.22 μm and ultrafiltration tube with the molecular weight of 100 KD were purchased from Millipore (USA). Exosomes-depleted FBS were purchased from System Biosciences (SBI, USA). N-methylmesoporphyrin IX (NMM) was provided by Frontier Scientific Inc. (Frontier Sci, USA). Before using, the concentration of NMM was measured by absorption spectrum [37]. The prepared solution of NMM (10 mM) was kept in dark at -20 °C and then diluted to the appropriate concentration depending on the needs of the experiment. Yeast tRNA and other chemicals were ordered from Sigma-Aldrich reagent Co., Ltd. (St. Louis, MO,

USA). All aqueous solutions were prepared using ⩾18.2 MΩ•cm ultrapure water (Milli-Q, Millipore).

2.2. Cells culture

CCRF-CEM cells (T cell line, human acute lymphoblastic leukemia) and Ramos cells (B cell line, human Burkitt's lymphoma) were kindly provided by Prof. Xiaoxiao Hu (Hunan University). Human cervical carcinoma cell (Hela), Mouse melanoma cells (B16F1) and Normal human liver cells (HL-7702) were supported by the Cell Center of FMBA lab (Zhengzhou University). All cells were cultured in DMEM medium supplemented with 10% exosomes-depteted FBS and 100 IU/mL double antibiotics (penicillin/streptomycin) and then incubated in a humidified incubator containing 5% CO_2 for two days at 37 °C. When the cells density grew to 80-90%, the supernatant of required cells were collected and processed.

2.3. Purification of exosomes

Exosomes were isolated from the supernatant of the cells above using centrifugal ultrafiltration according the previous report [38]. Briefly, the supernatant of cells was treated with centrifuge at 3,000×g for 30 min at low temperature and then filtered with 0.22 μm ultrafiltration membrane to wipe off the debris and intact cells. The treated supernatant was further concentrated with an 100 KDa ultrafiltration centrifuge tube at 5,000×g for 30 min at 4℃. In order to obtain the pure exosomes, the filtered media was then centrifuged at ultra-high speed of 160,000 ×g for 2 h at 4 °C. At last, after removing the supernatant, the exosomes pellet was redissolved in PBS (pH=7.2) and stored at -80℃for further use.

2.4. Detection of exosomes in buffer solution

To prepare G-R DNA, equal moles of G-4 DNA and blocker DNA were mixed together in PBS buffer at a final concentration of 1 μM. The mixed solution was annealed by heating at 95 °C for 10 min and then descending temperature to 4 °C at a rate of 1 °C min^{-1}. MB DNA was prepared according to the same procedures and the final concentration was also 1 μM. Under the optimized conditions, different concentrations of exosomes sample solution were incubated with 150 nM of MB DNA and 100 nM of G-R DNA solution at 4 °C for 1.5 h. Then, 1 μM of NMM was added into the above solution with a final volume of 100 μL at room temperature. After 30 min, the fluorescence signals of these solutions were recorded by a fluorescence measurement with an excitation wavelength of 399 nm. All of the measurements were performed three times and the standard

deviation was plotted as the error bar.

2.5. Detection of exosomes in real samples

10% Fetal bovine serum (FBS) (Gibco) was obtained by diluting into DMEM media and then ultracentrifuged at 100, 000 ×g for 2 h at 4 °C to discard the exosomes and extracellular vesicles, named it as ultracentrifuged FBS (UC FBS). To detect exosomes in real sample, the purified exosomes derived from CCRF-CEM cells were added in 10%, 20% and 30% (v/v) UC FBS, respectively. The process for detection exosomes in UC FBS samples was the same as that in the PBS buffer.

This strategy was further used to detect the exosomes from clinical blood diagnosis of human acute-lymphoblastic-leukemia patients. Whole blood samples from human acute-lymphoblastic-leukemia patients and healthy donors were provided by the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) and used under protocols approved by Life-Science Ethics Review Committee of Zhengzhou University. All patients in the human acute-lymphoblastic-leukemia group were examined using blood screening test, and all patients appeared specific clinical symptoms and clinical test results were positive. The range indicators by physical examination were in the normal value for test results of healthy donors. The blood serum was collected through centrifuging at 3,000 ×g for 15 min, and then the supernatant was filtered through a 0.22-μm pore filter. Exosomes detection in clinical serum samples was performed as described above.

3. Results and discussions

3.1. Investigation of exosome property

The morphologies of exosomes were performed using scanning electron microscope (SEM) and transmission electron microscopy (TEM). As can be seen from Fig. 1A and Fig. 1B, a uniform diameter with globoid vesicles is between 30-100 nm, which is consistent with previous literature reports [39]. The hydrodynamic diameter of exosomes recorded by dynamic light scattering (DLS) (Fig. 1C) is about 89.3 nm, which is consistent with TEM result. In addition, the zeta potential of exosomes is -19.7 ± 1.6 mV in PBS (Fig. S2B). From the nanoparticle tracking analysis (NTA) assay, we can see that the extractive exosomes has an average diameter of 96.6 ± 1.1 nm (Fig. S2A) and the corresponding concentration is $1.12 \times 10^{12} \pm 2.8 \times 10^{7}$ particles/mL (Fig. 1D). The size difference between TEM and NTA is probably due to the fact that the latter one is realized by monitoring the

hydrodynamic diameter of the exosomes in solution. In particular, these small particles contributed extensively to Brown Motion, which leads to a size shift compared to TEM. The concentrations of other different kinds of exosomes were also quantified by NTA assay and the results were shown in Fig. S3 and Table S2. All these findings corroborated the previous work for the characterization of exosomes [40, 41].

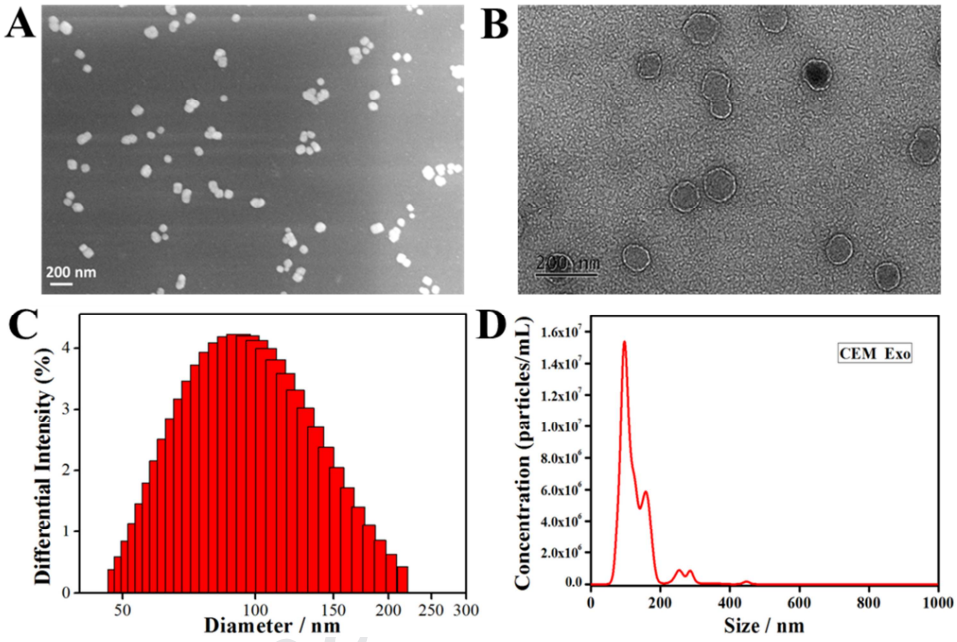


Fig. 1 Investigation of exosomes property. The images of (A) SEM and (B) TEM of CEM exosomes; (C) the size distribution histogram by DLS and (D) the quantitative analysis of CEM exosomes by NTA.

3.2. Feasibility of the proposed method

To evaluate the feasibility of this approach, the fluorescence intensity of the system was first performed to characterize reaction process in different conditions. It can be seen from Fig. 2 there is no obvious change of fluorescence intensity in the absence of target exosomes (red curve) and in the presence of control exosomes (blue curve), indicating a low fluorescence background and good selectivity. However, after introducing the target exosomes into the above mixture solution, a sharp fluorescence enhancement can be observed at 615 nm (green curve), which indicates that G-rich sequence has been exposed and folded into the quadrupled structures to modulate the fluorescence intensity. This result indicated that this platform can be used for the detection of exosomes.

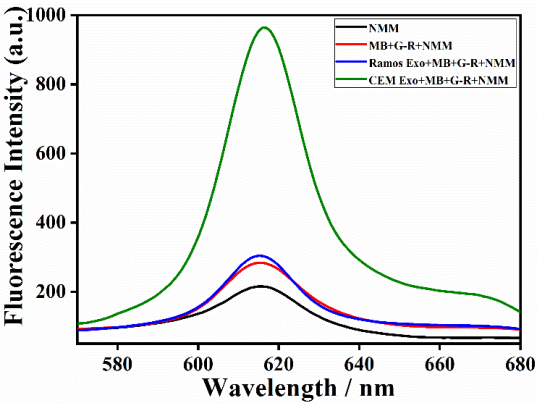


Fig. 2 Fluorescence emission spectra of NMM (black line), MB+G-R+NMM (red line), Ramos exosomes+MB+G-R+NMM (blue line), CEM exosomes+MB+G-R+NMM (green line).

3.3. Optimization of the experimental parameters

In order to achieve the best exosomes detection performance, experimental parameters in the detection protocol including the sequence of blocker DNA, the temperature and reaction time of binding between exosomes and MB, the concentration of MB, the concentration of NMM and the binding time between NMM and G4 structure were optimized. Since the base pairs between blocker DNA and G4-DNA play a crucial role in this assay, the sequence of blocked DNA was first optimized. Briefly, we designed a bare DNA strand containing a complete base of the trigger domain to trigger the chain displacement reaction and expose G4 sequence. Then the blocked base number of the blocker-DNA at 3′-end of G4-DNA was optimized. If there is insufficient base number of the blocker-DNA to block the 3′-end of G4-DNA, the higher background signal is obtained due to the partial free G4-DNA. In contrast, if there is excess base number of the blocker-DNA to block the 3′-end of G4-DNA, it is very difficult to expose the 3′-end of G4-DNA, resulting in the inhibition of the G-quadruplex. Similarly, the base number of the blocker-DNA at 5′-end of G4-DNA has been also optimized. It can be seen that, the largest fluorescence enhancement in the presence of bare DNA is obtained by using the blocker DNA-2, which is obviously higher than that of blocker DNA-1, blocker DNA-3, blocker DNA-4, blocker DNA-5, blocker DNA-6 and blocker DNA-7(Fig. S4A and S4B).Therefore, the blocker DNA-2 was chosen as the best block DNA named R-DNA for the later experiments. Next, the incubation time and reaction temperature between exosomes and MB strand were investigated. With increasing incubation time, the fluorescence signal-to-background increases gradually, and reaches to a maximum when the reaction temperature at 4 ℃ was used with incubation time of 90 min (Fig. S5). Subsequently, MB concentration, the reaction time of the NMM

with G4 and the NMM concentration were also optimized. As shown in Fig. S6, the best signal-to-background ratio can be obtained with 150 nM of MB, 30 min binding time of the NMM with G4-DNA and 1 μM of NMM.

3.4. Analytical performance of the sensor

Under the optimum conditions, this proposed method was used to detect exosomes in PBS buffer solution with 1% tRNA. As shown in Fig.3A, the fluorescence intensity increases with the concentrations of exosomes increasing from 5.0×10^5 to 7.5×10^7 particles/μL. Fig.3B shows the relationship between the intensity of NMM and the concentration of exosomes. The inset of Fig.3B illustrates that the fluorescence intensity increases linearly with the exosomes concentration range from 5.0×10^5 to 5.0×10^7 particles/μL. The correlation equation is Intensity =12.7[exosomes] + 318 (R^2 = 0.990). According to 3σ/slope rule, the LOD for exosomes is estimated to be 3.4×10^5 particles/μL. In addition, the linear range and LOD of this method was compared with other reported methods. As shown in Table S3, the LOD of this developed assay is comparable to most of the other methods (Table S3).

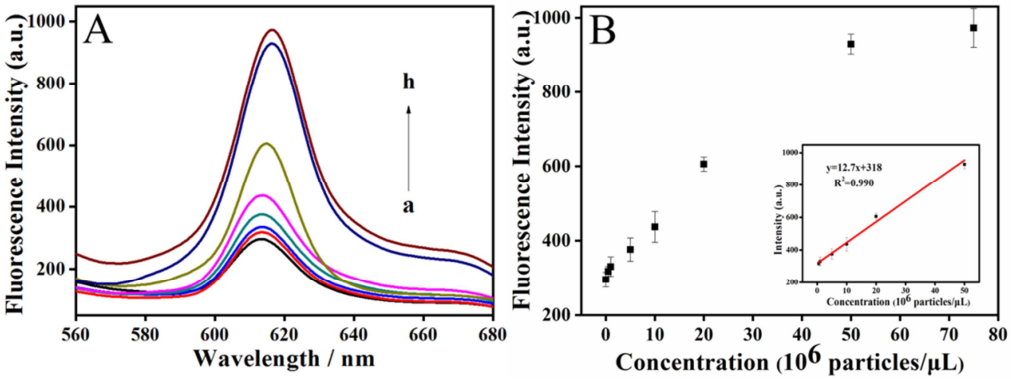


Fig. 3 (A) Fluorescence emission spectra of exosomes detection with different concentration: (a) 0, (b) 5.0×10^5, (c) 1.0×10^6, (d) 5.0×10^6, (e) 1.0×10^7, (f) 2.0×10^7, (g) 5.0×10^7, (h) 7.5×10^7 particle/uL. (B) Scatter plot of fluorescence intensity corresponding to (A) (0 - 7.5×10^7 particle/uL). Inset: The linear relationship between fluorescence intensity and concentration of exosomes ranging from 5.0×10^5 to 5.0×10^7 particle/uL for exosomes.

3.5. Selectivity evaluation

For a sensing system, a highly selective response to the target should be evaluated in the presence of other potential interferences. Therefore, the capability of this probe in discriminating the exosomes derived from different cell lines was investigated. According to reported literatures, the

expression level of PTK-7 in the CEM cells was higher than that of Ramos and HL-7702 cells. As shown in Fig. 4A, under the same concentration, the intensity from the exosomes produced from the CEM cells was much higher than that of the Ramos and HL-7702 cells and little higher than that of B16F1 cells and Hela cells. Meanwhile, a random was chosen to replace the aptamer as a control. The results showed that the fluorescence intensity was almost the same as that of the control (Fig. 4B). All these results demonstrate that this label-free and activatable strategy has a good selectivity due to the high specificity of aptamer.

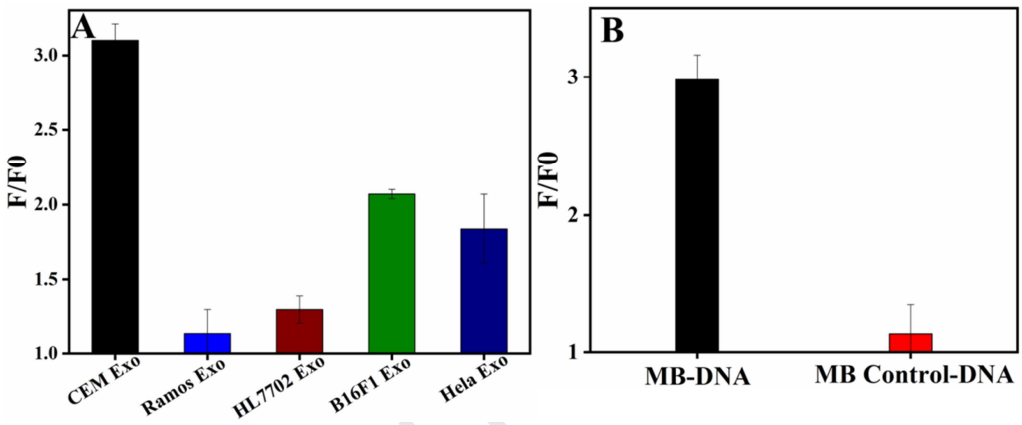


Fig. 4 (A) The specificity to exosomes derived from CEM cells, B16F1 cells, Ramos cells, Hela cells and HL-7702 cells. (B) The fluorescence intensity changes of aptamer MB and random control sequence after incubation with exosomes.

3.6. Analysis of ultracentrifuged FBS and plasma samples from clinics

To evaluate the practicality, ultracentrifuged FBS (UC FBS) was introduced as a real complex system to evaluate the performance of this method. The same number of exosomes was spiked in 10%, 20% and 30% UC FBS. As shown in Fig. 5A, the fluorescence response to target exosomes in 10%, 20% and 30% UC FBS are almost the same as that in PBS buffer solution. To further demonstrate the applicability of this assay in clinical samples, this assay was carried out in clinical plasma samples from seven human acute-lymphoblastic-leukemia patients and seven healthy individuals. As shown in Fig.5B and 5C, the fluorescence intensity of NMM in the patient plasmas is consistently higher than that in the healthy donors group. The results from plasma samples reveal that this platform can distinguish patients from healthy controls to a certain extent, which indicate that this label-free and activatable biosensor has a good performance in real sample and possesses great potential for future clinical applications.

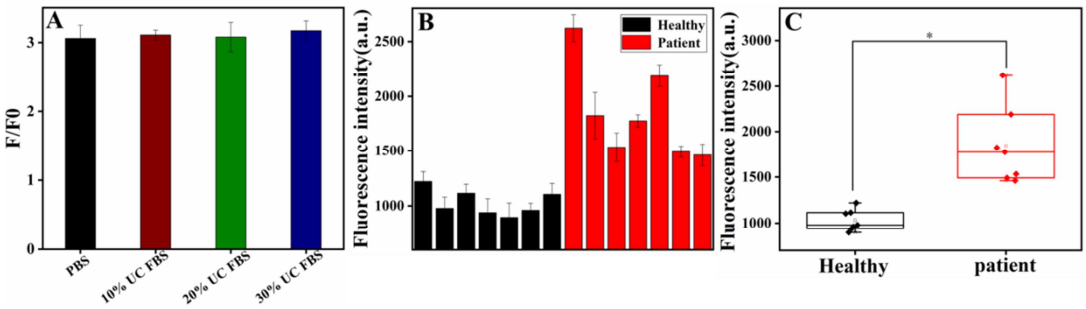


Fig. 5 (A) Detection of target exosomes in PBS and different complex samples. (B) Histogram and (C) box plots results of exosomes detection from the clinical sample.

4. Conclusion

In summary, we have constructed a unique activatable signal strategy by using a structure-switching aptamer for label-free detection of exosomes. This assay exhibits many advantages, such as high sensitivity and selectivity, low cost and easy operation. Under the optimized conditions, the developed platform has an excellent dynamic detection range from 5.0×10^5 to 5.0×10^7 particles/μL with a LOD as low as 3.4×10^5 particles/μL. Moreover, the label-free and activatable fluorescence assay can distinguish patients from a healthy group. The combination of activatable probes and other amplification technologies may produce efficient and simple sensing platforms for high sensitivity detection of diverse biological targets with low-cost instrumentation. With high sensitivity and specificity, we envisage that this diagnostic strategy for detecting exosomes may have a promising prospect in the future.

Acknowledgements

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Highlights

- The label-free and activatable strategy was first reported for exosomes detection.
- The aptamer probe exhibits high binding affinity and specificity toward exosomes.
- The sensor possesses advantages of high sensitivity, high selectivity and low cost.
- This platform can successfully distinguish patients from healthy groups.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: