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An Allosteric Probe Initiated Wash-Free Method for Sensitive Extracellular Vesicles (EVs) Detection through Dual-Cycle Assisted CRISPR-Cas12a

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ABSTRACT: Extracellular vehicles (EVs) are emerging as promising biomarkers for cancer diagnosis and therapy. Recognizing low abundance EVs from clinical samples in an easy-to-operate way is highly desired but remains a challenge. Herein, we established an Allosteric probe Initiated Dual-cycle amplification assisted CRISPR-Cas12a (AID-Cas) platform for sensitive detection of EVs in a wash-free way. In AID-Cas, the allosteric probe can specifically recognize and bind with target EVs, and thus initiate the following dual-cycle amplification. Subsequently, the amplified products were transcribed to generate numerous single-stranded RNAs, which could work as crRNA to trigger the *trans*-cleavage of CRISPR-Cas12a. Consequently, the proposed approach achieved a good linear response to extracted EVs in a concentration range from 10^2 to 10^6 particles/μL. Due to its high sensitivity, together with its wash-free convenience, the proposed strategy could have promising clinical potentials for early diagnosis of cancers.

KEYWORDS: Extracellular vesicles; CRISPR-Cas12a; Trans-cleavage; CD63 aptamer; Allosteric probe; Wash-free

Extracellular vesicles (EVs) are membrane structure vesicles released by a diverse of cell types. With growing evidence that these secreted EVs play an important role in many pathophysiological events of human being [1,2], such as carcinogenesis and severe metabolic disorder, their diagnostic and therapeutic exploitation is under intense investigation [3-5]. To discriminate EVs rapidly with high sensitivity in a complicated extracellular environment is becoming urgently desired.

Conventional prevalent techniques for EVs detection, such as enzyme-linked immunosorbent assay (ELISA), western blot (WB) and flow cytometry [6,7], mainly rely on the participation of EVs membrane protein specific antibodies, which is time-consuming, cumbersome, expensive. In detail, flow cytometry heavily requires expensive instruments and consumes large amounts of sample. Both WB and ELISA have encountered the intrinsic limitation of the detection sensitivity. In recent years, a diversity of target protein-based fluorescence [8,9], colorimetric [10,11], electrochemical, and electrochemiluminescence immunoassays [12,13] have been developed to cope with the problems of conventional approaches with direct identification of protein targets on the surface of EVs. Among them, fluorescence-based methods are most attractive owing to their advantages of convenient operation and commercially available fluorescent dyes. Recently, our team have developed several fluorescence-based approaches for EVs detection. In our previous attempts, we have proposed a rapid and sensitive exosome detection method by using CRISPR-Cas12a for signal amplification [10]. Nevertheless, Cas12 enzymes could only provide limited signal amplification capability which nearly

meet the requirement of differentiating EVs in extremely low abundance. Meanwhile, these fluorescence-based methods frequently encountered tedious works (nanoparticle synthesis [14], antibody conjugation [15,16], multiple washing steps etc.), which restrict their wide application in practical settings. For example, Bang-Ce Ye et al proposed a dual-signal amplification method for exosome detection via DNA dendrimer self-assembly [17]. Even through a high sensitivity was obtained, troublesome wash step to remove unwanted components is inevitable to ensure a high specificity. Therefore, it is of great importance to exploit facile and low-cost techniques capable of identifying and quantifying EVs with high specificity and sensitivity.

Aptamers (chemical antibodies) are stable DNA or RNA ligands that bind with high affinity and specificity to target antigens such as small molecules, peptides, proteins, cells, and tissues. As promising alternatives to antibodies, aptamer products are increasingly being used as research agents, diagnostics, biosensors, and tools for biomarker [18,19]. Recently Da Xing et al. proposed an allosteric probe by designing functional domains of oligonucleotide sequence to generate signal amplification after target recognition [20-22], but its sensitivity was still need to be improved. Herein, we propose an Allosteric probe Initiated Dual-cycle amplification assisted CRISPR-Cas12a (AID-Cas) approach for further improved sensitivity in EVs detection. In AID-Cas, the recognition of target CD63 positive EVs can catalytically initiates the allosteric transformation of the allosteric DNA probe, and thus trigger the dual-cycle signal amplification, generating numerous double-strand DNAs (dsDNA). The obtained dsDNA products are eventually transcribed into single-stranded RNAs, which could function as crRNA to guide CRISPR-Cas12a enzyme for target single strand DNA (ssDNA) recognition and cleavage. CRISPR-Cas12a that is composed by Cas12a enzyme and CRISPR RNA (crRNA) has recently been widely utilized for both ssDNA and dsDNA targeting [23,24]. It is also demonstrated that the activation of the Cas12a/crRNA system can nonspecifically cleave nearby ssDNA (*trans*-cleavage), which can be used as a method of signal amplification [25,26]. Therefore, the target ssDNA, whose initial fluorescence was quenched by a fluorescence donor-quencher pair, are collaterally cleaved. Consequently, the recovered FAM fluorescence after the destruction of FRET could be conveniently measured using spectroscopy. After optimizing the operating parameters, we demonstrated the feasibility of the proposed approach for quantifying EVs from both cell culture supernatant and clinical samples with extraordinary sensitivity.

EXPERIMENTAL SECTION

Reagents and materials

All the nucleic acids sequences (**Table S1**) were purified by HPLC and confirmed by mass spectrometry (Shanghai Sangon, China). Before the use, the synthesized sequences were firstly heated

to 90 °C for 10 min and gradually cooled down to 25 °C prior to any further applications. The T7 RNA polymerase, dNTPs (2.5 mM), Klenow Fragment exo, ribonucleotide (NTP) buffer, $MgCl_2$ (25 mM), RNase inhibitor, phosphate-buffered saline (PBS) solution and Nt.BbvCI nicking enzyme were all purchased from New England Biolabs (MN, US). The Cas12a enzymes were purchased from Inovogen Tech (Beijing, China). A549 cell line was obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PAGE related components were all purchased from Shanghai Sangon (Shanghai, China). The TEM imaging strategy was performed with a JEM-1400Plus. The nanoparticle tracking analysis (NTA) was performed with Nanosight NS300.

Cell culture, EVs isolation and characterization

The culture of the obtained A549 cells was in a mixture of RPMI-1640 medium with 10% fetal bovine serum (FBS), which is kept in an atmosphere with 5% CO_2 at 37°C. When the cells growth reached the 70% confluence, they were stored in a solution without any FBS for another 12 hours after being washed by PBS buffer. Afterwards, we collected the culture medium in ultra-centrifuge tubes and isolated the EVs through standard differential centrifugation separation strategy. After resuspended with PBS buffer, the size distribution of the obtained isolated EVs were characterized by TEM and NTA. The membrane proteins of EVs were characterized by Western Blot (WB) and CytoFLEX flow cytometry.

Investigation of allosteric probe-initiated hairpin assembly

The synthesized probes were dissolved in 1 × NEBuffer, containing $MgCl_2$ (10 mM), NaCl (50 mM), Tris-HCl (10 mM), DTT (1 mM), pH=7.9. Before detection, the extracted EVs were quantified by NTA method and diluted into different concentrations. Then 2.5 μL EVs was incubated with the synthesized florescent allosteric probe (5μL, 1μM) at room temperature for 30 min. Subsequently, florescence signals were detected by Hitachi fluorescence spectrophotometer F-7100 (Beijing, China) before and after the mixing of EVs. Meanwhile, the results were also recalculated by confocal microscope. In the process investigating hairpin assembly, four probes (D sequence, H1, H2, and H3 probe) with identical concentration (5μL, 5μM) were mixed and the resultant florescence were measured by Hitachi fluorescence spectrophotometer F-7100 (Beijing, China). Then the DNA extension was mixed with DNA templates, dNTP, and 1 × buffer solution containing Tris-HCl (40 mM), spermidine (2 mM), dithiothreitol (1 mM), $MgCl_2$ (11 mM) with pH 7.9 at 37 °C for 6 h. Nt.BbvCI nicking enzyme were diluted in buffer containing 10 mM Tris-HCl, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 200 μg/ml BSA and 50% Glycerol. Afterwards, the probes were mixed with Klenow DNA polymerase (2μL), dNTP (4μL) and Nt.BbvCI nicking enzyme (1μL) to perform further reaction. In the transcription process, 2μL DNA templates (2μM), 1μL T7 RNA polymerase, and 2μL NTP were mixed for 60 min.

AID-Cas for EVs detection

As for EVs detection, we mixed 1 × NEBuffer 2, 0.1 U μL^{-1} Klenow Fragment exo, 0.06 U μL^{-1} Nt.BbvCI, 200 μM dNTP and the related probes for 30 min for extension (37 °C). Then the transcription process was performed through mixing 1 ×buffer solution, 1 U μL^{-1} T7 RNA polymerase, 5 mM NTP mix, and 1 U μL^{-1} RNase inhibitor for 50 min (37 °C). Then the purified Cas12a (10 nM), synthesized ssDNA target (10 nM), florescence ssDNA reporter probe (200 nM) and the amplification product in 1 × buffer solution was mixed for 30 min (37 °C) and permitted for fluorescence measurement. prior to final detection, various enzyme concentration and incubation time were optimized.

Data analysis

Each test was independently repeated in no less than triplicate and the results were shown as mean ± standard deviation (SD). Data were then visualized using GraphPad Prism 8.0 (CA, USA). Student's t test was applied to analyze comparisons between two groups. Multiple groups were compared with one-way ANOVA and Least Significant Difference method (LDS). Differences were considered significant at values of $P < 0.05$.

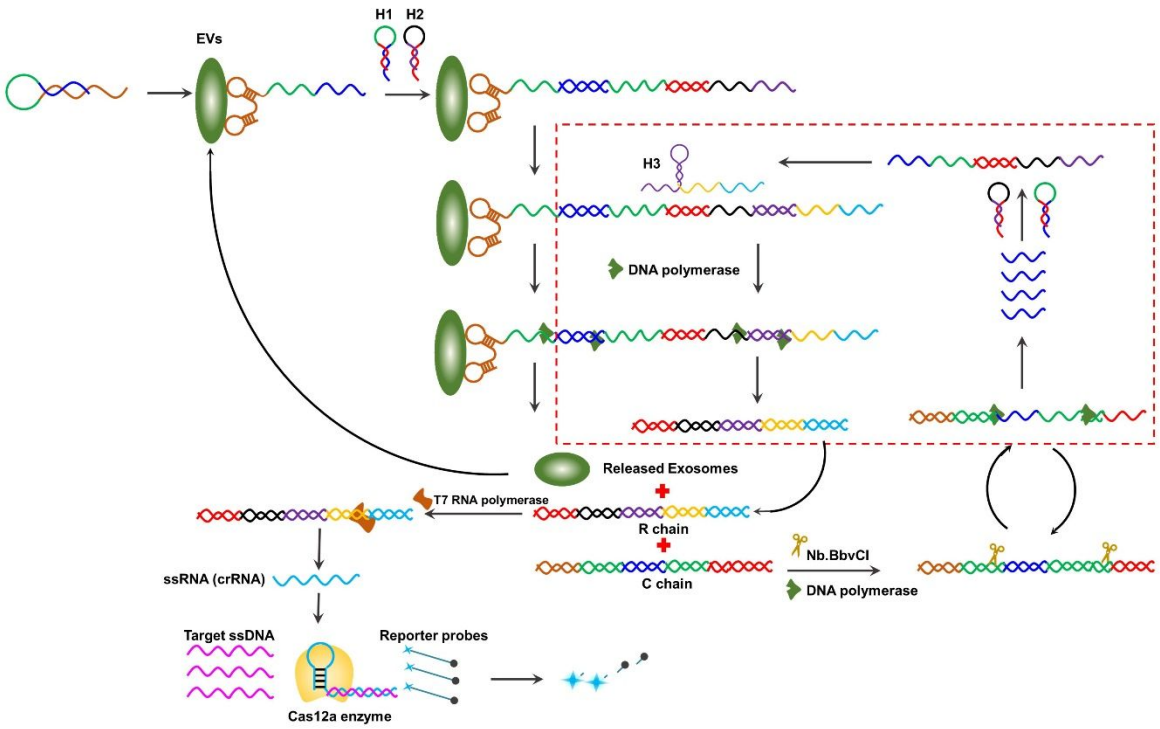
RESULTS AND DISCUSSION

Mechanism of the proposed approach

The mechanism of the proposed AID-Cas method is illustrated in **Scheme 1**. To obtain ultrasensitive EVs identification, four active components are employed: allosteric probe, H1 probe, H2 probe, and H3 probe. The allosteric probe is a ssDNA molecule composed of three functional domains: CD63 aptamer domain for EVs identification (brown), H1 initiator domain (blue) for hybridization chain reaction, and a nicking domain (green). By hybridization between the two complementary parts (underline, **Figure S1**) in allosteric probe, a hairpin structure is eventually formed after annealing. Three functional domains are designed in the H1 probe: H1 initiator binding site domain (blue), H2 initiator domain (red), and nicking domain (green). Notably, when recognizing complementary sequence (5'-CCTCAGC-3') of the nicking domain, the nicking enzyme (Nt.BbvCI) will cut it to generate a nick. H2 probe is composed by two domains: H2 initiator binding site domain (red) and H3 initiator domain (purple). The H3 probe is composed by an RNA transcription domain, a T7 promoter domain and a H2 initiator binding site domain that could transcript crRNA of Cas12a enzyme. The functional details of these designed probes are also illustrated in **Figure S1**.

When the EVs target is absent, the allosteric probe is in an inactive hairpin structure and H1 initiator domain is blocked so that the downstream reaction cannot be initiated. When the target EV is present, the CD63 aptamer domain in the allosteric probe can specifically identify and bind to the target EVs.

Therefore, the allosteric hairpin structure (inactive state) will be unwound and switched to an active state, initiating subsequent hairpin assembly by releasing the H1 initiator domain. After the catalyzed hairpin assembly between H1 and H2 probe, a DNA molecule complex consist of allosteric probe, H1 probe and H2 probe is formed and attached on the surface of EVs through CD63 aptamer. Meanwhile, the released H3 initiator domain unfold the hairpin structure of H3 probe and work as a primer for the following chain extension. Besides the H3 initiator domain, the H1 initiator domain, H1 initiator binding site domain and H3 initiator binding site domain can also function as primer for the following chain extension. Therefore, under the participation of DNA polymerase, two dsDNA chain could be produced with the following templates: Cycle chain (C chain) composed by double-strand allosteric probe and H1 probe, Reporter chain (R chain) composed by double-strand H2 probe and H3 probe. In the process of chain extension, target EVs are replaced to participate in the next amplification cycle (primary amplification) because of the polymerase extension reaction. After primer-initiated formation of C chain, Nt.BbvCI cleave the nicking domain in allosteric probe and H1 probe to generate a nick. Then, a DNA polymerase extend the 3'-end of the nicked site, which could simultaneously displace the former and newly synthesized H1 initiator domain. The released H1 initiator domains could then freely unfold H1 probes, initiating a new cycle of amplification, producing amounts of R chain. Besides the aforementioned cycles by released EVs, the Nt.BbvCI for C chain nicking and DNA polymerase for displacement constitute the second cycle of amplification. Afterwards, the T7 RNA polymerase can recognize the T7 promoter on the obtained R chain, and amplify the sequence through the transcription process to generate many single-stranded RNAs (ssRNAs). The crRNA is designed to be composed by a guide sequence which is about 21 nt in length and a recognition sequence which is about 10 nt in length. It can recognize the Cas12a protein through formation of secondary structure and thus develop the CRISPR-Cas12a sensing system. Finally, with the existing target ssDNA, the *trans*-cleavage capability of Cas12a/crRNA is initiated to cut the synthesized ssDNA reporter probes whose initial fluorescence was suppressed by a FAM-BHQ1 donor-quencher pair. As a result of cleavage, amplified fluorescence signals were generated for detection thereby. Besides the dual-cycle amplification, the RNA transcription from T7 RNA polymerase and trans-cleavage of Cas12a enzyme provide additional signal amplification and output.



Scheme 1. Working principle of the proposed strategy

Investigation of exosomes extraction and probe allosterism

To investigate the potential of proposed strategy for high-sensitive EVs quantification, we firstly isolate EVs from the culture media of NSCLC cells (A549) through differential velocity centrifugation. We then characterize the morphology of the extracted exosomes through TEM and NTA. The obtained TEM result presents a flat round shape of the isolated exosomes **(Figure 1a)**. The diameters distribution of the obtained exosomes is verified to varying from 78 nm to 280 nm **(Figure 1b)**; A peak value of 115 nm is observed from the NTA result which was consistent with the previous report. We then investigate several EVs-specific markers (CD9/CD63/CD81) which are overexpressed on the surfaces of exosomes and A549 cells though western blotting and flowcytometric analysis. **Figure 1c** and **Figure S2** clearly show a much higher levels of these specific proteins (CD9, CD63, and CD81) in the obtained NSCLC EVs than in A549 cells, while no obvious protein expression is observed in the negative control (fetal bovine serum-free RPMI-1640 medium).

To verify and visually demonstrate that the activate configuration of the designed allosteric probe can be specifically triggered through recognizing the target EVs, the allosteric probe is originally synthesized with a quenching moiety (BHQ-1) and a fluorescence (FAM) labelled at its terminals respectively, whose initial florescence is quenched due to the hairpin configuration. When recognized and bound to the extracted CD63 positive EVs, the fluorescence signal of the synthesized allosteric probe recovers (**Figure 1d**). As shown in **Figure 1e**, when mixed with extracted EVs, the fluorescence

signal (Em = 520 nm) increases to approximately 10.4 times stronger than the initial fluorescence intensity, suggesting that the allosteric probe successfully bind with EVs and switch to its active configuration. Meanwhile, neglectable fluorescence is observed in the absence of EVs, while many green fluorescence spots appear in the confocal result when EVs existed (**Figure 1f**). To verify the active allosteric probe can catalyze the downstream hairpin assembly, 12% polyacrylamide gel electrophoresis (PAGE) analysis and florescence assay are performed. In 12% PAGE, the DNA sequence (D) composed by H1 initiator domain and nicking domain of allosteric probe is synthesized to function as active allosteric probe. From the result we can see that the synthesized D sequence (band in lane 1) can unfold the H1 probe (lane 3, **Figure 1g**) and thus trigger the following hairpin assembly of H2 and H3 probe. It is worth noting that a 100~150 bp band (lane 5, **Figure 1g**) is observed appearing in the PAGE result for a solution buffer with the four probes (D+H1+H2+H3) contained, suggesting the successful hairpin assembly triggered by D sequence. The florescence assay is performed through synthesized probes (FH1, FH2 and FH3) with FAM labelled to the 3' end and BHQ-1 internally labeled to the 5' end, as illustrated in **Figure S3**. The result (**Figure 1h**) also demonstrate a successful hairpin assembly triggered by EVs recognition. Meanwhile, the H2 probe and H3 probe cannot be unfold by the H1 probe alone, suggesting their high selectivity towards the initiated H1 probe. Although previous reports have demonstrated that Cas12a enzyme can cleave dsDNA targets under the guidance of a single crRNA, the *trans*-cleavage activity of Cas12a triggered by ssDNA recognition remains elusive. Herein, we investigate the *trans*-cleavage activity of Cas12a by employing Cas12a to recognize synthesized short ssDNA and cleave the FAM-BHQ1-labeled reporter probes. **Figure 1i** shows that the average fluorescence intensity of the target is ~7-fold higher than that of the nontarget sample, suggesting high-efficiency *trans*-cleavage catalyzed by ssDNA recognition by Cas12a.

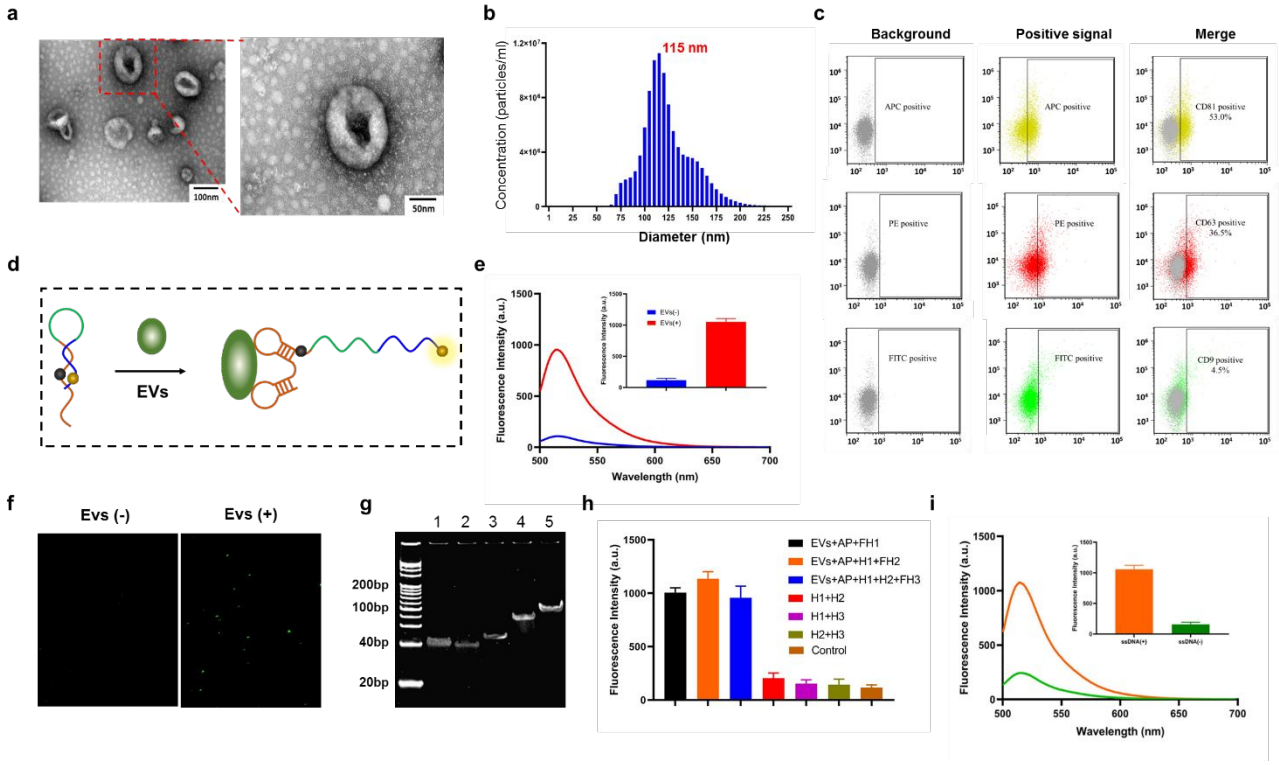


Figure 1. Investigation of EVs extraction and probe allosterism. *(a) TEM image of extracted EVs. The scale bar in the left image is 100 nm, and the right image is an enlarged section of the left image (scale bar=50 nm). (b) Histogram showing the size distribution of extracted EVs. The size of the extracted EVs ranged from 65 nm to 220 nm and peaked at approximately 115 nm. (c) Flow cytometric analysis of EV membrane proteins secreted from A549 cells. Background rates (gray) are from APC-labeled CD81, PE-labeled CD63, and FITC-labeled CD9. Pseudocolors in the picture indicate various positively stained EVs (yellow for CD81, red for CD63, and green for CD9). (d) Illustration of allosteric probe recognizing EVs. (e) Florescent spectrum of allosteric probe when EVs existed or not. Inserted is a histogram of the fluorescence intensity of the corresponding group at Em = 520 nm. (f) Confocal illumination result of allosteric probe when EVs existed or not. Scale bar = 1μm. (g) PAGE result of hairpin assembly. lane1: H1 probe; lane2: D sequence; lane3: D sequence+H1 probe; lane4: D sequence+H1 probe+H2 probe; lane5: D sequence+H1 probe+H2 probe+H3 probe. (h) florescence assay results of allosteric probe-initiated hairpin assembly. (i) The average fluorescence intensity of CRISPR-Cas12a when target ssDNA existed or not. Data are represented as the means ± SDs (n = 3).*

Optimizations of experimental parameters

We subsequently optimize the concentration of the designed allosteric probe, which plays a vitally catalytical role in the hairpin assembly, to improve the assay performance. The signal-to-background

ratio grows with the incremental allosteric probe concentration (until 0.8 μM) and begins to fall at a much higher concentration **(Figure 2a)**. In our original assumption, the reaction rate of catalytical process should be correspondingly enhanced with an increased allosteric probe concentration. However, an excessive concentration of allosteric probe induces a decreased amplification efficiency which might be explained by the formation of dimers contrarily blocked primer binding [20]. Consequently, 0.8 μM is taken as the most appropriate allosteric probe concentration for the following research. Besides, we optimize the incubation time of dual cycle amplification and RNA transcription. As shown in **Figure 2b**, no more significant signal-to-background ratio increasement for dual cycle amplification is observed after 30 min incubation. Meanwhile, the most appropriate incubation time of RNA transcription is 50 min when it will obtain enough signal-to-background ratio (**Figure S3**). After that, we verify the optimized concentration of the three included enzymes (DNA polymerase, Nt.BbvCI nicking enzyme, T7 RNA polymerase), respectively. Eventually, the optimal concentration of DNA polymerase, Nt.BbvCI nicking enzyme and T7 RNA polymerase are 0.1 U μL^{-1}, 0.06 U $\mu L^{-1}$and 1 U μL^{-1}, respectively (**Figure 2c, 2d and S4**).

Additionally, we optimize the incubation time of CRISPR-Cas12a by measuring differences in the signal-to-background ratio with increasing time. **Figure 2e** demonstrates that the signal-to-background ratio increases with time until peaking at a CRISPR/Cas12a incubation time of 10 min. No additional signal-to-background ratio is observed even when the incubation was extended an additional 15 min, revealing that a 10-min incubation is enough for Cas12a enzyme to reach cleavage equilibrium. Concerns regarding the off-target effects of CRISPR-Cas12a are raised prior to their large-scale clinical implementation. Among all the possible solutions to decrease these off-target effects, optimizing the complementary length of target ssDNA with crRNA plays an essential role in lowering these risks. In this experiment, we investigate the ability to active *trans*-cleavage abilities of 6 target ssDNA sequences (mat11, mat10, mat9, mat8, mat4 and mat0) that had 11,10, 9, 8, 4 and 0 bases totally complementary with the recognizing region of the designed crRNA. **Figure 2f** reveals the presence of mat10 could trigger *trans*-cleavage of CRISPR/Cas12a upon fluorescent probes, while the other four sequences (mat0, mat4, mat8, mat9) couldn't, suggesting that fully complementary with the recognizing region of the designed crRNA by 10 bases is necessary for signal amplification, ensuring highly specific target ssDNA recognition.

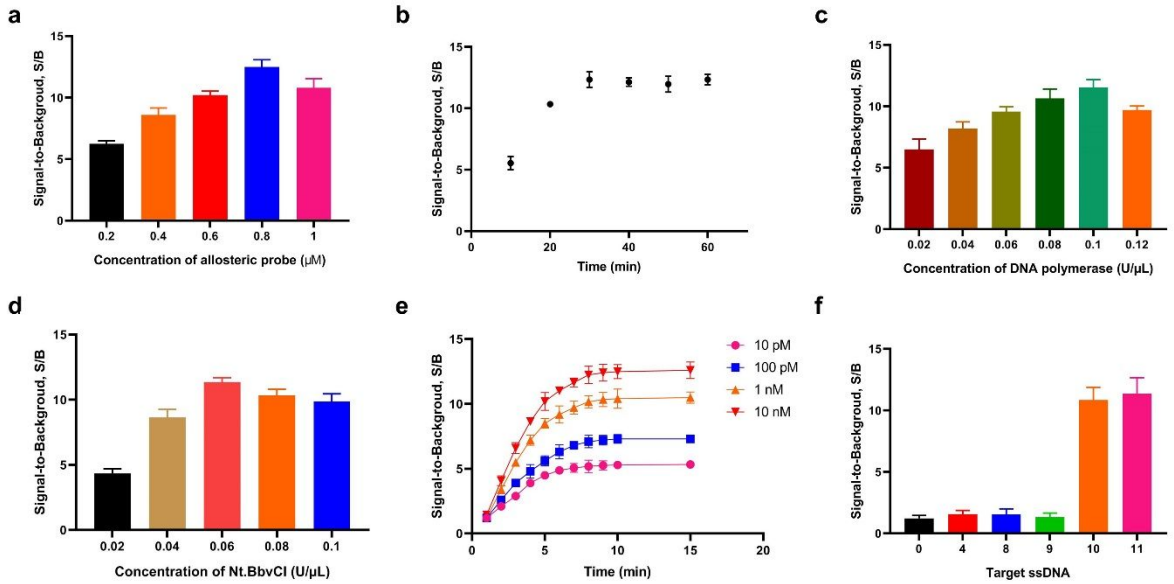


Figure 2. Optimizations of experimental parameters. *(a) Optimization of concentration of allosteric probe. (b) Optimization of the incubation time of three cycle amplification. (c) Optimization of concentration of DNA polymerase. (d) Optimization of concentration of Nt.BbvCI nicking enzyme. (e) Optimization of reporter probe incubation time with different concentration of reporter probe. Data are represented as the means ± SDs (n = 3). (f) Histogram showing florescence intensities of CRISPR-Cas12a system triggered from different target ssDNAs. Data are represented as the means ± SD (n = 3).*

Sensitive EVs detection through the proposed strategy

To verify the sensitivity of AID-Cas, the extracted EVs are quantified using NTA in advance and diluted into a series of concentrations. Signal-to-background ratio is observed linearly related to the logarithmic value of EVs ranging from 10^2 to 10^6 particles/μL (**Figure 3a**). The correlation equation between the obtained fluorescence intensity and the exosome concentration (C) was determined as Y = 4.880lg (C)-7.260 (R^2 = 0.9828; **Figure 3b**). To confirm the significance of each components in our proposed EVs sensing strategy, fluorescence assay was first performed. As shown in **Figure 3c**, a significantly high enhanced fluorescence responses are observed in the presence of extracted EVs (Figure 3c, Lines), indicating the successful initiation of AID-Cas method. The sensing system with Nb.BbvCI (Line 1) gave about 1.6 times fluorescence signal higher than that without Nb.BbvCI mediation (Line 2), which is consistent with the principle which nicking enzyme-mediated exponential cycle assist to improve the amplification efficiency. In contrast, none of the negative controls lacking

DNA polymerase, T7 RNA polymerase or Cas12a enzyme exhibit observable fluorescence signals (Lines 3-6). Then the repeatability of the proposed strategy is tested by detecting EVs for 10 consecutive repeats and the resultant signal to background showed a coefficient with a variation of 2.63%, indicating that the reproducibility of the proposed approach is enough for clinical detection (**Figure S6**). Eventually, the clinical feasibility is verified through applying both the proposed approach and NTA for the detection of EVs in collected serum samples from patients with non-small cell lung cancer (NSCLC). As expected, the results of serum EVs detected by this method are highly consistent with NTA results ($R^2 = 0.9592$, **Figure 3d**), indicating that this method has high application potential in the detection of clinical specimens.

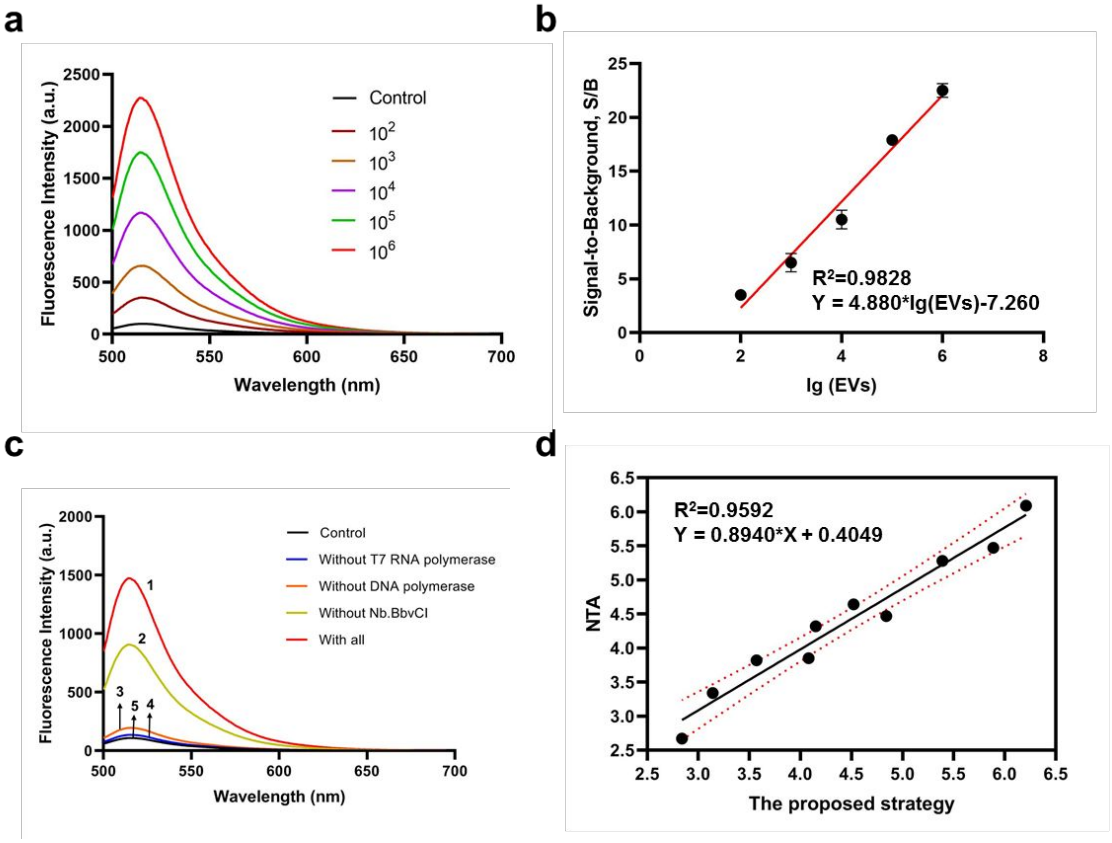


Figure 3. Feasibility of the proposed strategy. *(a) Florescence intensity of EVs with different concentrations. (b) Calibration curve of the target exosomes against measured signal to background result. Data are represented as the means ± SD (n = 3). (c) Fluorescence emission spectra of different sensing systems. (d) The fitting curves of regression analysis of NTA and the proposed method in EVs identification. n=10.*

Herein, we propose a new allosteric probe-initiated approach for high-sensitive EVs detection. By an elegantly design of dual cycle to induce signal amplification and CRISPR-Cas12a for signal output, several unique features are recognized: i) the introduction of allosteric probe greatly simplifies the

operation compared with the former reported aptamer based EVs sensing strategy; ii) under the participation of DNA polymerase, new DNA strands prolongate along the 5' -3' direction to yield dsDNAs and thus release the attached EVs for the next catalytic cycle (first cycle); iii) under the cooperation of Nt.BbvCI and DNA polymerase, H1 initiator domains are continuously produced with C chain as template (second cycle); iv) the continuously transcribed ssRNA can work as crRNA to initiate the following trans-cleavage of CRISPR-Cas12a; v) trans-cleavage activity of CRISPR-Cas12a provide the last amplification and signal output. Therefore, the proposed strategy exhibits a greatly improved amplification efficiency and EVs detection sensitivity. We then compared the principle and features of the proposed EVs sensing approach with several previously developed strategies (**Table 1**). The table showed that the proposed method has several unique advantages over conventional approaches, such as in sensitivity and easy-to-operate feature. Although the established method demonstrated a satisfactory performance in both laboratory and clinical applications, some deficiencies are potentially existed. Since not all exosomes have overexpressed CD63 protein, this method that based on CD63 aptamer for exosomes capture might have potential risk in capturing CD63-negative exosomes. Additionally, as we have illustrated in our former research, a noise signal triggered by free CD63 protein from ruptured EVs or cells may universally exist in EVs sensing strategies with merely CD63 as capture target.

Table1. Brief comparisons of various strategies for EVs sensing.

	Sensing target (s)	Signal amplification method	In vitro sensitivity	Time	Washing step	Protein interference	Ref
The proposed strategy	CD63 protein	Three cycle assisted CRISPR-Cas12a	10^2	1.5h	No	Yes	
Aptamer-Cas12a	CD63 protein	Cas12a	10^3	2h	Yes	Yes	[10]
DNA dendrimer self-assembly	CD63 protein	HDCR	10^3	More than 2h	Yes	Yes	[17]
Aptamer/AuNP Biosensor	CD63 protein	No amplification	Colorimetric	*	No	Yes	[8]
AcmPLA	CD63 protein & lipid bilayer	RCA	10^3	*	Yes	No	[11]

PLA-RPA-TMA	LMP1	RPA	10^2	1h and 50 min	Yes	Yes	[27]

"*": not mentioned. HDCR: hairpin DNA cascade reaction, RCA: rolling circle amplification, RPA: recombinase polymerase amplification

CONCLUSIONS

In summary, we report here a high-sensitive EVs detection method by allosteric probe-initiated dual cycle amplification assisted CRISPR-Cas12a. The proposed method stands out for its easy-to-operate feature and high sensitivity ensured by dual-cycle amplification assisted CRISPR-Cas12a. As a result, this method demonstrated a detection range of 10^2-10^6 particles/uL without the use of ultracentrifugation and can complete EVs detection within 1.5 hours. We hopefully expect that this strategy will provide a brand-new approach to the biological analysis of living systems, thus contributing to the diagnosis of diverse human diseases.

AUTHOR INFORMATION

Conflict of Interest: The authors declare no conflict of interest

ASSOCIATED CONTENT

Supporting Information: The Supporting Information is available free of charge on the ACS Publications website. The sequences of oligonucleotides used in this work; Functional details of allosteric probes; Western blot result of extracted EVs; Illustration of florescence assay; Optimization of RNA transcription time; Optimization of concentration of T7 RNA polymerase; Signal to background ratio result of EVs detected by the proposed strategy over 10 duplicates.

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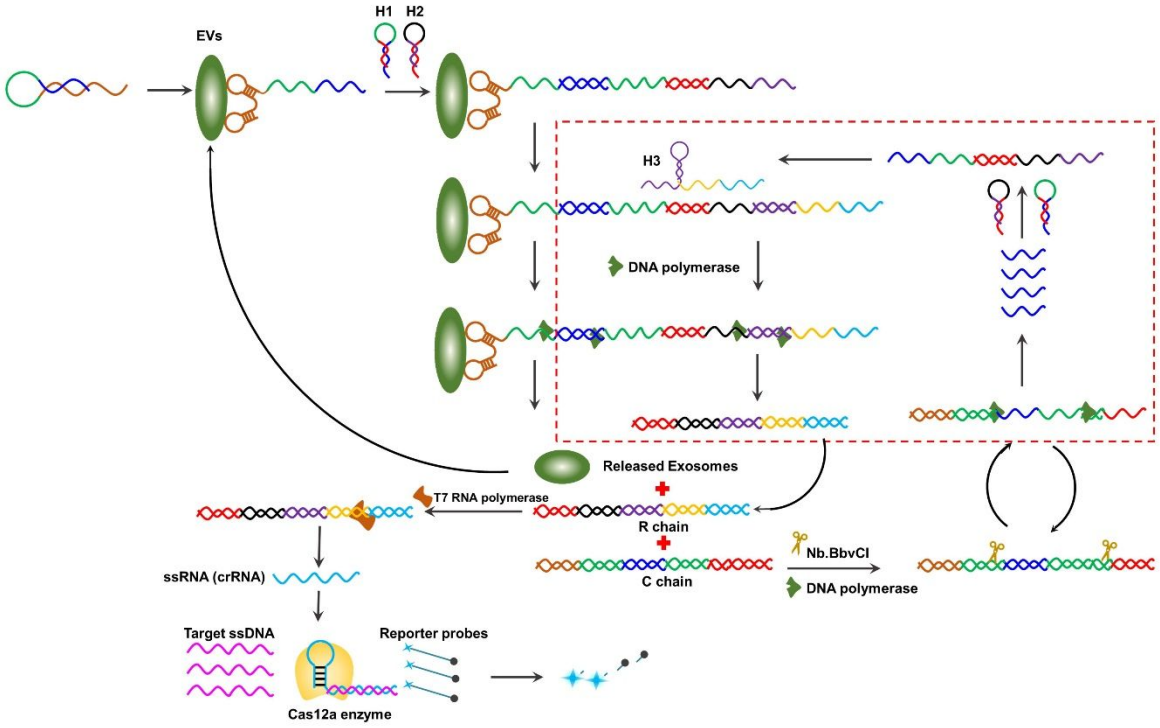
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