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Extraction-free, one-pot CRISPR/Cas12a detection of microRNAs directly from extracellular vesicles†

He Yan,[a] Song Han,[b] Steven Hughes[b] and Yong Zeng *[acd]

Current methods for extracellular vesicle (EV) miRNA analysis mostly require RNA extraction, which results in a multi-step, time-consuming workflow. This study reports an extraction-free method that combines thermolysis treatment of EVs with a one-pot EXTRA-CRISPR assay, enabling the vastly simplified analysis of EV miRNAs with a comparable performance to that of the extraction-based assays.

MicroRNAs (miRNAs) are a class of small non-coding endogenous RNAs that are involved in gene expression regulation *via* post-transcriptional messenger RNA (mRNA) suppression or degradation.[1] The understanding of miRNA's biological function has revealed their significant involvement in the development of diseases such as cancers.[2] Initially studied in tissues, miRNAs have also been identified in various body fluids such as blood and urine,[3] making the detection of circulating miRNAs a promising strategy in liquid biopsy for cancer diagnostics.[1,2] Extracellular vesicles (EVs), which play important roles in intercellular communication, offer valuable insights into physiological and pathological processes underlying diseases and have emerged as one of the most promising and rapidly evolving candidates in liquid biopsy.[4] EVs are considered as one of the major carriers of circulating miRNAs with high stability, and many studies have highlighted the potential clinical value of EV miRNAs.[5]

Since EV miRNAs are encapsulated in lipid membranes, efficient EV lysis and RNA extraction processes are required for accurate miRNA quantification. Chemical lysis of EVs using chaotropic salts is commonly used in bench-top protocols and has been integrated on chips,[6] however, it requires multiple steps for RNA extraction and the removal of interfering molecules, which is time-consuming. Besides, physical lysis methods such as acoustic wave lysis[7] and electric field lysis[8] have also been deployed in the EV lysis, but they require specialized devices and increase assay complexity. In contrast, thermolysis is capable of lysing lipid membranes by a simple and fast heating process without introducing potential amplification inhibitors or requiring complex instruments,[9–11] thus, it can remarkably simplify the detection of miRNAs in EVs.

We recently developed a CRISPR-based one-pot assay termed "EXTRA-CRISPR" (Endonucleolytically eXponenTiated Rolling circle Amplification with CRISPR-Cas12a) for the robust detection of miRNA.[12] EXTRA-CRISPR is a novel strategy that simultaneously leverages the *cis*-cleavage and *trans*-cleavage activities of Cas12a, where the *cis*-cleavage transforms the conventional linear RCA to the exponential amplification, and the *trans*-cleavage enables amplicon detection and signal generation. In this work, we sought to combine the EXTRA-CRISPR assay with a thermolysis-based sample-pretreatment method to enable the extraction-free, one-pot detection of miRNAs in small EVs (sEVs, 50–150 nm, also termed "exosomes") with a vastly streamlined workflow.

For the extraction-free detection of sEV miRNAs, the isolated sEVs are thermally lyzed for the release of miRNAs, followed by the one-pot EXTRA-CRISPR assay (Fig. 1A). Our assay is a tri-enzymatic cascade that contains padlock ligation, rolling circle amplification (RCA), and Cas12a-based nucleolytic cleavage (Fig. 1B) with both linear and exponential amplification mechanisms. Upon bridging the padlock by miRNA and mixing whole reagents, SplintR ligase starts to ligate the nick in the padlock to form a circular probe, followed by the initiation of RCA by Phi29 polymerase and the generation of RCA product. Then, ribonucleoprotein (RNP) will be activated by the RCA product and cut ssDNA reporters *via* its *trans*-cleavage function to produce the fluorescence signal, which is a linear process. Meanwhile, RNP can cut the RCA product into small fragments *via* its *cis*-cleavage function. These fragments contain single or multiple repeated sequences complementary to the padlock. They can trigger secondary ligation, RCA,

[a] *Department of Chemistry, University of Florida, Gainesville, FL 32611, USA. E-mail: zengy@ufl.edu*
[b] *Department of Surgery, University of Florida College of Medicine, Gainesville, FL 32610, USA*
[c] *J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL 32611, USA*
[d] *University of Florida Health Cancer Center, Gainesville, FL 32611, USA*

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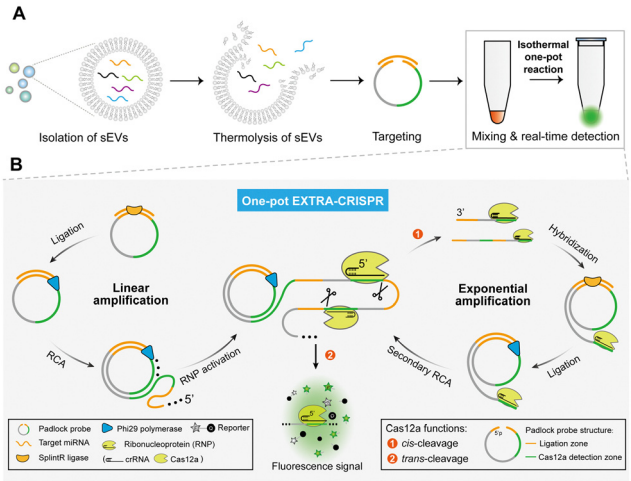

Fig. 1 Principle of the RNA extraction-free, one-pot detection of EV miRNAs. (A) Workflow that couples the thermolysis of EVs and EXTRA-CRISPR detection of miRNAs. (B) Mechanism of the EXTRA-CRISPR assay.

and signal generation, contributing to the exponential amplification of EXTRA-CRISPR.

To maximize the assay performance, we first optimized the EXTRA-CRISPR assays[12] that we initially developed to target four miRNAs associated with human pancreatic ductal adenocarcinoma (PDAC), namely miR-1246,[13] miR-21,[14] miR-451a,[14] miR-196a[13] (Table S1, ESI†). Systematic optimizations of several key parameters for the EXTRA-CRISPR assays were conducted, including the concentrations of reporter, RNP, SplintR ligase, and Phi29 polymerase, as reported in Fig. 2A–D. For example, the optimized assay conditions for miR-196a were determined to be 2 μM reporter, 1 nM RNP, 1.25 U μL^{-1} SplintR ligase and 0.1 U μL^{-1} Phi29 polymerase, considering the optimal combination of signal intensity and assay cost. This assay was performed in SplintR buffer with supplies such as dNTP and BSA, as summarized along with the protocols for three other miRNAs in Table S2 (ESI†). Fig. S1 (ESI†) demonstrates the success of RCA reaction and the *trans*- and *cis*-cleavage of the RCA amplicons by the activated Cas12a. Fig. 2E and Fig. S2 (ESI†) display the typical real-time curves obtained for the individual assays, suggesting a flexible reaction time from 20 min to 3 h depending on the target concentration.[12] Using the calibration curves (Fig. 2F), the limits of detection (LoDs) defined by the 3σ rule were determined to be 1.64 fM (5–500 fM linear range), 1.35 fM (5–500 fM linear range), 4.14 fM (5–500 fM linear range), and 7.96 fM (20–500 fM linear range) for miR-21, miR-196a, miR-451a, and miR-1246, respectively.

We then investigated the coupling of thermolysis treatment with EXTRA-CRISPR for direct detection of sEV miRNAs, using small EVs derived from PDAC cell lines. Using an ultracentrifugation (UC)-based protocol, sEVs were isolated from the culture media of PC1 cell, a patient-derived xenograft cell line of PDAC. Characterization by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) confirmed the morphological characteristics (Fig. 3A) and relatively small diameter within a range of ~50–250 nm (Fig. 3B), which are typically observed for UC-isolated EVs.[15] It is known that UC isolation, although very robust, results in significant amount of impurities in sEV isolates, such as lipid and proteins, which may adversely impact the EXTRA-CRISPR assay. Moreover, the high-level ionic species of PBS buffer used for EV isolation and storage may also affect the miRNA assay. Therefore, we first sought to mitigate these potential interfering effects by the simple dilution method. To this end, we targeted miR-451a which was found to present in PC1 sEVs at a very low endogenous level,[12] so that we can better assess the effects of the buffer and exogenous impurities in EV preparations *via* spiking in and measuring synthetic miR-451. In this experiment, PC1 sEV samples were first diluted with water to different levels and then spiked with synthetic miR-451a at the final concentration of 50 fM. Two μL of the diluted and spiked sEV samples were used for EXTRA-CRISPR detection as detailed in the Experimental Method section. Fig. 3C demonstrates that the miR-451a signal observed with the undiluted sEV sample was lower

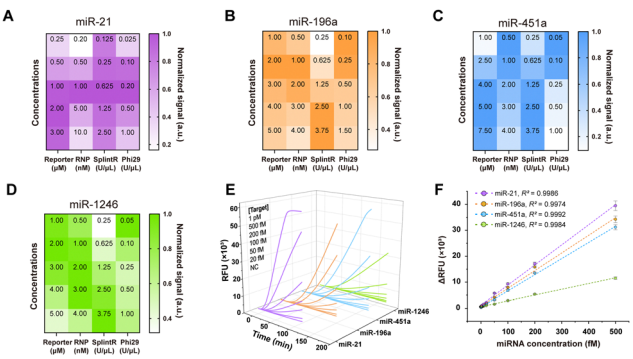

Fig. 2 Optimization of EXTRA-CRISPR assay. Optimization for the detection of miR-21 (A), miR-196a (B), miR-451a (C), and miR-1246 (D). (E) Real-time curves of EXTRA-CRISPR for miR-21, miR-196a, miR-451a, and miR-1246 with serial diluted concentrations. (F) Linear calibration curves for the four miRNAs.

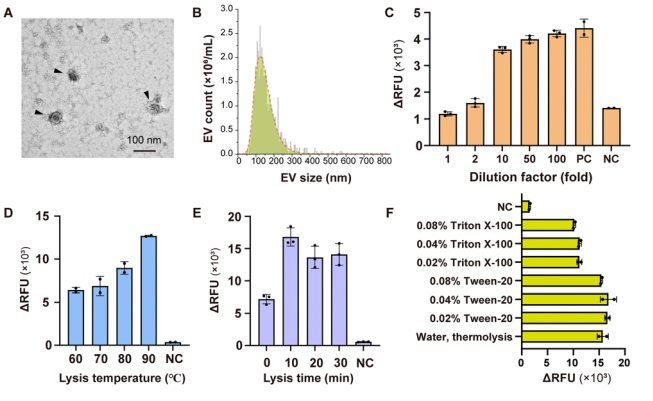

Fig. 3 Optimization of sEV thermolysis for direct EXTRA-CRISPR detection of sEV miRNAs. (A) TEM image of PC1 cell-derived sEVs isolated by UC. (B) Abundance and size distribution of PC1 EVs. (C) Effects of sample dilution on miRNA detection. (D) Optimization of lysis temperature. (E) Optimization of lysis time. (F) Effect of surfactants on sEV thermolysis and the EXTRA-CRISPR assay.

than that of the negative control (NC, the assay without miR-451a and sEVs), indicating the inhibitory matrix effects on the EXTRA-CRISPR reaction. The miR-451a signal was found to be restored by diluting the sEV preparation and reached a comparable level with that of the positive control (PC, the assay with only 50 fM miR-451a and no sEV sample) at the 10-fold dilution factor (equivalent to 81.8% of the PC signal) or higher. Despite the higher signals obtained with 50 and 100-fold dilution, such excessive dilution can vastly decrease the concentration of sEVs, thus reducing the detection sensitivity for endogenous sEV miRNAs. Hence, a 10-fold dilution of sEV preparations was adopted for subsequent experiments to suppress the matrix interferences while ensuring sensitive detection of sEV miRNA. We also tested the dilution method for sEV samples prepared from human plasma by UC. As expected, more dilution of the plasma sEV isolates (50 folds or higher) is required to substantially reduce the inhibitory matrix effects in comparison to the cell-line sEV samples (Fig. S3, ESI†), which can be attributed to the much more complex matrix of human plasma than cell culture media.

Next, we examined the temperatures for thermolysis in a range from 60 °C to 90 °C to ensure effective lysis of sEVs. As shown in Fig. 3D, higher temperatures resulted in stronger signals for detecting endogenous miR-1246 in PC1 sEVs, indicating more effective lysis of sEVs and release of miRNAs accessible for detection. Consequently, we determined 90 °C as the optimal temperature for thermolysis of sEVs, in line with the previous studies.[9,10] Subsequently, we investigated the effects of lysis time on the miRNA assay. As seen in Fig. 3E, a weak signal was detected even without sEV pre-treatment by thermolysis (*i.e.*, 0 min lysis time). This is because we employed a denaturing (80 °C, 5 min) and annealing procedure[12] to confer efficient hybridization of the padlock probe with its target, which leads to partial lysis of sEVs. The highest signal for miR-1246 was observed with incubating PC1 sEV samples at 90 °C for 10 min. Extending the thermolysis of sEVs to a longer period, such as 20 min or 30 min, was seen to suppress the detection signal, which could be attributed to the degradation of miRNAs due to prolonged exposure to heat.[16] Using NTA to compare the same sEV sample before and after thermolysis, we found that thermolysis resulted in a significant increase of particles smaller than the original vesicles in average (Fig. S4, ESI†), which are presumably attributed to the protein aggregates and membrane debris originating from EVs.

To further enhance sEV thermolysis, we have attempted to assess two surfactants commonly used for cell lysis: Tween-20 and Triton X-100. Surfactants at different concentrations were added to the sEV samples, followed by thermolysis and the EXTRA-CRISPR assay. As shown in Fig. 3F, addition of Triton X-100 in a concentration range of 0.02% to 0.08% (v/v) was seen to yield lower signal levels than that of the water-based thermolysis method, probably due to the inhibitory effect of Triton X-100 on the EXTRA-CRISPR reaction. In contrast, Tween-20 presented good compatibility with the one-pot assay but did not yield remarkable improvements in sEV thermolysis over the surfactant-free method. These results demonstrate the effectiveness of the simple surfactant-free thermal treatment method for lysis of sEV samples and its excellent compatibility with the one-pot EXTRA-CRISPR miRNA assay.

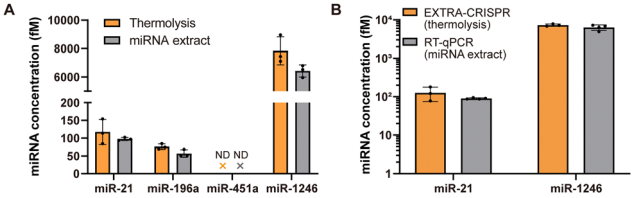

Fig. 4 Validation of thermolysis-coupled EXTRA-CRISPR. (A) EXTRA-CRISPR detection of sEV miRNAs based on the sample treatment by thermolysis and RNA extraction. (B) Comparison of miRNA expression levels in MIA sEVs determined by EXTRA-CRISPR and RT-qPCR. Thermolysis was used in the EXTRA-CRISPR assay, and total RNA extract was used in RT-qPCR reactions.

We proceeded to assess the performance of our RNA extraction-free, one-pot assay in parallel with the standard protocols involving RNA extraction. We first compared the EXTRA-CRISPR detection of sEV miRNAs without and with total RNA extraction. As shown in Fig. 4A, the thermolysis-enabled RNA extraction-free detection of four sEV miRNAs reports similar expression levels to those obtained with the total RNA extracts, suggesting the effectiveness and robustness of our thermolysis method for the EXTRA-CRISPR detection of sEV miRNAs. It was also observed that the extraction-free approach improves the detection signal over the RNA extraction-based assay (20.4% higher for miR-21, 34.2% higher for miR-196a, and 22.0% higher for miR-1246). Such improvement could be attributed to the advantage of our thermolysis method in minimizing sample loss and RNA degradation that could occur during the multi-step RNA extraction and elution procedure.

To further assess the analytical performance of our thermolysis-coupled EXTRA-CRISPR assay, we conducted the parallel comparison with a standard RT-qPCR assay involving RNA extraction for detecting two representative miRNAs, miR-21 (with a relatively low expression level) and miR-1246 (with a relatively high expression level), in sEVs derived from MIA PDAC cells. As depicted in Fig. 4B, the thermolysis-coupled one-pot assay yielded great consistency with RT-qPCR in quantifying these two sEV miRNAs (126.56 fM by thermolysis-coupled EXTRA-CRISPR *vs.* 90.13 fM by RT-qPCR) for miR-21, and 7.28 pM by thermolysis-coupled EXTRA-CRISPR *vs.* 6.35 pM by RNA extraction-coupled RT-qPCR). Together with Fig. 3, these results further verify that our thermolysis method afford effective lysis of sEVs to release miRNAs accessible for detection. Consistent with the observation described above, the miRNA concentrations measured with thermolysis-treated sEV samples were relatively higher than those by standard RT-qPCR, which corroborates the advantages of our method in simplifying sample preparation and mitigating sample loss and degradation. It is noted that UC isolation has been shown to produce lower yield and purity for EV isolation compared to many alternative approaches, including the precipitation-based methods. Direct analysis of UC-isolated EVs by the thermolysis-coupled EXTRA-CRISPR yields comparable or better analytical performance than that using the RNA extraction-

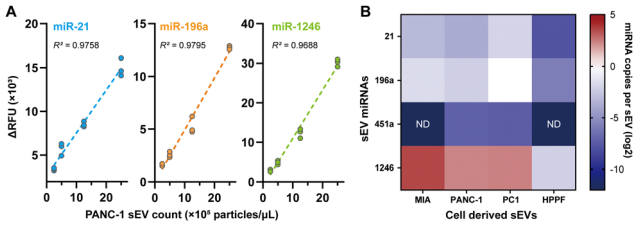

Fig. 5 Quantitative miRNA profiling of PDAC cell derived sEVs. (A) Linear relation between sEV count and signals using thermolysis-coupled EXTRA-CRISPR. (B) Expression level of miR-21, miR-196a, miR-451a, and miR-1246 in the sEVs from different cell lines.

based protocol and RT-qPCR, suggesting the robustness and broad adaptability of our approach for sensitive and quantitative analysis of miRNAs in sEV samples prepared with variable methods.

As a proof-of-concept study of potential biological applications, we adapted our assay to demonstrate quantitative miRNA profiling of PDAC-derived sEVs. We first conducted the calibration of our method for quantitative sEV miRNA analysis using sEVs derived from the PANC-1 cell line. Various sEV inputs from 2.5×10^5 to 2.5×10^6 particles per μL were tested by the thermolysis-coupled EXTRA-CRISPR assay. The detected signals showed solid linear relationship as a function of sEV count for three relatively abundant miRNAs, miR-21 (R^2 = 0.9758), miR-196a (R^2 = 0.9795), and miR-1246 (R^2 = 0.9868), demonstrating high sensitivity for quantitative detection with the LoD down to 2.5×10^5 EVs per μL (Fig. 5A). The miRNA abundance per sEV in different PDAC cell lines were further assessed by the thermolysis-coupled EXTRA-CRISPR (Fig. 5B). It was seen that three sEV miRNAs (miR-21, miR-196a, and miR-1246) were upregulated in PDAC-derived cell lines, MIA, PANC-1, and PC1, compared to the normal control, a human primary pancreatic fibroblast (HPPF) cell line. We also discovered that miR-1246 exhibited the highest abundance among the miRNAs in PDAC cell-derived sEVs, while miR-451a was undetectable in the MIA and HPPF sEVs and had very low levels in PANC-1 and PC1 sEVs (<0.007 copies per sEV). The relatively high expression levels of miR-196a and miR-1246 in PDAC cell lines are consistent with the previous study,[13] and the large span of sEV miRNA levels supports the complexity of miRNAs in biological samples. Moreover, the thermolysis-coupled EXTRA-CRISPR assay for stoichiometric analysis of sEV microRNA content yielded similar results to the extraction-based EXTRA-CRISPR method.[12] Altogether, our results suggest that the thermolysis-coupled EXTRA-CRISPR offers a sensitive and robust approach for simplified and expedited detection of miRNAs in sEVs.

In summary, we have developed a simple extraction-free technology for sEV miRNA analysis that couples thermolysis and the one-pot EXTRA-CRISPR assay, mitigating sample loss, RNA degradation, and the risk of analytical variations and cross-contaminations. Using this assay, we demonstrated quantitative detection of miR-21, miR-196a, and miR-1246 in PDAC PANC-1 derived sEVs at an input as low as 2.5×10^5 particles per μL, and we confirmed the upregulated expression of miR-21, miR-196, and miR-1246 in PDAC cells. We envision that this simple assay would provide a potentially impactful tool for EV miRNA analysis and open opportunities for developing integrated systems for highly multiplexed sEV profiling to address the needs in a broad array of fields, including biological research, clinical lab diagnosis and point-of-care testing.

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Conflicts of interest

H. Y. and Y. Z. are co-inventors on a US provisional patent application based on this work (PCT/US2023/063351). Y. Z. holds equity interest in Clara Biotech Inc. and serves on its scientific advisory board. All other authors declare no competing interest.

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