Supporting Information

In situ detection of miRNA-21 in MCF-7 cell-derived extracellular vesicles using the red blood cells membrane vesicles strategy

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EXPERIMENTAL SECTION

Chemicals and regents

All oligonucleotides were synthesized by Shanghai Bioengineering Co Ltd (Shanghai, China). Their sequences are all shown in Table S1. All of the DNA sequences were dissolved and diluted to a final concentration of 10 μM in PBS buffer and stored at 4°C until use. All MCF-7 breast cancer cells involved in the experiments were obtained from Wuhan Pronset Life Sciences Co. All other reagents were analytically pure and could be used directly without further purification. All water used in this experiment was sterilised ultrapure water.

Instrument

In the course of this experiment, the nanomaterials were morphologically characterised by transmission electron microscopy (JEM-2100, JEOL). Particle size and zeta potential were measured using a Zeta-Size Nano instrument (Zen 3600, Malvern Instruments Ltd.). Nanoparticle tracking analysis (NTA) was performed with Zetaview (Particle Metrix, Germany). Absorbance measurements for the BCA protein experiments were achieved using a BioTek Epoch full wavelength enzyme labeller, USA. Fluorescence data from the experiments were detected and recorded by an F-4600 fluorescence spectrophotometer (Hitachi). Quantitative analysis of fluorescent images using image J software.

Synthesis of DNA nanospheres

The required miRNA and three hairpin probe stocks were added sequentially to the PCR tubes according to the probe concentration required for the experiment and fixed to 100 μL with PBS buffer. after 120 minutes of reaction at 37°C, the reaction products were stored at 4°C and prepared for subsequent experimental analysis. Each reaction is preceded by annealing of the oligonucleotide chain. According to the procedures in the literature, the oligonucleotide solution was first heated at 95 ° C for 5 minutes and then slowly cooled to room temperature. [1]

Cell culture experiments

The medium used for the MCF-7 cells in this experiment was DMEM containing 10% fetal bovine serum and 1% double antibody (penicillin-streptomycin.) During the cell culture, the temperature in the cell incubator was maintained at 37°C and the CO_2 concentration was 5%.

DNA in situ self-assembly reaction for the detection of miRNA-21

In Fig. S1, a palindromic complementary sequence was added to the 3' end of each hairpin probe, which allowed the probes to be linked head to tail. These Y-shaped DNA structural units can also be linked to each other by this complementary sequence to form a larger DNA structure, and these linkages are not fixed in the same plane but are an assembly process in three dimensions, resulting in a three-dimensional DNA nanosphere. As shown in Fig. S1, miRNA-21 and the three probes are incubated together at 37°C to react. miRNA-21 can only open Probe A but not Probe B and Probe C. The next DNA self-assembly

reaction can only occur when Probe A is opened. Specifically, miRNA-21 opens the hairpin probe A-Cy5 and then forms the 21-A-Cy5 structure. A-Cy5 then hybridizes with probe B to produce a 21-A-Cy5-B triple-stranded structure containing more sticky ends. Next, C-Cy5 continues to be opened and hybridised to 21-A-Cy5-B, creating a four-stranded structure of 21-A-Cy5-B-C-Cy5. However, this RNA strand is displaced and released by the action of C-Cy5 so that it can re-engage in the next assembly reaction. This results in the formation of the A-Cy5-B-C-Cy5 Y-structure unit. Finally, a large number of Y-structural units are cross-linked by complementary sticky ends and assembled into the final DNA NS product. During this entire reaction, the opening of the A-Cy5 probe emits a fluorescent signal, while the opening of C-Cy5 enhances the fluorescent signal and enables signal amplification. To observe the DNA self-assembly process for fluorescence detection purposes, we modified the Cy5 fluorescent group and the BQH-2 quenching group on probes A and C, respectively, to obtain fluorescent probes A-Cy5 and C-Cy5, all of which are listed in Table S1.

Polyacrylamide gel electrophoresis

DNA reaction samples were subjected to 10% polyacrylamide gel electrophoresis (PAGE) at a fixation potential of 90 V, using 1 x TAE buffer as the electrophoresis buffer. The ratio of DNA solution to loading buffer was 5:1 during the sample pre-treatment phase. after electrophoresis, the electrophoresis gel was stained for 1 h using Super Red dye and then imaged on a gel imager.

Extraction of EVs

The EVs were isolated from MCF-7 cell culture supernatant by using the Cell Culture Supernatant Exosome Rapid Extraction Kit (Yisheng Biotechnology (Shanghai) Co. According to the experimental operation procedure in the literature, the normal fetal bovine serum in the cell medium was replaced with fetal bovine serum without exosomes for three passages before EVs extraction, and the extraction experiment was carried out.[2,3] Finally, the isolated exosomes were dispersed in PBS and stored at −80 °C, which was used as EVs standard for the following experiments.

Preparation of RVs

According to the preparation process in the literature, RVs with a diameter of about 200 nm were prepared by extrusion method in this study.[4,5] First, 5 times the volume of deionised water was added to the purchased 2 % mouse erythrocyte suspension, mixed and left for 1h at 4°C to allow the erythrocytes to absorb water and open up. The haemoglobin was then removed from the suspension by centrifugation at 14,000 R for 10 min, the supernatant was discarded and the bottom precipitate was washed three times with PBS. The obtained light pink RBCs precipitate was dispersed with 1 mL of PBS solution, and then the dispersion was repeatedly extruded 5 and 10 times through 0.45 μm filters and 0.22 μm filters, respectively, to finally obtain RVs with a particle size of around 200 nm. Alternatively, the RVs encapsulating the hairpin probe were prepared by co-extrusion: the hairpin probe was mixed with the RBCs suspension and

repeatedly extruded through the filter at the same time, and the free probe was removed from the extruded solution using an ultrafiltration tube.

Fluorescence measurements

Fluorescence measurements were performed on samples from the DNA self-assembly reaction to investigate the feasibility and specificity of DNA nanosphere-based miRNA-21 detection. All DNA samples were synthesized and prepared according to the previously mentioned steps, and each sample to be tested was diluted separately to 100 μL with PBS buffer for subsequent detection. The fluorescence spectra were then recorded from 650 nm to 750 nm using an F-4600 fluorescence spectrophotometer with a xenon lamp as the excitation source and 635 nm as the excitation wavelength. Prior to the fluorescence measurement experiment, the same concentrations of A-Cy5, B and C-Cy5, and different concentrations of the target miRNA-21 were added to the PCR tubes and fixed to 100 μL. After incubation at 37°C for 120 min, the fluorescence spectra were then analysed separately. However, for a more detailed analysis of the fluorescence growth trend, we also added five other groups of miRNA-21 concentrations and measured the fluorescence intensity at 20 nM, 30 nM, 40 nM, 150 nM and 200 nM.

Fluorescence confocal experiment

To further verify the successful fusion of EVs with RVs membranes, we photographed the membrane fusion effect with incubation time using confocal. We stained the previously prepared EVs and RVs with DiO (green) and DiI (red)

dyes for 20 min, and then incubated both at 37°C. Then, we took fluorescence confocal images at 0 h, 1 h and 2 h using Nikon confocal (Fig. 2), respectively. As shown in the figure, before incubation (0 h), no co-localization of red fluorescence and green fluorescence occurred. In other words, it is clear that the fusion of EVs and RVs has not yet occurred in the membrane. After incubation for 1 h, the figure demonstrates that partial fusion has occurred, but there is still some free EVs and RVs. When we continue to incubate the EVs and RVs until 2 h, almost all of the red fluorescence and green fluorescence have co-localized and resynthesized into yellow. This data set can not only prove that the success of membrane fusion of EVs and RVs, but also indicate that the optimal time for incubation of these two is 2 h.

Table S1 All DNA oligonucleotide sequences used in this work.

Name	Sequence (5'-3')
MiRNA-21	UAG CUU AUC AGA CUG AUG UUG A
A	TT TTT CAA CAT CAG TCT GAT AAG CTA GCC TTT AAT ACG TAG CTT ATC AGA TTT TTT T *ACCAAG CTTGGT*
B	T TTT GAT AAG CTA CGT ATT AAA GGC ATG ATG AAC TGA GCC TTT AAT ACG TTT TTT *ACCAAG CTTGGT*
C	TTT ATT AAA GGC TCA GTT CAT CAG TAG CTT ATC AGA CTG ATC AAC TGA GCT TTT T *ACCAAG CTTGGT*
A-Cy5	TT TTT CAA CA/iBHQ2dT/CAG TCT GAT AAG CTA GCC TTT AAT ACG TAG CTT ATC AGA TT/iCy5dT/ TTT T *ACCAAG CTTGGT*
C-Cy5	TTT AT/iCy5dT/ AAA GGC TCA GTT CAT CAG TAG CTT ATC AGA CTG ATC AAC TGA GCT TT/iBHQ2dT/ T *ACCAAG CTTGGT*
MiRNA-27	UUC ACA GUG GCU AAG UUC CGC
MiRNA-155	UUA AUG CUA AUC GUG AUA GGG GUU
MiRNA-373	GAA GUG CUU CGA UUU UGG GGU GU
SM MiRNA-21	UAG CUU AUC AGA CUG AUG UUC A
DM MiRNA-21	UAG CUU AUC AGA CUG AUA UUC A
TM MiRNA-21	UAG CUU AUC AGA UUG AUA UUC A

(**Note**: Underlined fragments indicate the own complementary part of the hairpin probe. Fragments in italics are complementary palindromic sequences and those with a grey background are fluorescent and quenched motifs modified on the probe.)

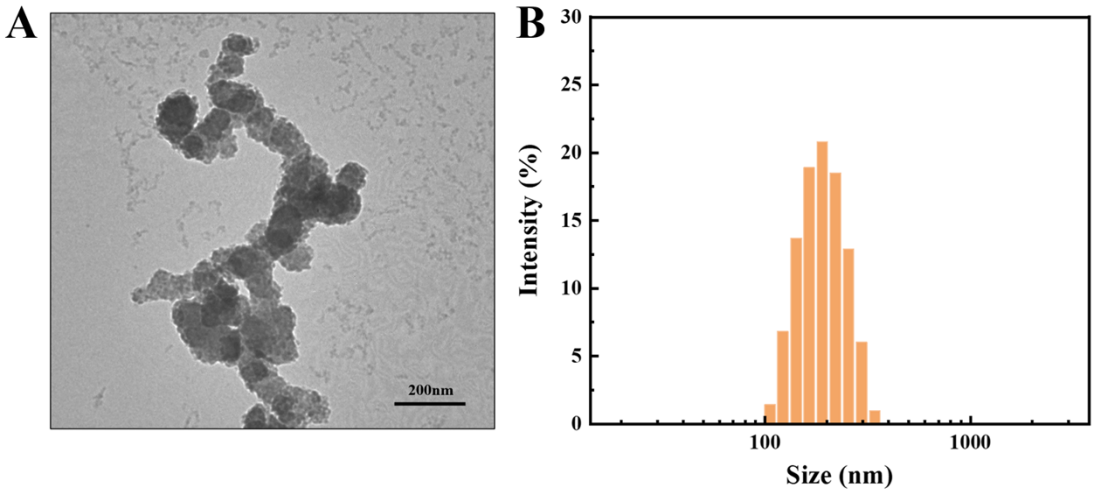

Fig. S1 TEM image (A) and particle size distribution (B) of DNA NS.

We designed three DNA hairpin probes on the base sequence of the target miRNA-21. They are named probe A, B and C. The sequences of the three hairpin probes were complementary to each other in order to form a Y-shaped DNA structural unit (Fig. S1). These Y-shaped units continue to assemble until they form a DNA nanosphere (DNA NS).

We have taken transmission electron microscopy (TEM) images of the prepared DNA NS (Fig. S1A), which show the successful synthesis of DND NS using the DNA self-assembly synthesis method, which shows that the particle size of the DNA NS is around 200 nm and appears to be spherical. In addition to this, the particle size distribution (Fig. S1B) shows a particle size of around 194.5 nm, which together with the TEM results confirms the size of the DNA NS synthesised.

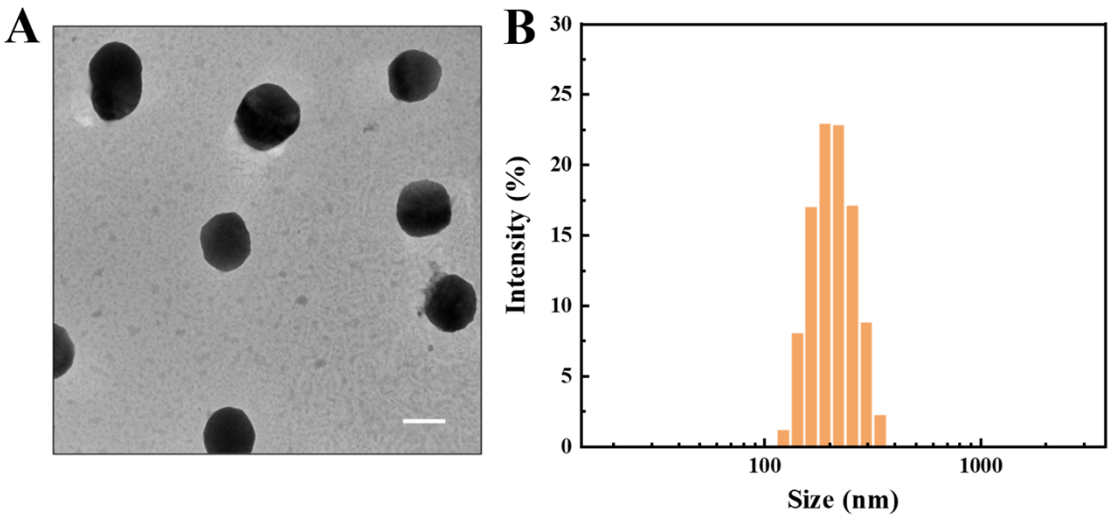

Fig. S2 TEM image (A) and particle size distribution (B) of EVs. Scale bar, 200 nm.

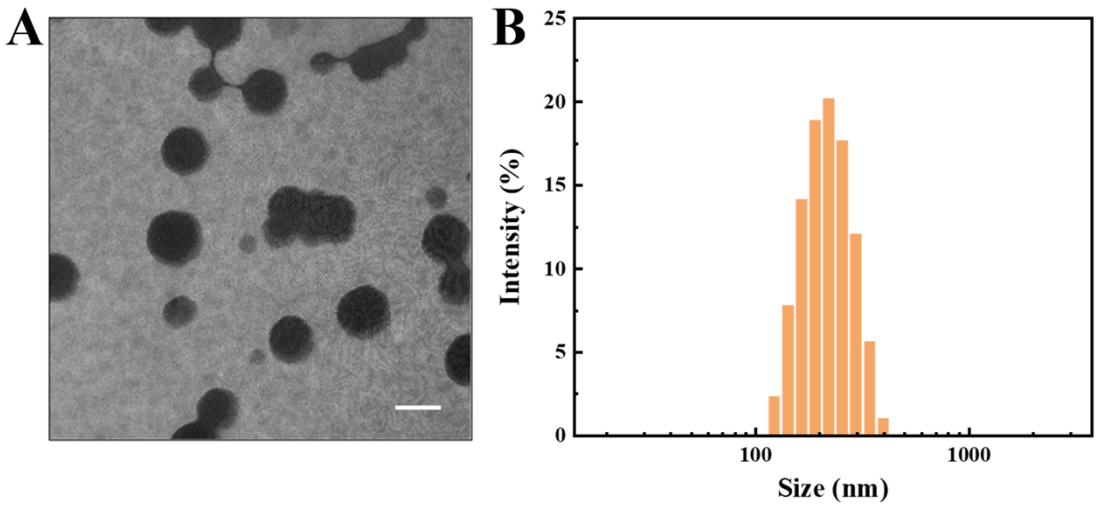

Fig. S3 TEM image (A) and particle size distribution (B) of RVs. Scale bar, 200 nm.

In this study, we extracted EVs from MCF-7 cell culture supernatants and characterised their morphology by TEM images (Fig. S2A). It can be clearly observed that the EVs are homogeneously dispersed and spherical, with a diameter of approximately 200 nm. The particle size distribution (Fig. S2B) demonstrates a particle size distribution of around 211.6 nm, which proves that the extracted EVs are homogeneous and well dispersed. In addition, TEM images

(Fig. S3A) and particle size analysis of the prepared RVs showed that the size distribution of the RVs was about 222 nm (Fig. S3B). This proved the successful preparation of RVs.

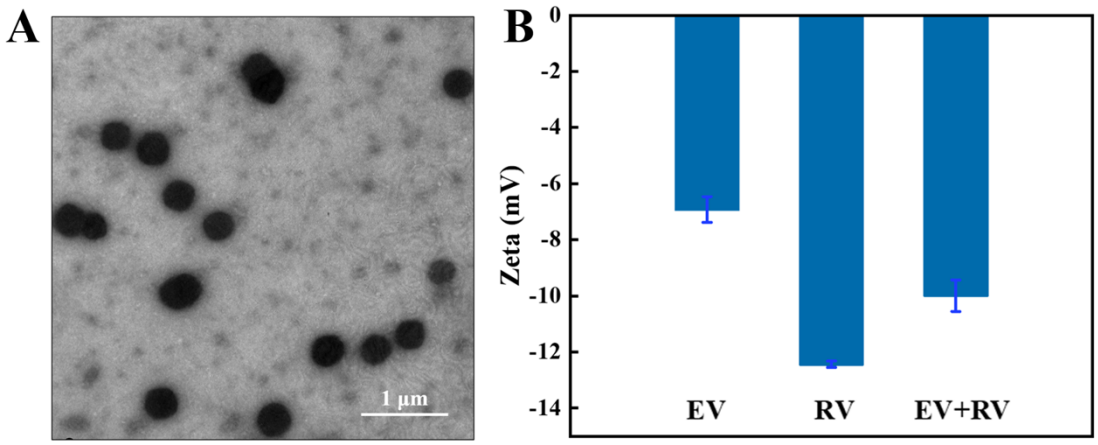

Fig. S4 (A) TEM image after incubation of EVs and RVs; (B)Images of EVs, RVs and the potentials of both after incubation.

In order to further demonstrate the successful fusion of EVs and RVs more directly, on the one hand, TEM image was performed on the vesicles obtained after EVs and RVs incubation. Prior to incubation, both EVs and RVs sizes were about 200 nm, respectively. Then EVs and RVs were incubated at 37 ℃ for 2 h, and the membrane fusion products were characterized by TEM. The TEM image (Fig. S4A) showed an increase in vesicle size to about 500 nm after incubation, which was a good indication of successful membrane fusion of RVs and EVs. On the other hand, we also confirmed the success of membrane fusion through the change of the electric potential before and after incubation (Fig. S4B). Before incubation, the zeta potentials of EVs and RVs were -6.923 mV and -12.43 mV respectively, but after incubation the potentials became -9.99 mV, as the

membranes had fused and the fused membrane potential was in between the two. Therefore, this two results proves that the membrane fusion reaction between EVs and RVs occurred after incubation.

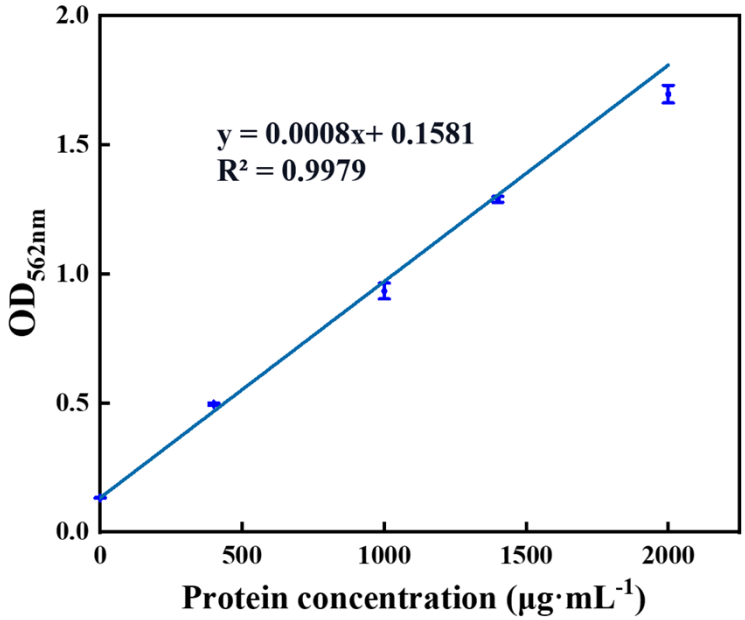

Fig. S5 Standard regression line of absorbance at 562 nm for different concentrations of protein solutions.

At the same time, in order to quantify the EVs extracted from each batch, we used the BCA protein quantification method to quantify the proteins on the EVs, thus achieving a standard quantification of the EVs so that subsequent experiments could standardise the amount of EVs used. We measured the absorbance at 562 nm for five different protein concentrations and made a standard regression line (Fig. S4) to obtain the equation for the relationship between protein concentration and absorbance. The absorbance of each new batch of extracted EVs was measured in subsequent experiments to obtain the

corresponding amount of protein contained, which also corresponds to the amount of EVs.

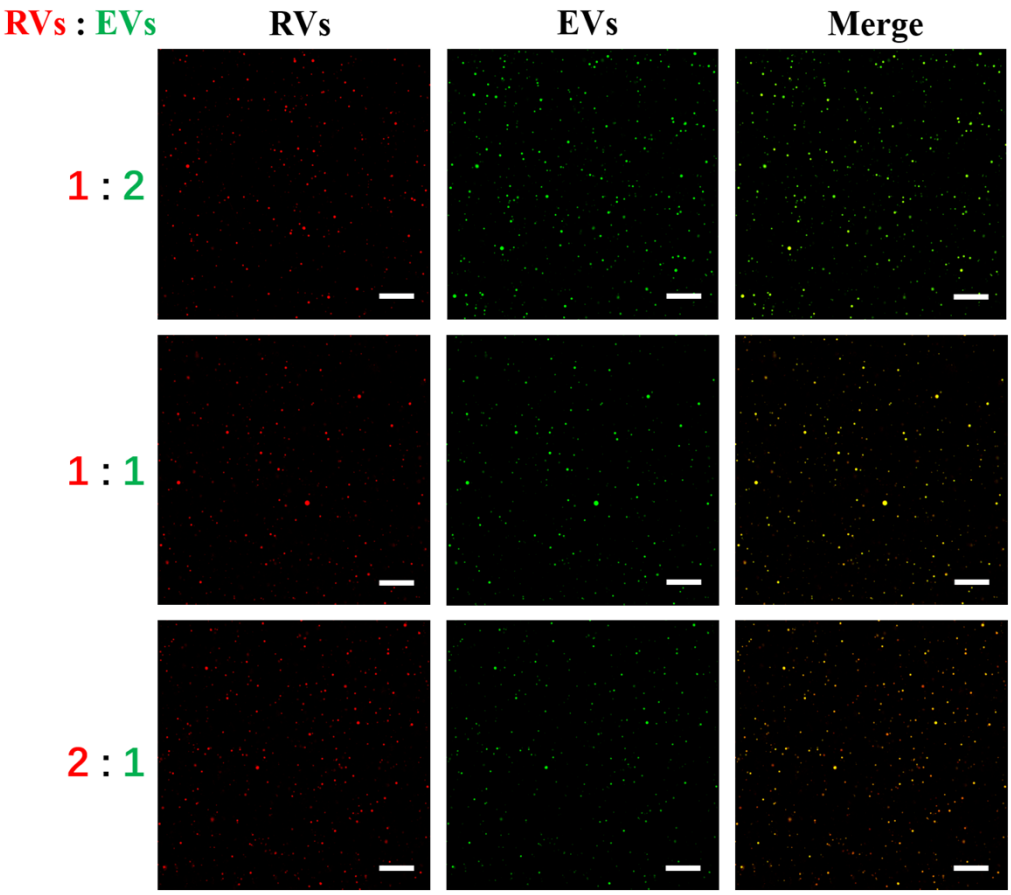

Fig. S6 CLSM images showing the fusion efficiency of the EVs with RVs at different ratios. Scale bar, 25μm.

In order to verify the ability of EVs to fuse with RVs in different quantity ratios, RVs and EVs were labeled with DiI and DiO, respectively. Fluorescence images of the fusion process were obtained using Nikon confocal microscopy. As shown in Fig. S6, the efficiency of membrane fusion varies after incubation of reactants in different proportions for 2 hours. The experimental results showed

that when the ratio of RVs to EVs was 1:1, the fluorescence recombination of red and green was higher, and the fusion efficiency of the two was maintained at a higher level.

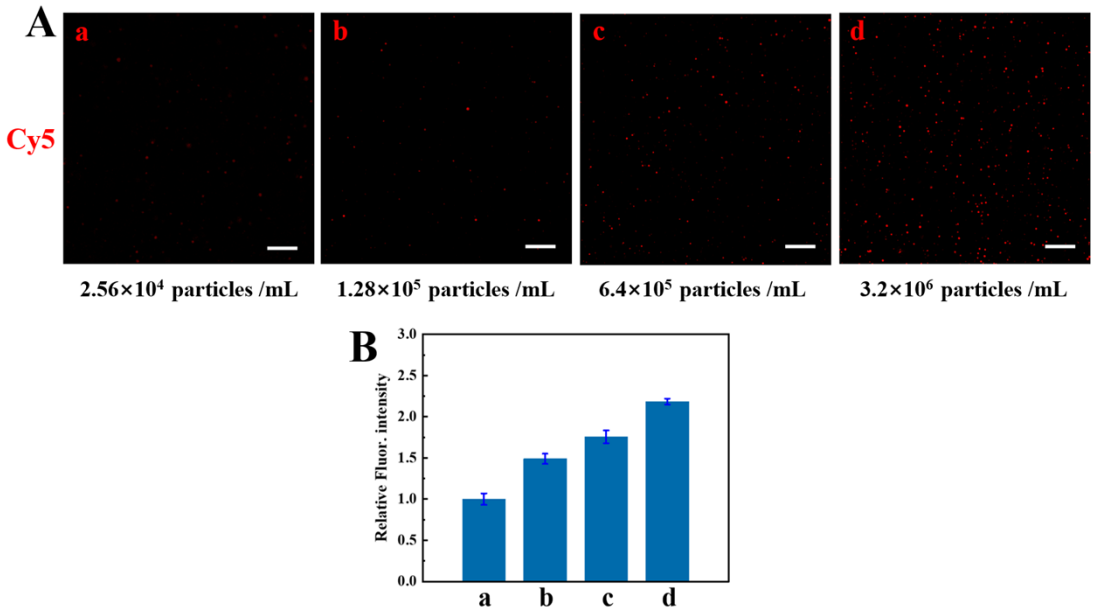

Fig. S7 In situ detection of miRNA-21 induced fluorescence confocal imaging image within EVs. Scale bar, 25μm. (A) Confocal fluorescence image of miRNA-21 in situ detection after adding different amounts of EVs;(B) Histogram of the quantitative analysis of fluorescence intensity for the four experimental groups within Fig. S7A.

It is evident from Fig. S7A that, from left to right, the number of fluorescent bright spots produced by Cy5 on the DNA probe increases sequentially as the concentration of added EVs particles increases. This is because the more EVs added, the more DNA self-assembly reactions occurred between miR-21 and the probe after membrane fusion, resulting in more red fluorescent spots. In situ fluorescence imaging was performed to detect miR-21 using the strategy of

erythrocyte membrane fusion. In addition, we also used image J software for quantitative analysis of fluorescent images and compared the fluorescence intensity of the four experimental groups. The corresponding relative fluorescence intensity in Fig. S7B can also prove the trend that the number of fluorescent dots is positively correlated with the number of EVs added.

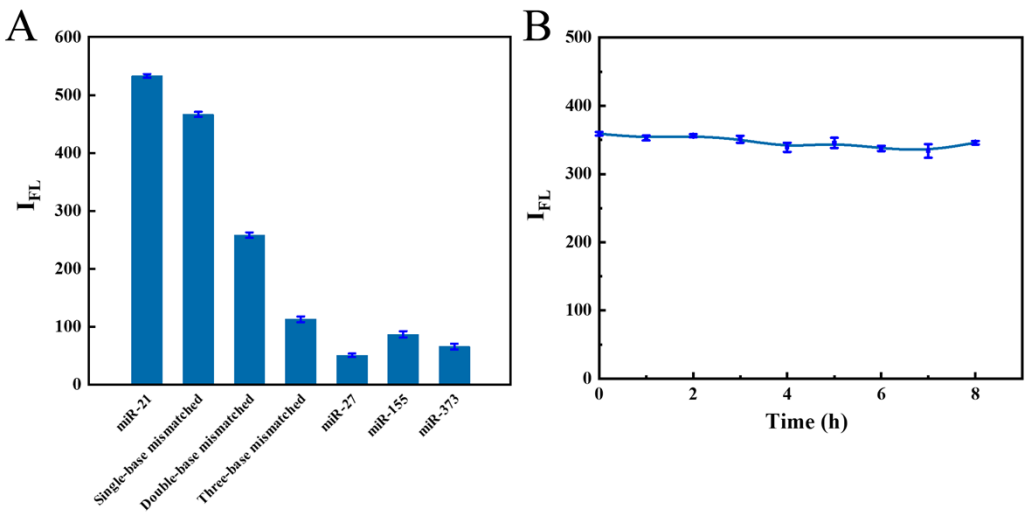

Fig. S8 Fluorescence intensity plots of seven different miRNAs detected using the erythrocyte membrane vesicle strategy (A) and stability assay of the DNA self-assembly reaction over 8 hours (B).

To explore the specificity of the DNA self-assembly reaction triggered by the three hairpin probes we designed for miRNA-21 for other different types of detector miRNAs, we selected six different miRNAs and miRNA-21 to react with each of the three probes and examined the fluorescence intensity of each group (Fig. S5A). As shown, the six controls were single base mismatch, double base mismatch, triple base mismatch, miR-27, miR-155 and miR-373. In these tests for the probes against miRNA-21, only miRNA-21 effectively triggered the

reaction and showed strong fluorescence, while all other miRNAs had fluorescence responses that fell well short of this intensity. This demonstrates that the DNA self-assembly reactions of the three hairpin probes we designed are highly specific to the target detectors they target. This also inspired us to design the DNA self-assembly reactions of the hairpin probes according to the sequences of different detectors to achieve fluorescence signal detection. In addition, the stability of the fluorescence signal of the product formed at the end of the DNA self-assembly reaction at 4 °C was also examined (Fig. S5B).

References

1. D. Mao, M. Zheng, W. Li, Y. Xu, C. Wang, Q. Qian, S. Li, G. Chen, X. Zhu and X. Mi, *Biosens. Bioelectron*., 2022, **204**, 114077.
2. H. Shao, H. Im, C. M. Castro, X. Breakefield, R. Weissleder and H. Lee, *Chem. Rev.*, 2018, **118**, 1917-1950.
3. N. P. Hessvik and A. Llorente, *Cell. Mol. Life Sci.*, 2018, **75**, 193-208.
4. R. H. Fang, C.-M. J. Hu, K. N. H. Chen, B. T. Luk, C. W. Carpenter, W. Gao, S. Li, D.-E. Zhang, W. Lu and L. Zhang, *Nanoscale*, 2013, **5**, 8884-8888.
5. M. Li, H. Fang, Q. Liu, Y. Gai, L. Yuan, S. Wang, H. Li, Y. Hou, M. Gao and X. Lan, *Biomater. Sci.*, 2020, **8**, 1802-1814.