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A three-dimensional multipedal DNA walker for ultrasensitive detection of tumor exosomes

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A three-dimensional (3D) multipedal DNA walker triggered by the specific recognition between aptamer and proteins was developed for ultrasensitive tumor exosome sensing. A detection limit of 1 particle μL^{-1} was obtained with high selectivity. With the advantages of simple, cost-effective and ultrasensitive, the biosensor offers potential for the early clinical diagnosis of tumors.

Exosomes (EXs) are small extracellular vesicles (EVs) with sizes distribution of 30–150 nm, which are secreted from various cells by exocytosis and circulate in most body fluids.[1] They carry much key molecular information (e.g., nucleic acids, mRNA, microRNAs and protein) from originating cells and transport them to recipient cells via cell membrane fusion, which is important in intercellular communication.[2] Compelling evidence elucidated that tumor EXs reflect plenty of information about various physiological conditions and facilitate tumor progression and metastasis.[3] Besides, owing to their high expressions in body fluids as well as minimal invasiveness during sample collection, EXs are emerging as a promising noninvasive biomarker in liquid biopsy.[4] Conventional detection methods for EXs are enzyme-linked immunosorbent assay (ELISA), western blot and mass spectrometry. Currently, many other new approaches have been developed for EXs detection, including fluorescence, colorimetry, nanoplasmonic sensors, electrogenerated chemiluminescence (ECL), etc.[5] However, most of these methods are restricted by their tedious, time-consuming operations and low sensitivity. Considering the relatively low concentrations of EXs in the early stage of the disease, especially tumors, the development of highly sensitive detection methods for EXs is still a great challenge.

DNA walker, which is based on the flexibility, specificity and predictability of Watson-Crick base pairing, can autonomously travel on the designed nanoscale tracks to conduct the amplification of signals.[6] As a powerful molecular nanomachine, DNA walker has shown its great application potential in sensing analysis of EXs. For example, Xu et al. achieved the sensitive electrochemiluminescence (ECL) detection of tumor EXs with a detection limit of 60 particle μL^{-1} by aptamer-binding DNA walker, which moved continuously and controllably on two-dimensional (2-D) electrode surface with the help of Nb.BbvCI nicking endonuclease.[7] Compared with the 2D surface, DNA walker conjugating DNA substrates on three-dimensional (3D) nanomaterials (e.g., gold or magnetic nanoparticles) possess a better amplification efficiency due to their high surface-to-volume ratio and large capacity.[8] By employing the magnetic bead (MB) as a 3D scaffold, together with two aptamers targeting CD63 and (epithelial cell adhesion molecule) EpCAM as the capture and recognition probes, Zhang's group developed a one-leg DNA walker for ultrasensitive analysis of tumor EXs by coupling exonuclease III (Exo III)-assisted recycling.[9] The detection sensitivity (a detection limit down to 13 particle μL^{-1}) was indeed obviously improved by this proposed 3D DNA walker, yet the walking steps with one-leg were not sufficient, which was not favorable for the development of high-efficiency biosensors.

Recently, multipedal DNA walker has gained extensive attention because of its high efficient automatic walking. By using avidin to simultaneously immobilize four DNA legs, Ellington's group demonstrated that DNA walker with tetra-legs appeared to have higher adherence to 3D microparticle as well as a longer proceeding, which resulted in higher analytical performance compared with one-leg DNA walker.[10] Therefore, various multipedal DNA walkers have been developed, including a high-speed multipedal DNA walker powered by Ribonuclease H (RNase H), AuNPs-based multipedal DNA walker and so on.[11] However, these assemblies of DNA legs require multiple separation and washing steps, tedious chemical modification and release process. Moreover, multipedal DNA walkers are rarely found in the analytical detection of EXs. Aptamer, as an emerging type of immuno-affinity moiety, is becoming an

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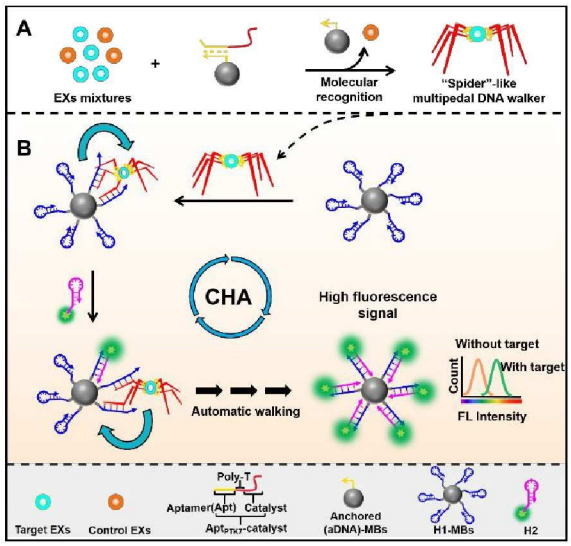

Scheme 1 Schematic diagram of aptamer recognition-based 3D multipedal DNA walker for ultrasensitive detection of tumor exosomes.

attractive molecular recognition element with potential in designing DNA walkers for EXs sensing due to their excellent merits, such as high affinity and specificity against targets, low cost, better stability, easy preparation and modification, and a wide range of target molecules.[7,9,12] Moreover, many aptamers against tumor cells can effectively recognize their EXs because of the existence of similar surface membrane proteins.[13] Therefore, multipedal DNA walker integrating targeted molecular recognition of aptamers, as an ideal alternative, is supposed to be beneficial for higher sensitivity.

Herein, we have developed an aptamer-binding 3D multipedal DNA walker for ultrasensitive detection of tumor EXs using enzyme-free signal amplification technology of catalyzed hairpin assembly (CHA). As depicted in **Scheme 1**, the mechanism of this 3D multipedal DNA walker involved two steps: the construction of multipedal DNA walker by conformational switch of aptamer and 3D movement of multipedal DNA walker by catalytic recycling. As a proof of concept, CEM (human acute lymphoblastic leukemia) EXs with a high abundance of PTK7 (protein tyrosine kinase 7) proteins, a protein closely associated with tumors, are chosen as model targets.[13,14] In this strategy, PTK7-targeting aptamer tailored at the 5' end of a catalyst by a poly-T linker, which was referred to as Apt$_{PTK7}$-catalyst, is first trapped by partial hybridization with anchored DNA (aDNA) conjugated on micrometer-scale sepharose beads (MBs). MBs were used here to capture a large number of PTK7 aptamers. Upon addition of the target EXs, Apt$_{PTK7}$-catalysts were released from MBs by the stable binding between PTK7 receptors and PTK7 aptamer. Since PTK7 receptors are over-expressed on CEM EXs,[13, 14] per CEM EXs would be able to bind more Apt$_{PTK7}$-catalysts effectively, during which a multipedal DNA walker just like a spider was constructed (part A in **Scheme 1**). After a low-speed centrifugation by vortex shaker, the multipedal DNA walkers resulted from supernatant were obtained and then applied to the next traveling. During the free-running process on MBs track, the DNA legs of the multipedal DNA walker, as initiators, could hybridize to the toehold of hairpin DNA1 (H1) conjugated on the surface of MBs (H1-MBs) and open the structure of H1 via a toehold-mediated strand exchange. The newly exposed toehold region of H1 then interacted with a toehold within 5-carboxy fluorescein (FAM)-labeled hairpin DNA2 (FAM-H2), which resulted in a DNA tripartite complex between H1, H2 and DNA legs through branch migration. As the DNA strand displacement proceeds, the DNA tripartite complex would transform into the most thermodynamically favorable configuration of H1-H2 duplex. When one leg was displaced by FAM-H2, the other leg would then search adjacent H1 to trigger the next cycle, which ensured the legs traversed directionally along H1-MBs for a long time (the continuous walking process was powered by a catalyzed hairpin assembly, abbreviated as CHA, part B in **Scheme 1**). Meanwhile, MBs, a 3D track covalently conjugated with a high density of H1, made the movement locally ordered on the MBs surface, thereby maintaining a high local concentration of H1 and accelerating the movement efficiency of multipedal DNA walker. In this way, a very small number of target EXs would initiate the cycles with more production of FAM-H2-H1 DNA duplex conjugated to the MBs, which provided amplified detection of target EXs.

Prior to EXs detection, CEM and Ramos (human Burkitt's lymphoma) EXs were chosen as model targets and controls due to a much higher level of PTK7 in CEM EXs than that of Ramos.[13] Therefore, Ramos EXs are relatively ideal controls of CEM EXs. Then, both EXs were isolated from cell culture medium, respectively, by differential ultracentrifugation according to previously described protocols.[15] The concentration of CEM EXs and Ramos EXs were measured using NanoSight, which were about 9.4×10^7 particles μL^{-1} and 2.8×10^7 particles μL^{-1}. Transmission electron microscopy (TEM) image of purified CEM EXs exhibited a typical phospholipid bilayer membrane structure with an average diameter of 110 nm (**Fig. S1A, ESI†**), which was comparable to the size determined by NanoSight. In another validation, EXs-enriched protein markers, CD-63 and Alix, which were highly expressed on the membrane and in the lumen of EXs, respectively, were clearly identified by western blotting (WB) (**Fig. S1B, ESI†**). These outcomes were well corroborated the previous evidence,[14,16] validating the high-efficiency isolation of CEM EXs.

After verifying the high purity of isolated EXs, we proceeded to apply EXs in constructing the multipedal DNA walker. With the addition of target CEM EXs, the PTK7 aptamer in Apt$_{PTK7}$-catalyst that conjugated on MBs bound the PTK7 proteins on CEM EXs surface to construct multipedal DNA walkers. After a low speed-separation, the multipedal DNA walkers from supernatant were obtained. To investigate whether the multipedal DNA walker was formed, AuNPs modified with cDNA1 (cDNA1-AuNPs) was used, which could be intuitively observed by TEM. This designed cDNA1 was completely complementary to the catalytic sequence in Apt$_{PTK7}$-catalyst. Besides, control DNA was used as a negative control. If a multipedal DNA walker was formed, there would be many cDNA1-AuNPs around the EXs because of the strong binding between cDNA and catalytic sequence. As expected, when using cDNA1-AuNPs, a large number of AuNPs were bound on EXs surface (**Fig. S1D, ESI†**). By contrast, negligible assembly was observed with control DNA-modified AuNPs (**Fig. S1E, ESI†**). These results indicated that the multipedal DNA walker could be successfully prepared due to the stable binding between aptamer in Apt$_{PTK7}$-catalyst and proteins on EXs surface. Moreover, the number of DNA "legs" on EXs was determined to be 302 molecules per EXs, which guaranteed the multipedal DNA walker would be more stable on the 3D track (**Fig. S2, Table S2, ESI†**).

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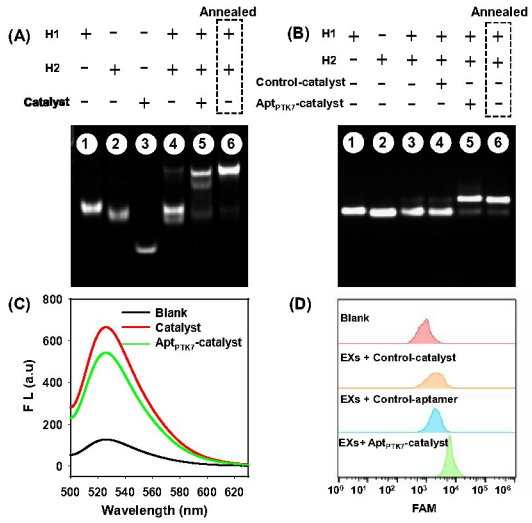

Fig. 1 Gel electrophoresis analysis of catalytic reaction after incubation with free catalyst (A) and Apt$_{PTK7}$-catalyst (B). Annealed assembly (dotted rectangle, lane 6 in A and B). (C) Fluorescence intensities of catalytic reaction after incubation with free catalyst and Apt$_{PTK7}$-catalyst. (D) Flow cytometry assay of Apt$_{PTK7}$-catalyst and control subjects (control-catalyst and control-aptamer) after incubation with CEM EXs (9.4×10^3 particles μL^{-1}). All measurements were performed in triplicate.

Therefore, the multipedal DNA walker could be directly used for the following experiments.

To explore the potential of Apt$_{PTK7}$-catalyst to propel the movement of multipedal DNA walker through CHA mechanism, the feasibilities of free catalyst and Apt$_{PTK7}$-catalyst were first validated by gel electrophoresis. As shown in **Fig. 1A**, in the absence of catalyst, a mixture of H1 and H2 did not produce a new band with lower migration (lane 4). In contrast, a newly band was formed from a mixture of H1 and H2 (lane 5) after adding the catalyst, which was consistent with the annealed assembly of H1-H2 duplex (lane 6). Having confirmed the successful CHA reaction triggered by a free catalyst, we next tested this amplification process using extended Apt$_{PTK7}$-catalyst. Gel electrophoresis in **Fig. 1B** displayed that a distinct band of H1-H2 duplex was examined with the presence of Apt$_{PTK7}$-catalyst (lane 5). Whereas, no assembly was observed with the control-catalyst, which was designed with the catalytic region of Apt$_{PTK7}$-catalyst subjected to an arbitrary change such that it did not trigger the CHA process. Furthermore, the fluorescence intensity of Apt$_{PTK7}$-catalyst was much higher than that of the blank **(Fig. 1C)**. These results indicated that the Apt$_{PTK7}$-catalyst could successfully trigger the CHA reaction. Prior to the application of Apt$_{PTK7}$-catalyst in detecting EXs, the binding force between PTK7 aptamer in Apt$_{PTK7}$-catalyst and anchored DNA over different hybridization lengths have be verified (**Fig. S3, ESI†**). In order to ensure that the target EXs had a better recognition effect, we first chose 12 bp of hybridization length to roughly test the detection capability of this method. In addition, the saturated concentrations of anchored DNA on MBs were determined to be 7.2×10^{10} units on average, which considered to be an important factor for enhancing the detection sensitivity (**Fig. S4, ESI†**). After that, we then employed Apt$_{PTK7}$-catalyst in detecting EXs. Meanwhile, two control probes, including control aptamer and control catalyst, were designed to investigate the specificity of the proposed assay. Control aptamer was designed by changing the PTK7 aptamer of Apt$_{PTK7}$-catalyst into random sequences that it had little affinity to target EXs. As a result, strong fluorescence intensity from

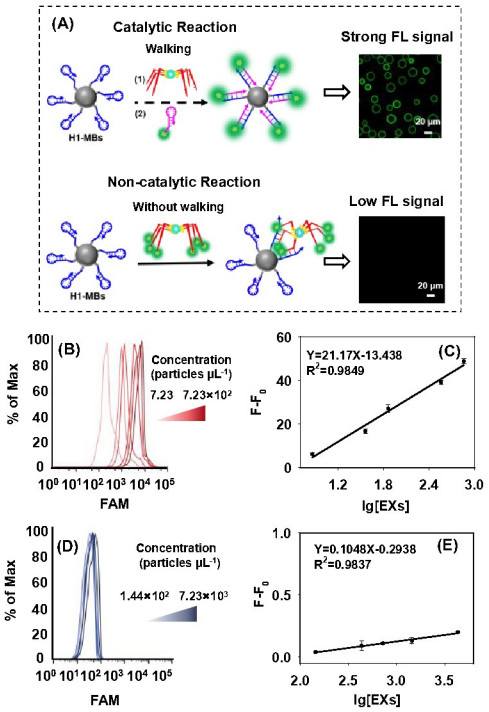

Fig. 2 (A) Schematic and fluorescence confocal image of catalytic reaction and non-catalytic reaction after incubation with CEM EXs. Fluorescence histograms of catalytic reaction (B) and non-catalytic reaction (D) over various concentrations of CEM EXs and their corresponding calibration curves (C and E). Error bars: SD (n = 3).

Apt$_{PTK7}$-catalyst was observed after the addition of CEM EXs. Conversely, control aptamer and control catalyst showed negligible fluorescence signals (**Fig. 1D, Fig. S5, ESI†**). These results proved that the prepared multipedal DNA walker had a high specificity.

To obtain the best assay performance, some critical parameters were optimized. In the EVs-triggered multipedal DNA walker system as shown in **Fig. S6-S9** (ESI†), the optimized experiment conditions were as follows: 17 bp of the hybridization length was selected for EXs recognition, concentration of FAM-H2 was 1 nM and the length of poly-T linker in Apt$_{PTK7}$-catalyst was 21 nt. Besides, the incubation temperature and walking time were 25 °C and 4 h, respectively.

Then, under the above optimal conditions, the proposed DNA walker was employed for the quantitative analysis of CEM EXs. Fluorescent confocal images demonstrated the higher amplification efficiency of the proposed multipedal DNA walker than that of non-catalytic reaction (**Fig. 2A**). Meanwhile, the comparison of detection performance between catalytic multipedal DNA walker and non-catalytic reaction after adding different concentrations of EXs was carried out. As depicted in **Fig. 2B and D**, with increasing amounts of EXs, the fluorescence signals from the DNA walker improved remarkably, whereas a tiny enhancement was obtained by the non-catalytic reaction. **Fig. 2C** and **E** showed their corresponding calibration curves. The limit of detection (LOD) of the catalytic multipedal DNA walker and non-catalytic reaction were determined to be 1 and 66 particles μL^{-1}, respectively, based on $3\sigma/S$, in which the LOD of DNA walker increased by a factor of about 66-fold to that of non-catalytic reaction, generating nearly two orders of magnitude magnification ($LOD=3\sigma/S$, σ=standard deviation of the blank probe values, S=slope of the calibration curve). Additionally, the detection limit of our developed method has been compared with other

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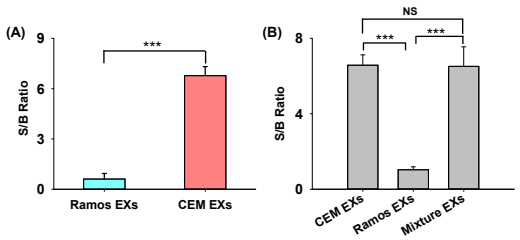

Fig. 3 (A) Selectivity studies of the DNA walker toward target CEM EXs and control Ramos EXs. (B) Investigation of detection performance of the DNA walker after incubation with CEM, Ramos and Mixture EXs. Mixture EXs: 7.23×10^3 particles μL^{-1} CEM EXs + 5 × 7.23 × 10^3 particles μL^{-1} Ramos EXs. Compared with the Ramos EXs group, ***P<0.001. Compared with the CEM EXs, NS: P>0.05. Error bars: SD (n = 3).

previously reported biosensors, suggesting its high sensitivity with good potential in early diagnosis of tumors (**Table S3, ESI†**).

We then evaluated the selectivity of the proposed assay. As shown in **Fig. 3A**, the fluorescent intensity after incubation with CEM EXs was significantly increased, while control Ramos EXs exhibited a weak fluorescence intensity, suggesting the PTK7 expression on CEM EXs was much higher than those from Ramos EXs. This result was consistent with the previous report.[13,14] Furthermore, the mixture of CEM EXs and five times concentrations of Ramos EXs presented a similar fluorescence intensity with that of CEM EXs, indicating that the detection ability of this method was almost unaffected even in the mixtures of higher concentrations of control EXs (**Fig. 3B**).

Given the challenge of the practical application of this multipedal DNA walker, ultracentrifuged EXs-free fetal bovine serum (UC FBS) was chosen as a mimetic biological environment. As shown in **Fig. S10**, the S/B ratio in 5%, 10%, 30% and 60% UC FBS were almost the same as those in buffer, suggesting that the strategy was suitable in a complex environment. Then, we further determined the LOD of the method in 5% UC FBS (**Fig. S11, ESI†**). It was nearly 3 particles μL^{-1}, which was a little higher than that in buffer, indicating the binding affinity of aptamer will be affected to some extended in the complex samples. Besides, the recovery experiment was carried out by spiking different amounts of CEM EXs in 60% serum sample. Good recoveries (79.31 to 98.71%) and low relative standard deviations (RSD) (3.09 to 8.75%) were received **(Table S4, ESI†**). Compared with some reported literatures in higher-fold diluted serum,[2a,17] this strategy had a better recovery. To further test the clinical use of this DNA walker, human serum was collected from leukemia patients (n =2) and lymphoma patients (n = 4) (**Fig S12, ESI†**). Significance analysis of the fluorescence signal between two groups was performed, implying its high feasibility and reliability of this method in clinical samples. Furthermore, by changing the PTK7-target aptamer into MUCI (Mucin 1)-target aptamer, this biosensor can also detect MCF-7 (breast cancer) EXs, suggesting it can be a versatile platform for sensitive detection of other targets EXs (**Fig. S13, ESI†**).

In summary, we have developed a 3D multipedal DNA walker for ultrasensitive detection of tumor EXs. Due to the high expressions of PTK7 protein on CEM EXs, the multipedal DNA walker can be easily acquired by using EXs to bind more DNA "legs" via aptamer recognition, avoiding the requirement of multiple separation and washing steps, tedious chemical modification, and subsequent release process. Moreover, propelled by CHA, a single target can activate the directional autonomously movement with the production of a larger number of signal molecules, which provide a strong amplification signal with a low detection limit of 1 particle μL^{-1}. In addition, the introduction of PTK7-target aptamer offers an ideal alternative to distinguish the tumor EXs from the control EXs with high selectivity. Meanwhile, the designed multipedal DNA walker can be also employed in complex serum samples with high feasibility and reliability. Therefore, with the advantages of simple, cost-effective and sensitive, the developed biosensors display great potential in clinical applications.

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Conflicts of interest

There are no conflicts to declare.

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TOC only: Herein, we developed a three-dimensional (3D) multipedal DNA walker based on the aptamer-binding for ultrasensitive exosomes sensing.