Supporting Information

Sensitive and sustained imaging of intracellular microRNAs in living cells by a high biocompatible liposomal vehicle introduced isothermal symmetric exponential amplification reaction

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Content

S1. Reagents and materials

Egg lecithin was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cholesterol was purchased from Sigma-Aldrich Co. (America). Bovine serum albumin-FITC (BSA-FITC) was purchased from Beijing Bossbio Bio-Technology Co. Ltd. (Beijing, China). Absolute ethyl alcohol was purchased from Guangzhou Chemical Reagent Factory (Guangdong, China). DNAs were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). MiRNAs were purchased from TaKaRa Biotech Company (Dalian, China). The sequences of the used oligos are shown in **Table S1**. Before use, the molecular beacon (MB) probes were diluted to 10 μM by a 10 mM Tris-HCl buffer (pH 8.0, containing 1.5 mM $MgCl_2$). The MB probes were heated at 95 ^{o}C for 5 min and then cooled slowly to room temperature over 30 min to have these probes perfectly folded in hairpin structure.

The A549 cells and the HLF cells were obtained from the Cell Resource Center, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. T4 DNA ligase, exonuclease I (*E. coli*), exonuclease III (*E. coli*), Klenow fragment polymerase (3'→5' exo-, KF polymerase), Nt.BsmAI, 10 × T4 DNA ligase reaction buffer, RNase Inhibitor were bought from New England Biolabs (Beijing, China). SYBR Green I was obtained from Xiamen Bio-Vision Biotech. Co. Ltd. (Xiamen, China). Diethyl oxydiformate (DEPC), Deoxyribonucleotides mixture (dNTPs), RPMI-1640 medium, penicillin-streptomycin, 0.25% Trypsin-EDTA (1×), fetal bovine serum (FBS) were obtained from Guangzhou Qiyun Biotech. Co., Ltd. (Guangzhou, China). Lipofectamine 3000 Transfection Kit was purchased from ThermoFisher Scientific Co. (Shanghai, China). Annexin V-FITC Apoptosis Detection Kit and MTT Cell Proliferation and Cytotoxicity Assay Kit were purchased from Beyotime Biotechnology Co. (Shanghai, China). Other regents with analytical grade were obtained from Beijing Chemical Co. (Beijing, China) and used without further purification. All the solutions and deionized water used were treated with DEPC and autoclaved to protect from RNase degradation.

The buffer solutions used in the assay were as follows. T4 DNA ligase reaction buffer: 400 mM pH 7.8 Tris-HCl buffer containing 100 mM $MgCl_2$, 100 mM dithiothreitol and 5 mM ATP. 1 × NEB buffer 2: 10 mM pH 7.9 Tris-HCl buffer containing 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol. 1 × NEB CutSmart buffer: 20 mM pH 7.9 Tris-acetate buffer containing 50 mM potassium acetate, 10 mM magnesium acetate and 0.1 mg mL^{-1} BSA.

Table S1 Sequences of the used oligonucleotides

Name	Sequences (5'-3')
miR-27a	UUC ACA GUG GCU AAG UUC CGC
miR-27b	UUC ACA GUG GCU AAG UUC UGC
STD-template	**(PO_3)** GCG GAA CTT AGC CAC TGT GAA AGA GAC CCT CCA GTT CCG CGC GGA ACT TAG CCA CTG TGA AAG AGA CCC TCC AGT TCC GC
FAM/BHQ1 labeled molecular beacon (FAM/BHQ1-MB)	**(FAM)** GCG CGC AAG TGT CAC CGA TTC AAG GCG CGC **(BHQ1)**
Oligo-Cy5	**(Cy5)** UAG UAG GUU GUA UAG UUU UAC UAC CUC AUG UGU GUG UGU GUU GAG G

S2. Experiments

S2.1 Preparation of STD-template

The STD-template was prepared from its 5'-phosphorylated STD-template precursor with the aid of ligation template. Ligation reaction was conducted at 16 ℃ for 2 h in a volume of 20 μL containing 1 μL 5'-phosphorylated STD-template precursor (100 μM), 2 μL 10 × T4 DNA ligase reaction buffer, 16 μL DEPC-treated H_2O and 1 μL of T4 DNA ligase (100 U μL^{-1}). The ligation reaction was terminated by heating the mixture at 65 ℃ for 10 min. The resultant mixture was then added with exonuclease I (20 U μL^{-1}) and exonuclease III (100 U μL^{-1}) to digest the leftovers ssDNA and dsDNA, and heated at 80 ℃ for 20 min to denature enzymes.

The concentration of the STD-template was determined by measuring the absorbance at 260 nm with a NanoDrop-2000c spectrophotometer (NanoDrop Technologies, USA). The STD-template were diluted with DEPC-treated water (RNase free) to 1 μM and stored at －20 ℃ before use.

S2.2 SEXPAR protocol

Under optimal conditions of 0.2 U μL^{-1} KF polymerase, 0.2 U μL^{-1} ENase, 1 mM Mg^{2+}, and 0.1 μM STD-template, the SEXPAR was performed by the mixture of three solutions of Part A, B, and C. Part A consisted of 10 μL of 5 μM STD-template and 10 μL of 2.5 mM dNTPs. Part B consisted of 2.5 μL of 5 U μL^{-1} KF DNA polymerase, 5 μL of 10 × NE Buffer, 2.5 μL of 5 U μL^{-1} Nt.BsmAI nicking enzyme, and 2.5 μL of 10 × CutSmart buffer. Part C consisted of 10 μL of 10 μM MB probes. These three parts were respectively encapsulated into the liposomes and transferred into A549 and HLF cells for SEXPAR.

S2.3 Preparation of Liposomal Vehicles

Liposomes were prepared by film dispersion and freeze-thaw method. Egg

lecithin and cholesterol (w/w = 3 : 1) were dissolved in absolute ethyl alcohol, evaporating the solvent, then hydrating the mixture with PBS buffer (0.1 M, pH 7.4) to a final concentration of 6 mg mL^{-1}. The mixture was sonicated for 3 min to obtain the liposome suspension.

Oligos or proteins were added to the liposome suspension at a lipid/cargoes volume ratio of 1 : 1. The mixtures were frozen at –20 ^{o}C overnight and thawed slowly at room temperature. After repeating the above frozen-thaw process for three times, the oligos or proteins were encapsulated into the liposomes (noted as liposome-oligo or liposome- protein).

S2.4 Characterization of Liposomal Vehicles and Lipo3000

A droplet of liposome or Lipo3000 suspension was spread over tin foil paper placed on an aluminum stub and dried in a vacuum for 1 h. The morphologies of liposomes and Lipo3000 were studied by observation of these dried samples on a Field Emission-Scanning Electron Microscope (FE-SEM, HITACHI SU8010, Japan). The particle sizes and concentration of liposomes and Lipo3000 were tested by diluting them to 1 mL with PBS buffer and analyzed on a NanoSight (Malvern, Britain). The zeta potentials of liposomes and Lipo3000 were determined by a Zetasizer (Brookhaven, USA).

The encapsulation efficiency (EE) of liposomes for oligos or proteins was evaluated from the model oligo (Oligo-Cy5) and protein (BSA-FITC). The standard curves of Oligo-Cy5 and BSA-FITC were obtained by the measurements of the fluorescence intensities of Oligo-Cy5 (0.001, 0.005, 0.010, 0.020, 0.040 and 0.060 mg mL^{-1}) and BSA-FITC (0.01, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.00 mg mL^{-1}) respectively on an F-7000 fluorophotometer (HITACHI, Japan). Before the freeze-thaw process, the Oligo-Cy5/liposome or BSA-FITC/liposome mixtures were centrifuged at 12,000 rpm at 4 ^{o}C for 1 h. The fluorescence intensities of the supernatants were detected, representing the total concentration of Oligo-Cy5 or BSA-FITC. After repeating freeze-thaw cycles of the mixtures, the above processes

were conducted again to quantify the free concentration of Oligo-Cy5 or BSA-FITC. The concentrations of total (c_{total}) and free (c_{free}) Oligo-Cy5 or BSA-FITC were obtained from the calibration curves of Oligo-Cy5 and BSA-FITC. The EE was calculated by the following Eq. (1).

$$\text{EE}(\%) = \left(1 - \frac{c_{free}}{c_{total}}\right) \times 100\% \quad (1)$$

S2.5 Investigation of Liposomal Vehicles Transport Capacity and Cytotoxicity

The A549 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (ThermoFisher, Shanghai, China) at 37 ^{o}C in a humidified atmosphere of 5% CO_2. The cells (1×10^4 cells $well^{-1}$) were placed in 48-well-culture boards to a final growth medium volume of 200 μL and cultured for 24 h. The medium was taken out, then 80 μL of flesh RPMI-1640 medium and 20 μL of BSA-FITC-liposome or Oligo-Cy5-liposome were added to each well. After incubation at 37 ^{o}C for 2, 4, 8 ,12 and 24 h, the resultant cells were washed with PBS buffer for three times and then observed on a laser scanning confocal microscope (Zeiss LSM710, Germany). The BSA-FITC was excited at 488 nm and emitted in the range of 520-530 nm, and the Oligo-Cy5 was excited at 633 nm and emitted in the range of 665-675 nm. As a contrast, the Lipo3000 -BSA-FITC and Lipo3000-Oligo-Cy5 were comparatively tested as above.

Flow cytometry (Becton Dickinson) and confocal microscopy (Zeiss LSM710, Germany) were used to detect apoptosis of liposomes and Lipo3000 via an Annexin V-FITC Apoptosis Detection Kit (Beyotime). After incubated with liposomes and Lipo3000 for 6 and 24h, the adherent cells were dissociated by 0.25% Trypsin-EDTA (1×) (Life Technologies) and collected. The cell sediment was resuspended with 195 μL of Annexin V-FITC binding buffer, and incubated with 5 μL of Annexin V-FITC and 10μL of Propidium Iodide (PI) avoid light for 15min and placed on ice.

SpectraMax M2 (Molecular Devices) was used to detect the cell viability of liposomes, Liposome-27a, Lipo3000 and Lipo3000-27a in the MTT assay. The A549

cells were cultured in 96-well-culture boards to a final growth medium volume of 100 μL for 24 h. The medium was taken out and replaced with 100 μL of flesh RPMI-1640 medium, then 10 μL of the liposomes or Liposome-27a, or 2 μL of Lipo3000 or Lipo3000-27a (1 μM miR-27a mimic) were added to each well. After incubation for 6 and 24h, the resultant cells were washed with PBS buffer for three times and treated by the MTT Cell Proliferation and Cytotoxicity Assay Kit according to the instruction manual. The absorbance was measured at 570 nm by SpectraMax M2.

S2.6 Visualization of miR-27a in living cells

The SEXPAR substances were encapsulated into the liposomes via freeze-thaw method. 40 μL of liposome-A, 25 μL of liposome-B and 40 μL of liposome-C were successively added incubated with A549 and HLF cells to a final volume of 200 μL at 37 ^{o}C for 2h. In addition, besides the above SEXPAR substances, miR-27a mimic-liposomes (0.1, 1, 5μM) were added to A549 cells in advance to simulate the up-regulation of miR-27a. Finally, A549 and HLF cells were washed with PBS buffer for three times and detected by confocal microscopy (Zeiss LSM710, Germany).

S3. Structure of STD-template

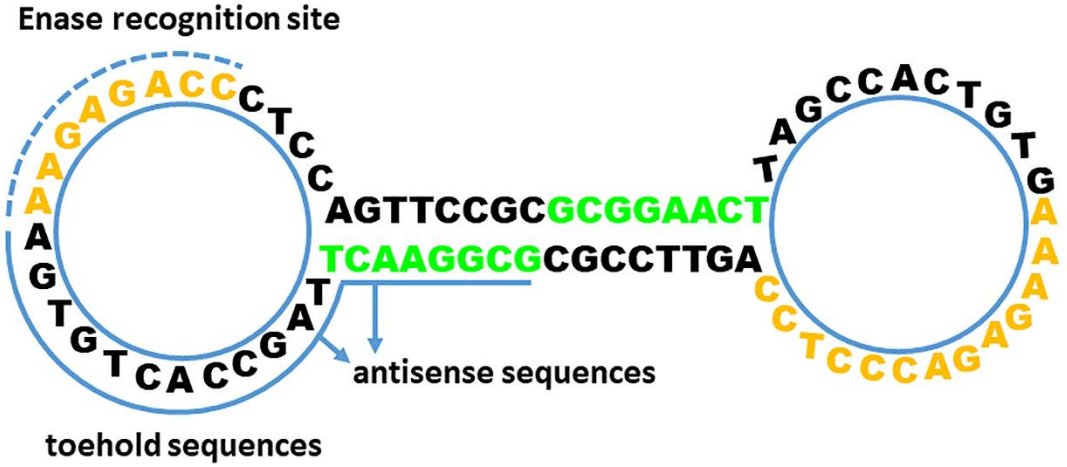

Fig. S1. Structure of STD-template

S4. Calculation of encapsulation efficiency

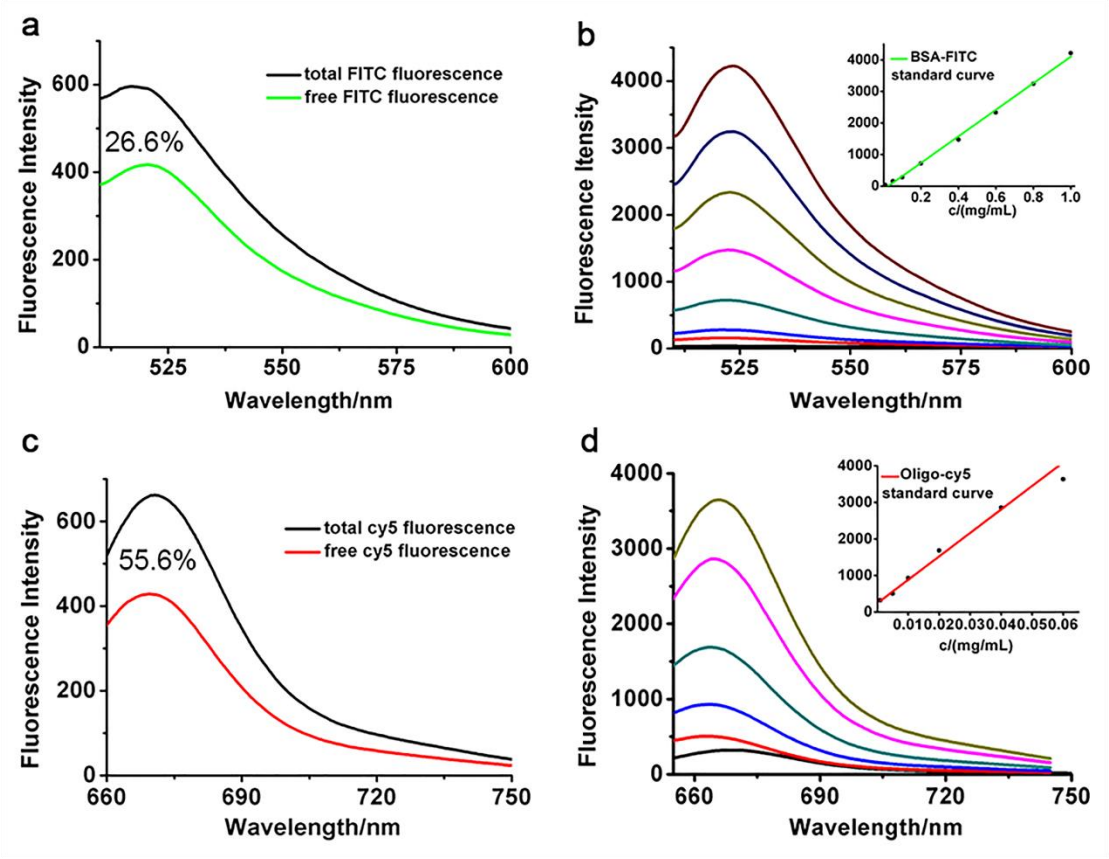

Fig. S2 Encapsulation curves, fluorescence curves and standard curves of (a, b) BSA-FITC and (c, d) Oligo-Cy5.

S5. Verification of transport capacity of liposomes and Lipo3000

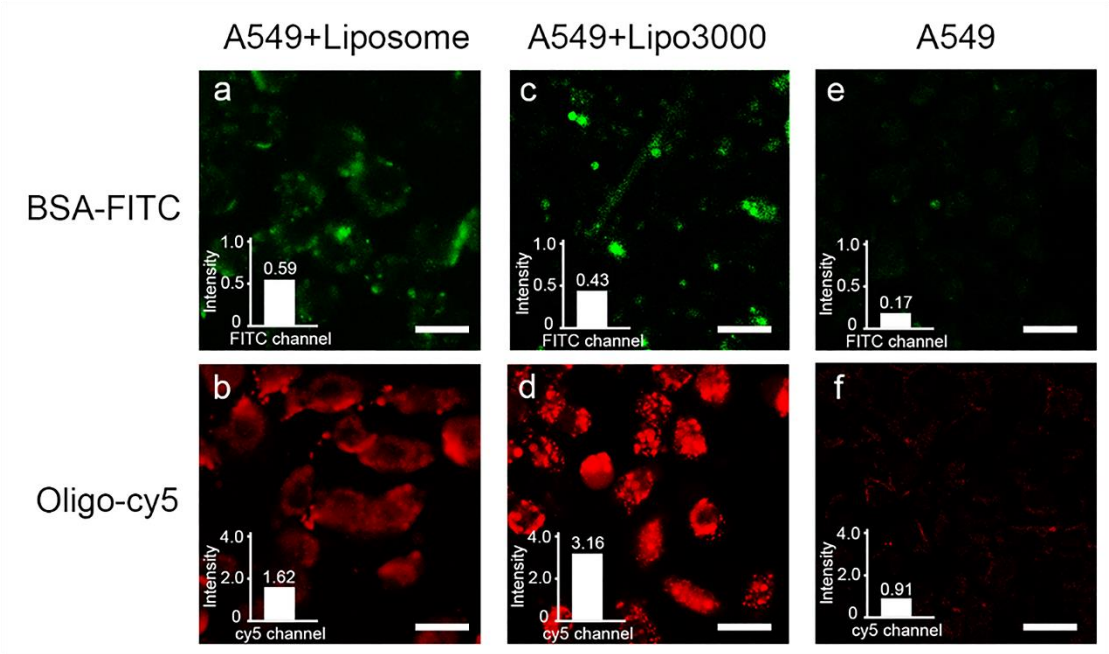

Fig. S3 Confocal images of A549 cells after delivery of BSA-FITC and Oligo-Cy5 into cells in the presence of (a, b) liposomes or (c, d) Lipo3000 and (e, f) absence of vehicles. Scale bar: 20 μm. Insets: the arithmetic mean intensities of FITC and Cy5 channel measured by the standard software of the LSM710.

S6. Analytical performance of SEXPAR

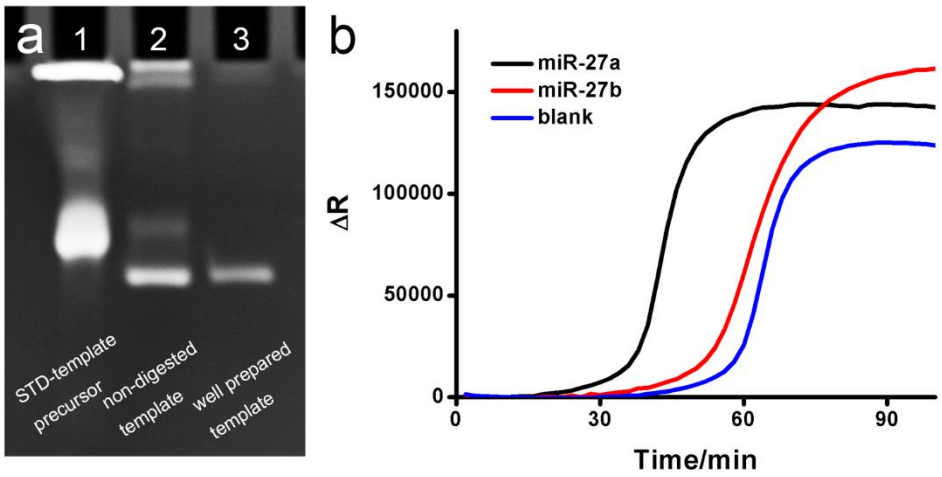

Fig. S4 Analytical performance of SEXPAR on STD-template. (a) A gel electrophoresis assay of non-ligated STD-template (STD-template precursor, lane 1), non-digested template (STD-template precursor after ligation by T4 DNA ligase, lane 2), and well prepared STD-template (the ligated STD-template after digestion by Exonuclease I and Exonuclease III, lane 3). (b) Real-time fluorescence curves of SEXPAR in response to miR-27a, miR-27b and blank at 37 ^{o}C.