SUPPORTING INFORMATION

Probing exosome internalization pathways through confocal microscopic imaging

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Experimental section

Reagents and materials

All oligonucleotides designed in this study were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The sequences of oligonucleotides are shown in Table 1. Phosphate buffer solution (PBS), Dulbecco's modified Eagle medium (DMEM), adenosine triphosphate (ATP), trypsin-EDTA, and RIPA lysis buffer were obtained from Solarbio Science and Technology Co., Ltd (Beijing, China). Horseradish peroxidase (HRP)-conjugated secondary antibody, DiI, Rabbit anti-human CD63 polyclonal antibody and BCA protein assay kit was obtained from Beyotime Biotechnology Co., Ltd (Shanghai, China). Opti-MEM and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Co., Ltd. (Waltham, MA, USA). Cell counting kit-8 (CCK-8) was purchased from Yeasen Biotech Co., Ltd. (Shanghai, China). ExoEasy Mid kit was purchased from Qiagen Inc. (Dusseldorf, Germany). Cytochalasin D and methyl-β-cyclodextrin (MβCD) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). Cell lines, including hepatocellular carcinoma cell line (HepG2), human breast cancer cell line (MCF-7), and human cervical cancer cell line (HeLa) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The 24-well cell culture plate with bottom glass was purchased from In Vitro Scientific (Sunnyvale, CA, USA). The 12-well cell culture plate, 96-well cell culture plate, and black 384-well micro-plate were purchased from Corning Inc. (NY, USA). Centrifugal filter units (MWCO 100 kDa) and 0.22 μm filters were purchased from Millipore Corp. (Bedford, MA, USA). Ultrapure water (≥18 MΩ) was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used in all experiments.

Table S1. **Sequence information for oligonucleotides used in this study**

Name	[a] Sequence (5'-3')
A	BHQ1-TTCCTCCGCAATACTCCCCCAGGTGCGGAGGAATT/i6FAMdT/TTT-Cholesterol
S	ACCTGGGGGAGTATTGCGGAGGAAGGT-Cy3
A'	TTCCTCCGCAATACTCCCCCAGGTGCGGAGGAATTTTT-Cholesterol
S'	ACCTGGGGGAGTATTGCGGAGGAAGGT
F	FAM-T_{30}-Cholesteryl
R	BHQ1-GGCATAGGCTATAGGCCATGACCAGCCTATGCCTT/i6FAMdT/TTT-Cholesterol
S*	TGGTCATGGCCTATAGCCTATGCCCCA-Cy3

Instrumentation

The fluorescence spectra and absorbance values were measured by a microplate reader (Bio-Tek Instrument, Winooski, USA) using a black 384-well microplate and a transparent 96-well microplate, respectively. The transmission electron microscopy (TEM) image of exosome was obtained on a Jeol JEM-1400 instrument (JEOL Ltd., Japan). The size distribution of exosomes before and after probe labelling was analysed using a NanoSight system (Malvern Instruments Ltd., U.K.). The gel was imaged on a Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology, Shanghai, China). The cell images were performed by a Nikon confocal laser scanning microscope system (Nikon Corp., Tokyo, Japan). Flow cytometry data were collected with a CytoFLEX S FCM (Beckman Inc., CA, USA) and analysed by CytExpert software.

Cell culture and exosome collection

All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C, and maintained in DMEM supplemented with 10% FBS to 50% confluence followed by washing three times with PBS, and then incubation for 48 h in serum-free DMEM medium. After that, the cell culture supernatant was collected and centrifuged at 4 °C (480 g for 5 min followed by 2000 g for 10 min) to remove the intact cells and cell debris. Then the supernatant was filtered through a 0.22 μm filter to remove large contaminating vesicles. The pre-treated cell culture supernatant was used to obtain exosomes using an ExoEasy Mid kit according to the manufacturer's instructions.

Characterization of exosomes

The collected exosomes from HepG2 cell culture supernatant were loaded on Cu grids. After staining with 1% phosphotungstic acid, the grid was dry completely at room temperature. Transmission electron microscopy (TEM) was then executed to characterize the exosome morphology. The protein concentrations of exosomes were measured using a BCA protein assay kit. The size changes of exosome before and after probe modification were measured using a NanoSight system. The surface adhesive protein of exosomes was analysed by Western blot according to previous literature.[1] In brief, the purified exosomes were first lysed by RIPA buffer on ice for 15 min. Then, the proteins were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter membrane. The membrane was then blocked in 5% BSA blocking buffer, followed by incubating with primary antibodies against exosome proteins at 4 °C for 12 h. After that, the membranes were washed and incubated with HRP-labelled secondary antibodies for 1 h at 25 °C. Finally, the signal was measured by an ECL kit on a Gel Imaging System.

Labelling of exosomes with probe AS

The labelling process was divided into three steps. First, to form the fluorescent probe AS, anchor strand (A) and signal strand (S) were prehybridized by cooling from 90 °C to 22 °C at a rate of 1.5 °C /min in PBS to give a final concentration of 20 μM. Then, 3 μL of AS (20 μM) was added to an exosome aliquot containing 20 μg protein and diluted to 200 μL with PBS before incubation for 30 min in the dark at 25 °C. Finally, the centrifugal filter unit was used to remove unincorporated AS probe from AS-labelled exosomes. The reaction mixture of exosomes and AS was added to the column and diluted to 400 μL with PBS. Then, the column was placed in the collection tube and centrifuged for 2 min (5000 g) to remove unincorporated probe. The eluate was discarded. This process was repeated four times. Afterwards, the column was inverted in a new collection tube and centrifuged for 1 min (600 g) to recover the labelled exosomes.

Cellular uptake of AS-labelled exosomes in HepG2 cells

For internalization assay, HepG2 cells were seeded in 24-well cell culture plate with 500 μL DMEM and incubated for 24 h to reach 80-90% confluency. After that, HepG2 cells were washed three times with PBS. Then, an aliquot of 500 μL Opti-MEM containing 40 μg/mL labelled exosomes was added to each well and incubated for 3 h before confocal microscopy was performed. After incubation, the well was washed three times with PBS followed by imaging. For each analysis, cells with unlabelled exosomes addition were imaged in parallel and analysed as negative control.

Investigation of the cytotoxicity of endocytosis inhibitors in HepG2 cells with the CCK-8 assay

The cytotoxicity of endocytosis inhibitors in cells was analysed according to the manufacturer's

instructions. In brief, HepG2 cells were seeded in a 96-well cell culture plate with 1×10^4 cells per well and incubated for 24 h in a humidified incubator containing 5% CO_2 at 37 °C. After incubation and washing, an aliquot of 100 μL Opti-MEM containing cytochalasin D or methyl-β-cyclodextrin (MβCD) with different concentrations was added to the wells of the treatment group and incubated for 30 min. Meanwhile, an aliquot of 100 μL Opti-MEM without cytochalasin D and MβCD was added to another cell well as a control, and a clear well without cells was treated with 100 μL Opti-MEM without any inhibitors as a blank. An aliquot of 10 μL CCK-8 reagent was then added to each of the above wells. After 1.5 h incubation, the absorbance value of the treatment group, control group, and blank group at 450 nm (OD_{450}) was measured to obtain as A_t, A_c, and A_b, respectively. The cell viability at different inhibitor concentrations was calculated based on the equation:

Cell viability (100%) = $[(A_t–A_b)/(A_c–A_b)] \times 100$.

Incubation of cells with endocytosis inhibitors

HepG2 cells were seeded in a 24-well cell culture plate with 500 μL DMEM for 24 h to reach 80–90% confluency. They were then washed three times with PBS. After that, 500 μL Opti-MEM containing 20 μM cytochalasin D or 5 mM MβCD was added to the wells and pre-incubated for 30 min. HepG2 cells were washed three times with PBS before incubating with AS-labelled exosomes (60 μg/mL) for 3 h. They were then washed three times with PBS prior to performing cell confocal microscopy.

Fluorescence microscopy analysis

Fluorescent images were obtained using a Nikon confocal scanning system with a 40× objective. The fluorescence of FAM and Cy3 was obtained by an Argon laser emitting a 488 nm wavelength and a helium-neon laser emitting a 561 nm wavelength, respectively. The cells with the addition of unlabelled exosomes were set as the blank control.

Flow cytometry analysis

HepG2 cells were seeded in a 12-well cell culture plate with 1 mL DMEM for 24 h to reach 80-90% confluency. They were then washed three times with PBS. After that, the probe labelled exosomes were incubated with the cells. Incubation was performed at 37 °C with 5% of CO_2 for 3 h. Elapsed the incubation time, cells were washed three times with PBS and detached with trypsin. Cells were then pelleted at 800 g for 2 min and washed twice with PBS. The pellet was finally resuspended in 500 μL of PBS and analysed by flow cytometry. The obtained data were then analysed by CytExpert software.

Supporting figures：

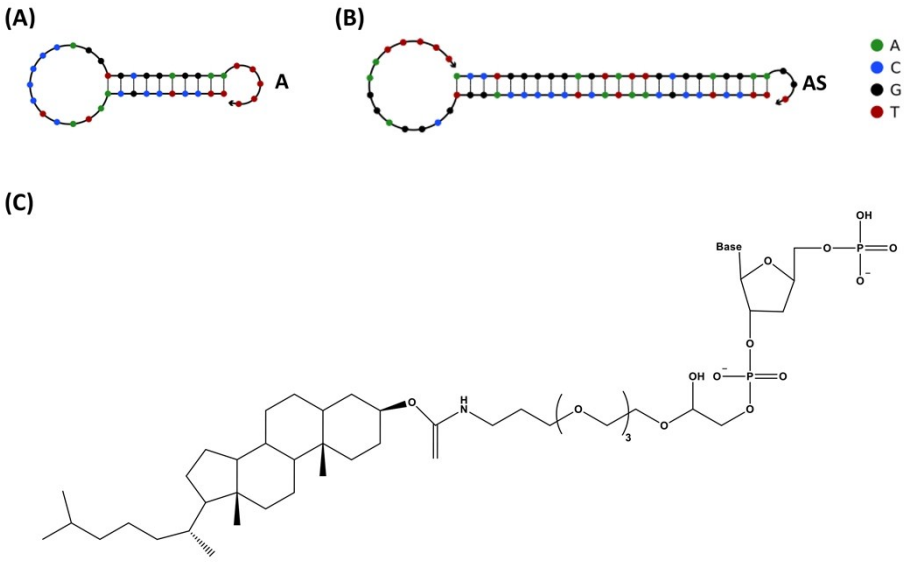

Fig. S1 The secondary structures of probe A (A) and AS complex (B) predicted by the NUPACK software. (C) Chemical structure of cholesterol moiety, which is labeled with DNA.

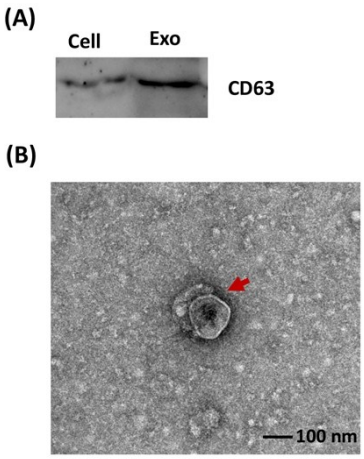

Fig. S2 Characterization of exosomes. (A) Western blot image of CD63 protein from HepG2 cell lysates and purified exosomes using a ExoEasy kit. Twenty μg proteins extracted from cell lysates and exosomes were tested, respectively. (B) TEM image of exosomes purified from HepG2 cell culture supernatant.

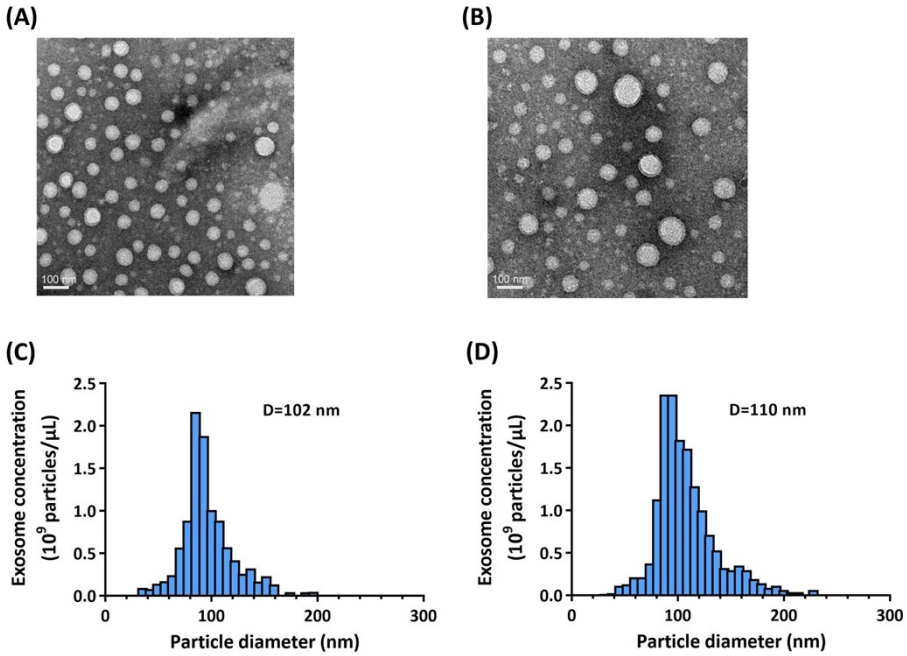

Fig. S3 Characterization of exosomes. TEM images of exosomes before (A) and after (B) probe labelling. Size distributions of exosomes before (C) and after (D) probe labelling by NTA.

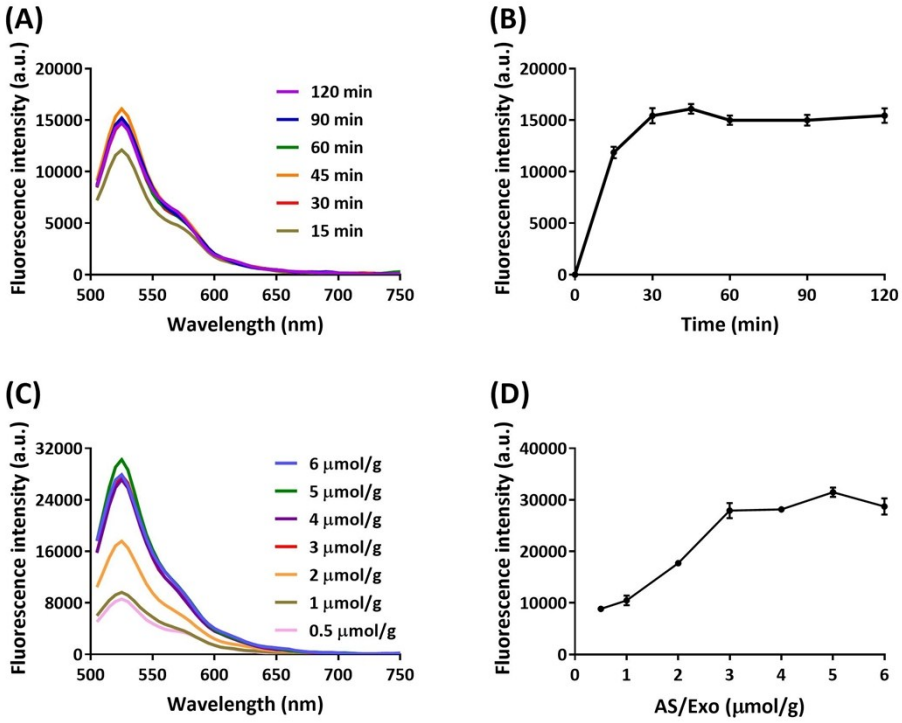

Fig. S4 Optimization of incubation time and AS concentration of the proposed labelling method for exosomes from HepG2 cells. (A) Fluorescence emission spectra responses to different incubation times (15, 30, 45, 60, 90, and 120 min). (B) The relationship of the fluorescence enhancement with different incubation times. (C) Fluorescence emission spectra in the presence of different concentrations of AS, ranging from 0.5 to 6 μmol/g (n_{AS}/m_{exo}). (D) The relationship of the fluorescence enhancement with different concentrations of AS. Error bars show the standard deviations of three experiments.

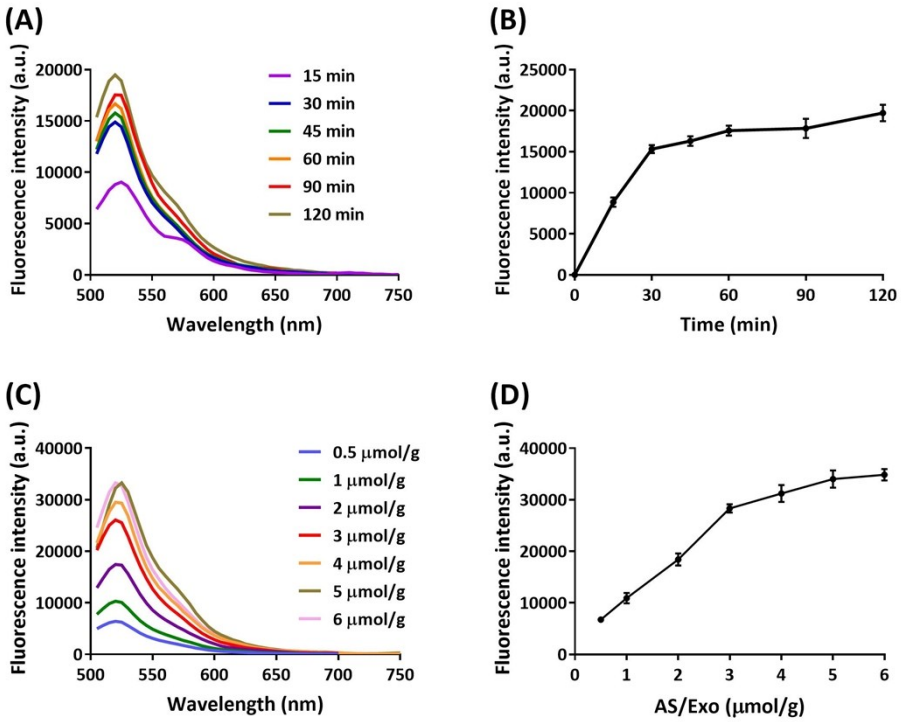

Fig. S5 Optimization of incubation time and AS concentration of the proposed labelling method for exosomes from MCF-7 cells. (A) Fluorescence emission spectra responses to different incubation times (15, 30, 45, 60, 90, and 120 min). (B) The relationship of the fluorescence enhancement with different incubation times. (C) Fluorescence emission spectra in the presence of different concentrations of AS, ranging from 0.5 to 6 μmol/g (n_{AS}/m_{exo}). (D) The relationship of the fluorescence enhancement with different concentrations of AS. Error bars show the standard deviations of three experiments.

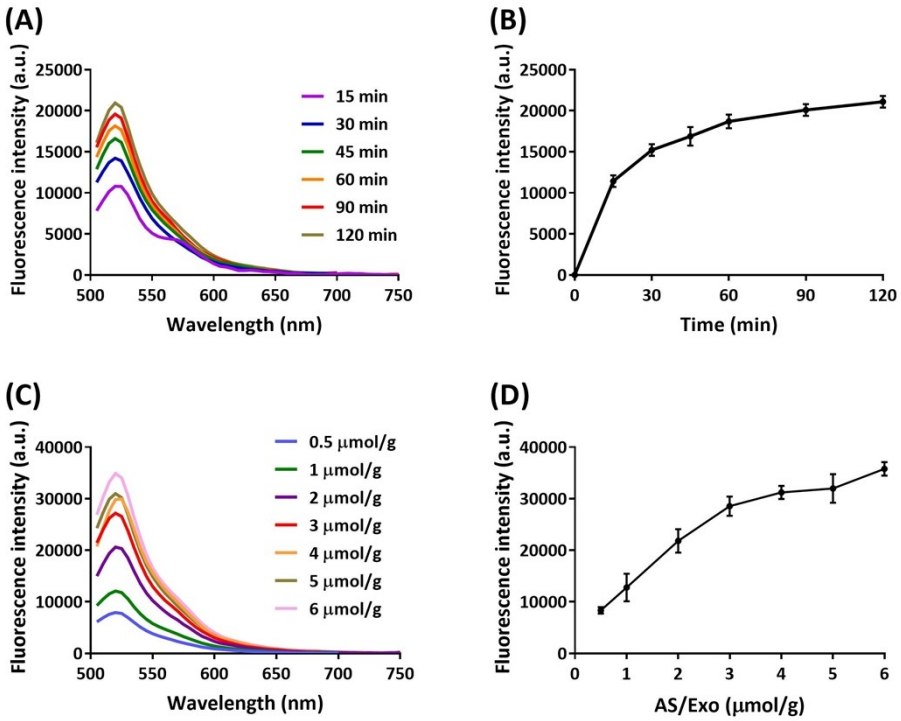

Fig. S6 Optimization of incubation time and AS concentration of the proposed labelling method for exosomes from HeLa cells. (A) Fluorescence emission spectra responses to different incubation times (15, 30, 45, 60, 90, and 120 min). (B) The relationship of the fluorescence enhancement with different incubation times. (C) Fluorescence emission spectra in the presence of different concentrations of AS, ranging from 0.5 to 6 μmol/g (n_{AS}/m_{exo}). (D) The relationship of the fluorescence enhancement with different concentrations of AS. Error bars show the standard deviations of three experiments.

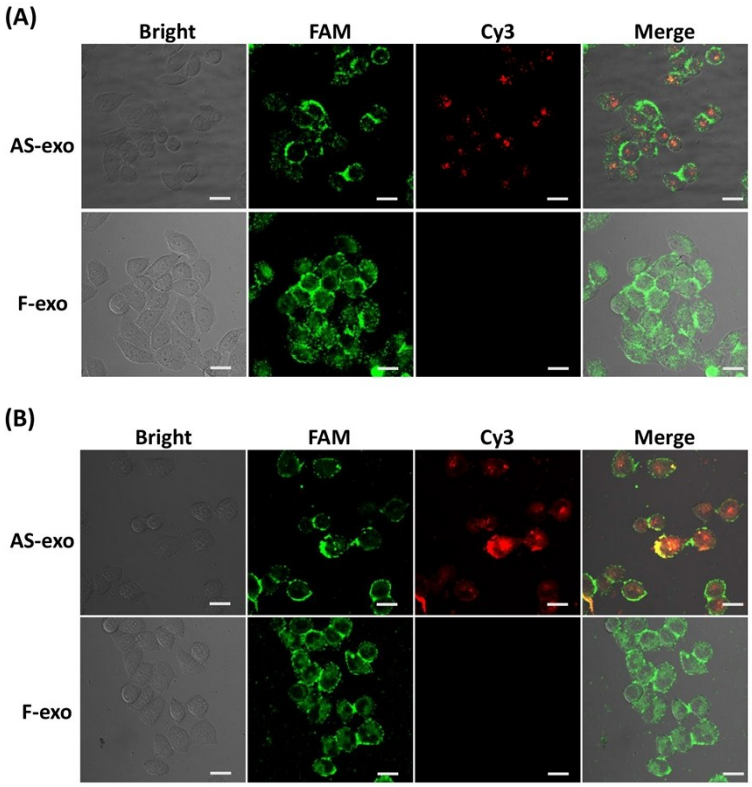

Fig. S7 Comparison of AS-labelled exosome and FAM tagged DNA strand (F) labelled exosome uptake in HepG2 cells. (A) Confocal microscopy images of AS- and F-labelled exosomes (40 μg/mL) that derived from HepG2 cells uptake in HepG2 cells. (B) Confocal microscopy images of AS- and F-labelled exosomes (40 μg/mL) that derived from MCF-7 cells uptake in HepG2 cells. Scale bars: 20 μm.

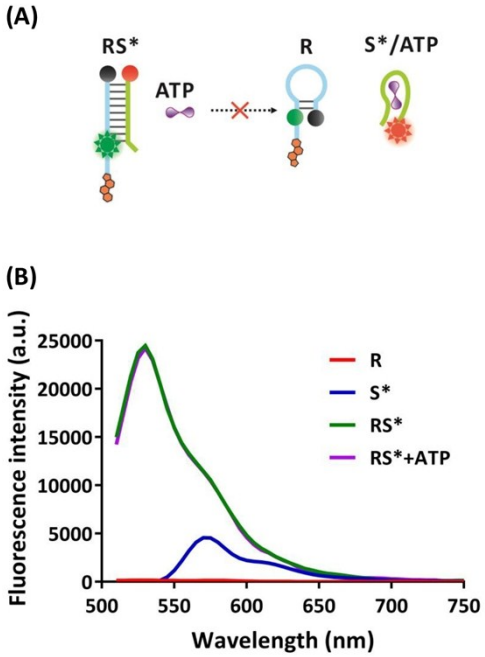

Fig. S8 Investigation of the reaction between RS* and ATP molecule. (A) Schematic of the reaction between RS* and ATP molecule. (B) Fluorescence emission spectra (λ_{ex} = 485 nm, λ_{em} = 518 nm) of random sequence (S*), anchor chain (R), RS* complex, and RS* complex with ATP.

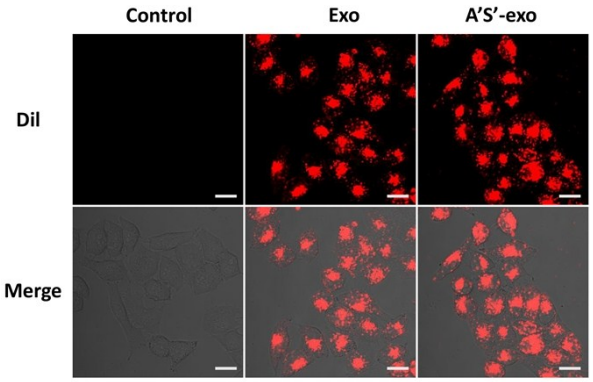

Fig. S9 Investigation on the effect of probe modification on exosome internalization. Exosomes were modified with no fluorescence labelled A'S' probe (A'S'-exo), which has the same sequence as AS probe. Confocal fluorescence microscopy images comparison of DiI-labelled exosomes and DiI labelled A'S'-exo uptake in HepG2 cells. Scale bars: 20 μm.

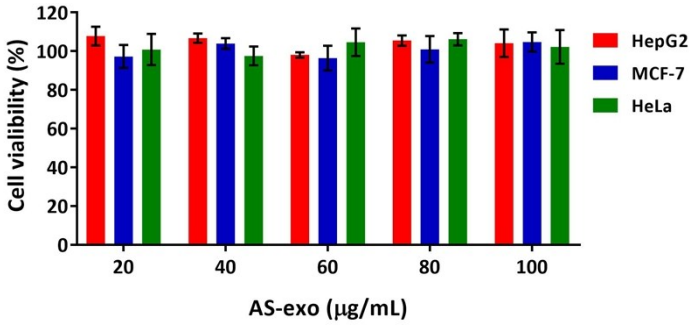

Fig. S10 Cytotoxicity investigation of AS-labelled exosomes on HepG2 cells using the CCK-8 assay. The

exosomes from HepG2 cells, MCF-7 cells and HeLa cells were tested with various concentrations (20, 40, 60, 80 and 100 μg/mL), respectively.

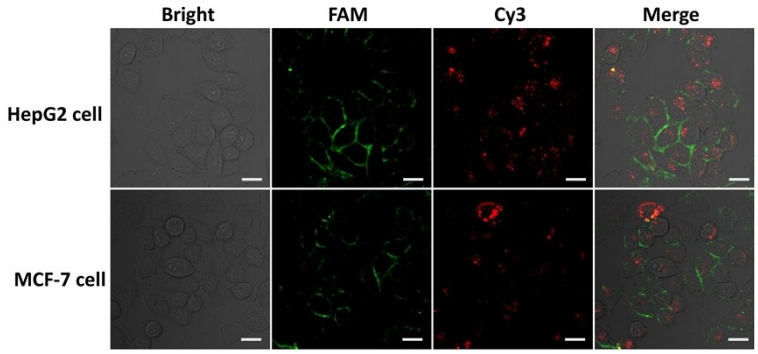

Fig. S11 Investigation the effects of different cell lines on exosome uptake. Confocal microscopy images of AS-labelled exosomes (40 μg/mL) that derived from HepG2 cells uptake in HepG2 cells and MCF-7 cells, respectively. Scale bars: 20 μm.

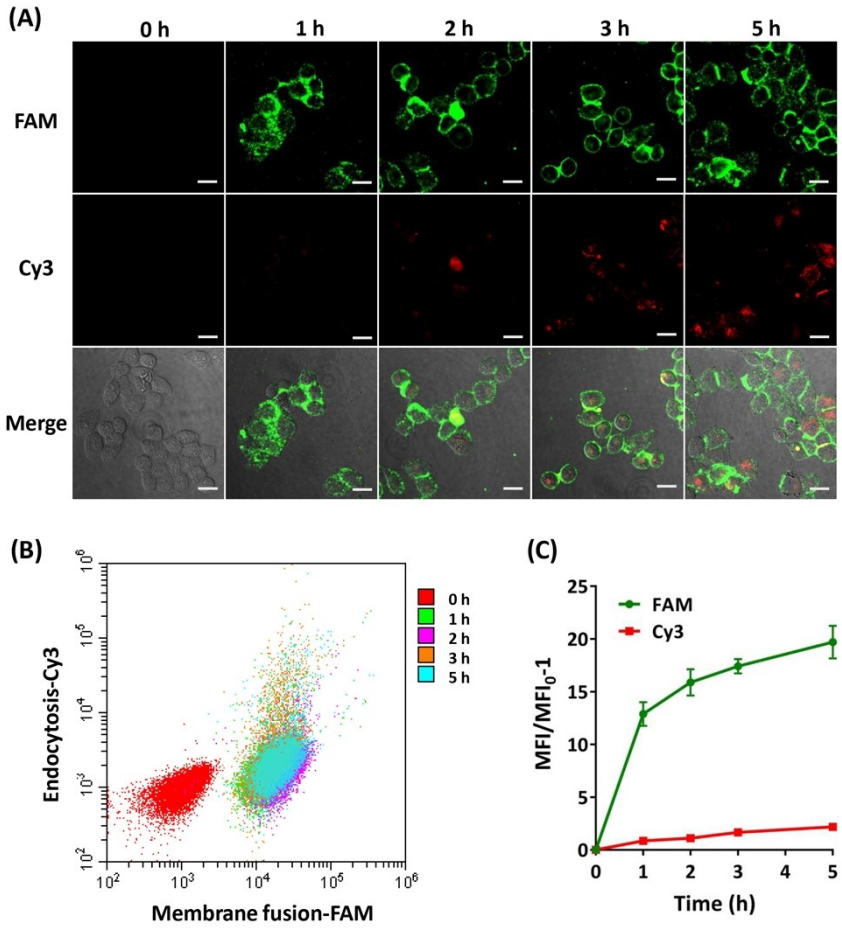

Fig. S12 Investigation the effects of incubation time on exosome uptake. (A) Confocal microscopy images of HepG2 cells incubated with AS-labelled exosomes (40 μg/ml) for 1, 2, 3 and 5 h. Scale bars: 20 μm. (B) Flow cytometry analysis of HepG2 cells incubated with AS-labelled exosomes for different times. (C) Mean fluorescence intensity (MFI) change ratio (MFI/MFI_0-1) corresponding to different incubation time. MFI and MFI_0 are mean fluorescence intensity of FAM or Cy3 in the presence and absence of AS-labelled exosomes, respectively.

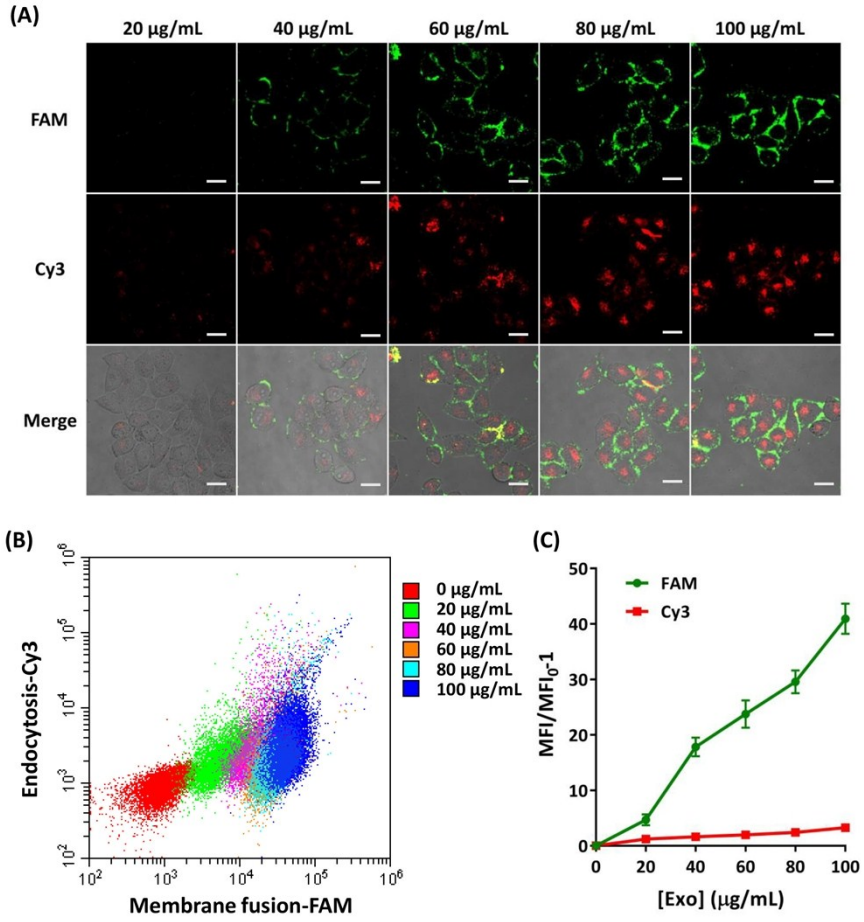

Fig. S13 Investigation the effects of exosome concentration on cellular uptake. (A) Confocal microscopy images of HepG2 cells incubated with different concentrations of AS-labelled exosomes originating from HepG2 cells for 3 h. Scale bars: 20 μm. (B) Flow cytometry analysis of HepG2 cells after 3 h incubation with different concentration of AS-labelled exosomes. (C) Mean fluorescence intensity change ratio corresponding to different concentration of AS-labelled exosomes. MFI and MFI_0 are mean fluorescence intensity of FAM or Cy3 in the presence and absence of AS-labelled exosomes, respectively.

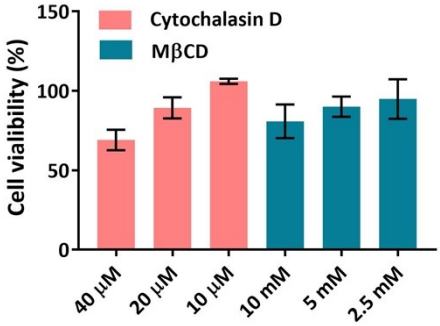

Fig. S14 The effect of endocytosis inhibitors on cellular viability. HepG2 cells were treated with the indicated endocytosis inhibitors for 30 min, and then cell viability was determined by the CCK-8 assay. Relative cell viability was normalized to the control (no inhibitor addition).

References

1. F. He, J. Wang, B. C. Yin and B. C. Ye, *Anal. Chem.*, 2018, **90**, 8072-8079.