

Modulation of intracellular transport pathways to enhance the transcytosis of nanodrugs across the blood-brain barrier

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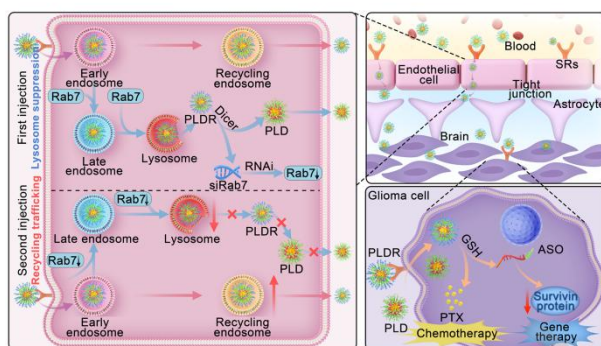
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Spherical nucleic acids (SNAs)-based nanocarriers (PLDR) enhance blood-brain barrier transcytosis by modulating the intracellular transport pathway of nanocarriers in brain microvessel endothelial cells, shifting from the lysosomal degradation pathway to the recycling pathway through the use of siRNA-mediated regulation of Rab GTPase protein expression. Consequently, this strategy enables enhanced synergistic chemo/gene therapy for glioma.

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ABSTRACT: Effective treatment of brain diseases is limited by poor blood-brain barrier (BBB) penetration of drugs and their nanosized carriers. The intracellular transport of nanocarriers in brain microvessel endothelial cells (BMECs) significantly affects BBB-crossing efficiency. Most nanocarriers follow the lysosomal degradation pathway, where they are trapped and degraded, limiting BBB penetration. Here, we develop spherical nucleic acids (SNAs)-based nanocarriers that enhance BBB traversal by redirecting intracellular transport in BMECs from lysosomal degradation pathway to recycling pathway. A hydrophobic redox-responsive paclitaxel (PTX) prodrug is covalently linked to a hydrophilic nucleic acid complex formed by connecting antisense oligonucleotides targeting survivin mRNA and siRNA targeting Rab7 via a DNA bridge. This produces an amphiphilic conjugate capable of self-assembling into SNAs. These SNAs enter BMECs via scavenger receptors and release siRNA, which reduces Rab7 expression and lysosome formation. Upon re-administration, SNAs are mainly transported through the recycling pathway rather than lysosomal degradation pathway, enhancing BBB crossing and tumor accumulation. High glutathione levels in tumor cells trigger the release of PTX and antisense oligonucleotides, enabling synergistic therapy of glioma. Our strategy addresses the challenge of limited BBB transcytosis and holds promise for improving drug delivery in neurological disorders.

KEYWORDS: blood-brain barrier, intracellular transport, spherical nucleic acids, drug delivery, amphiphilic conjugates

1 Introduction

Achieving efficient delivery of drugs to the brain is critical for advancing neuroscience research and improving the management of neurological disorders [1, 2]. However, the restrictive properties of the blood-brain barrier (BBB) pose a significant challenge to the effective and sufficient delivery of therapeutic agents to the central nervous system [3]. The BBB functions as a dynamic interface between the blood circulatory system and the central nervous system, comprising tightly junctioned brain microvascular endothelial cells (BMECs), pericytes, and astrocytic endfeet, which collectively regulate the translocation of substances into and out of the brain [4]. It has been reported that approximately 98 % of small-molecule drugs and the majority of macromolecular drugs are unable to penetrate the BBB effectively, thereby hindering the attainment of therapeutic concentrations at the tumor site [5]. Consequently, there is a critical need to develop innovative drug delivery systems capable of efficiently traversing the BBB and delivering therapeutic agents to the brain.

Currently, the predominant strategy for enabling drug nanocarriers to traverse the BBB is receptor-mediated transcytosis (RMT) [6]. This process primarily comprises

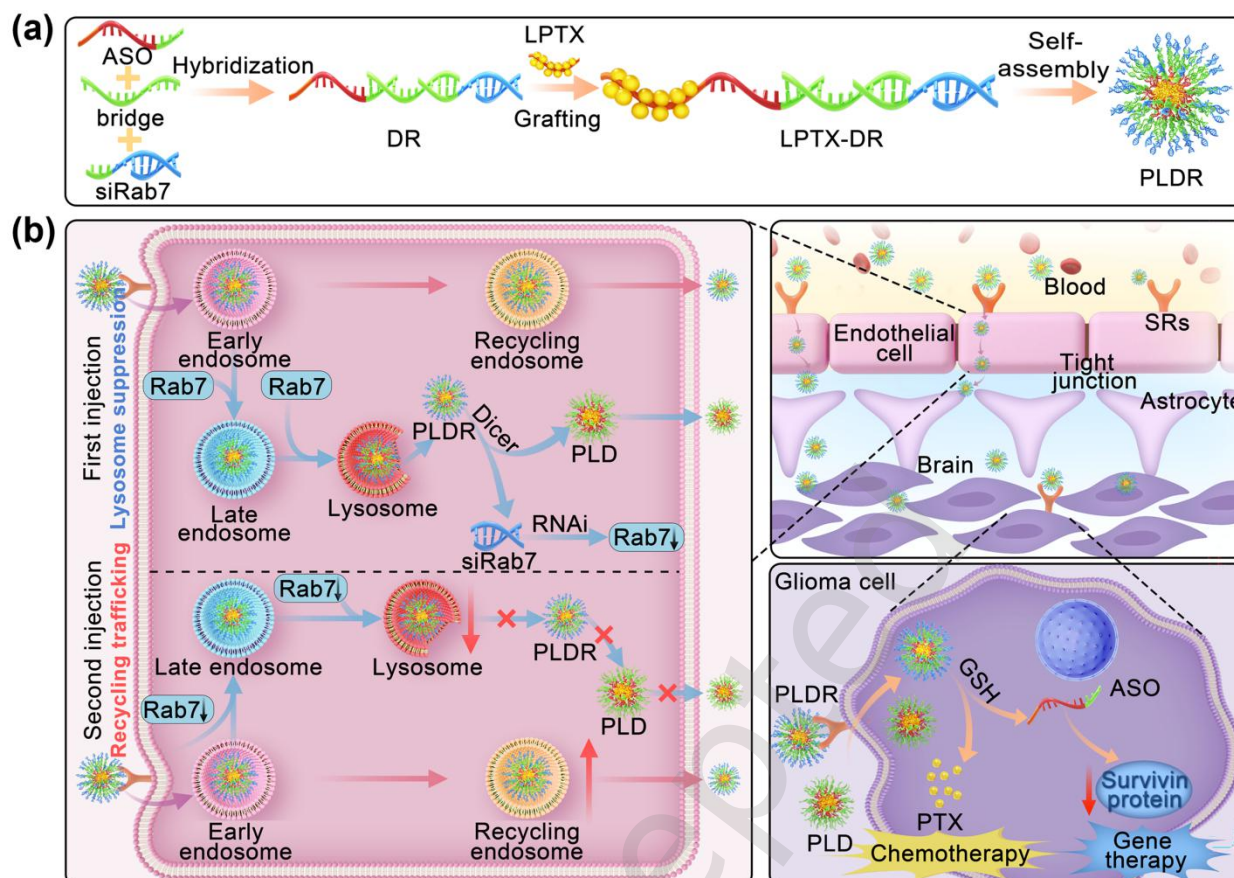
three sequential steps: (1) the recognition and binding of receptors on the luminal (blood) side of the BBB endothelium by ligands present on the nanocarriers, followed by endocytosis into the cell, (2) the intracellular transport of nanocarriers mediated by vesicles, and (3) the exocytosis of nanocarriers from the abluminal (brain) side of the BBB endothelium into the brain parenchyma [7, 8]. The intracellular transport of nanocarriers in BMECs is closely associated with the efficiency of BBB transcytosis [7, 9]. The transport process of nanocarriers within cells involves a series of complex subcellular vesicles, including early endosomes, recycling endosomes, late endosomes, and lysosomes, etc [10]. Following endocytosis into the BMECs cells, the majority of nanocarriers are initially transported to early endosomes, where they are subsequently sorted into various intracellular trafficking pathways [11]. A significant portion of these nanocarriers are routed to late endosomes and subsequently transported to lysosomes, a process commonly referred to as the lysosomal degradation pathway [11]. Due to the presence of various hydrolases within lysosomes and the unique acidic microenvironment they maintain, nanocarriers are prone to entrapment and degradation in this environment [12], which subsequently inhibits exocytosis and reduces their efficiency in crossing

the BBB. Conversely, a small fraction of nanocarriers enters the recycling pathway, wherein they are directed to recycling endosomes and subsequently transported to the plasma membrane, facilitating transcytosis [13]. Therefore, modulating intracellular transport pathways in the BMECs cells may serve as a promising strategy to enhance the BBB-crossing efficiency of nanocarriers, specifically by reducing the prevalence of the lysosomal degradation pathway and promoting alternative transport routes, such as the recycling pathway.

Rab GTPases are capable of specifically regulating intracellular vesicle transport [14]. Rab7 plays a key role in the trafficking of molecules from early endosomes to late endosomes, as well as from late endosomes to lysosomes [14]. Additionally, the Rab7 protein contributes to the maturation of late endosomes and lysosomes [15]. Therefore, we hypothesize that reducing lysosome formation by down-regulating Rab7 expression may decrease the transport of nanocarriers through the degradative pathway. As a result, nanocarriers may be less prone to degradation and more likely to be transported via alternative pathways, particularly the recycling pathway, thereby enhancing the efficiency of BBB transcytosis.

To this end, we herein prepared a spherical nucleic acids (SNAs)-based nanocarrier capable of delivering the chemotherapy drug paclitaxel (PTX) and genetic materials, specifically antisense oligodeoxynucleotide and siRNA, across the BBB for the treatment of brain tumors. The redox-responsive paclitaxel prodrug (designated LPTX), serving as the hydrophobic component, was covalently grafted onto a nucleic acid complex (designated DR), which acted as the hydrophilic segment, to yield an amphiphilic conjugate (designated LPTX-DR) (Scheme 1). This conjugate subsequently self-assembled into spherical nucleic acids,

termed PLDR. The DR complex was composed of an antisense oligodeoxynucleotide (ASO) and a siRNA duplex strand (siRab7), which specifically targeted the survivin and Rab7 proteins, respectively. Spherical nucleic acids (SNAs) exhibit a unique SNA effect, attributed to the densely packed and highly oriented nucleic acid structures on their surface [16]. Compared to free nucleic acids, SNAs demonstrate superior stability during blood circulation [17, 18]. Moreover, the SNA nanocarrier (PLDR) can be efficiently internalized by brain microvascular endothelial cells (BMECs) through the recognition of scavenger receptors (SRs), which were abundantly expressed on the surface of BMECs. Following cellular internalization, the siRNA duplex was released from SNAs by ribonuclease Dicer in the cytoplasm and subsequently suppressed Rab7 protein expression via the classical RNA interference (RNAi) pathway, thereby reducing lysosome formation. This reduction decreased SNAs transport through the lysosomal degradation pathway. Upon the second administration of spherical nucleic acids, they were more easily transported via recycling pathways, thereby enhancing the efficiency of BBB penetration and facilitating the effective delivery of therapeutic agents to the brain parenchyma. As a proof-of-concept, we selected glioblastoma (a malignant brain tumor) as the model for evaluating drug delivery efficacy and therapeutic outcomes. After traversing the BBB, SNAs specifically targeted scavenger receptors that were highly expressed on glioma cells, thereby enhancing their accumulation at the tumor site. Subsequently, the elevated glutathione levels within tumor cells triggered the release of two model therapeutic agents, paclitaxel (PTX) and antisense oligonucleotides (ASO). The downregulation of survivin expression by ASO synergized with PTX to effectively inhibit tumor growth.



Scheme 1 Design and synthesis of spherical nucleic acids to cross the BBB for the synergistic treatment of brain tumors. (a) Synthesis of the amphiphilic conjugate (LPTX-DR) and self-Assembly of spherical nucleic acids (PLDR). (b) Schematic illustration of the mechanism of spherical nucleic acids across the BBB to brain tumor for chemotherapy and gene therapy. The first injection of PLDR inhibited lysosome formation by down-regulating the expression of the Rab7 protein. The second injection of spherical nucleic acids preferentially crossed the BBB via the recycling pathway rather than the lysosomal degradation pathway.

2 Experimental

2.1 Materials

3,3'-Dithiodipropionic acid, acetic anhydride, toluene, dichloromethane (DCM), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), acetone, dithiothreitol (DTT), dimethyl sulfoxide (DMSO), polyinosinic acid (poly I), glutathione (GSH), pyrene and FITC-labeled dextrans (4, 10 and 70 kDa) were purchased from Aladdin Reagent Co., Ltd (Shanghai, China). (4-(bromomethyl)phenyl)methanol, 4-dimethylaminopyridine (DMAP) and paclitaxel (PTX) were purchased from Energy Chemical Technology Co., Ltd. (Shanghai, China). DNA and RNA strands were obtained by Sangon Biotech Co., Ltd (Shanghai, China). Calcein acetoxymethyl ester (calcein AM), propidium iodide (PI), lyso-tracker green, Hoechst 33342, penicillin streptomycin (PS), non-essential amino acids (NEAA), sodium pyruvate (SP), puromycin and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Beyotime Biotechnology (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. Dulbecco's Modified Eagle Medium (DMEM) with 1.5 g/L sodium bicarbonate was obtained from iCell Bioscience Inc. (Shanghai, China). Trypsin was purchased from Shanghai Xiaopeng Biotechnology Co., Ltd. The anti-EEA1, anti-VAMP 3 and anti-SNAP 23 antibodies were purchased from

Proteintech Group, Inc. All reagents were analytical grade or higher and used without further purification.

2.2 Cell culture and animals

The bEnd.3 cells (murine brain microvascular endothelial cells) were cultured in DMEM containing NaHCO_3 (1.5 g/L), 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (PS). U87MG cells (human glioblastoma tumor cells) were incubated in DMEM medium containing 10% FBS, 1% PS, and 1% non-essential amino acids (NEAA) and 1% sodium pyruvate (SP). For in vivo bioluminescence monitoring of glioma growth, U87MG-Luc cells — a luciferase-expressing human glioblastoma cell line derived from parental U87MG cells via lentiviral transduction — were used to establish the orthotopic glioblastoma mouse model. U87MG-Luc cells were cultured in DMEM medium supplemented with 10% FBS, 1% PS, 1% NEAA, 1% SP, and 0.01% puromycin. All the above cell lines were obtained from Procell Life Science & Technology Co., Ltd. (Hubei, China) and incubated at 37 °C in a humidified incubator with 5% CO_2 .

Female BALB/c nude mice (6-7 weeks old) were purchased from China Three Gorges University (China).

2.3 Preparation and characterization of PLDR

Synthesis of Linker

The GSH-responsive linker was synthesized according to

previous literature [19]. Specifically, the 3,3'-dithiodipropionic acid (4 mmol) was dissolved in acetic anhydride (3 mL) and stirred under the nitrogen atmosphere at room temperature. After 2 h, toluene (20 mL) was added to the above mixture solution to remove the acetic acid, and the solvent was subsequently evaporated using a rotary evaporator. The resulting intermediate product was dissolved in anhydrous DCM (30 mL), followed by the addition of 4-(bromomethyl)benzyl alcohol (4 mmol) and DMAP (0.4 mmol). The reaction mixture was stirred at 30 °C overnight. The crude product was purified via silica gel column chromatography using a petroleum ether/ethyl acetate mobile phase to afford the GSH-responsive linker. The chemical structure of the linker was confirmed by ^1H -NMR spectroscopy, and the corresponding spectrum was presented in Fig. S1 in the ESM.

Synthesis of LPTX

The obtained linker (21 mg), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (12 mg), PTX (30 mg) and DMAP (6 mg) were added to the anhydrous DCM and stirred for 24 h at room temperature. Then, the mixture solution was purified by silica gel column chromatography using petroleum ether/ethyl acetate mobile phase to obtain LPTX. The chemical structure of the LPTX was confirmed by ^1H -NMR spectroscopy, and the corresponding spectrum was presented in Fig. S2 in the ESM.

Synthesis of PLDR

The ASO (3 μL , 50 μM), DNA bridge (3 μL , 50 μM) and siRab7 (3 μL , 50 μM) were added to PBS (pH=7.4, 3 μL) and shaken for 5 h at 37 °C to obtain DR. Subsequently, LPTX was dissolved in DMSO solution (20 mg/mL, 2 μL) and added to the above-mentioned DR solution. The resulting mixed solution was reacted overnight in a shaker at 55 °C. Then, the reaction solution was subjected to centrifugal ultrafiltration to remove the unreacted LPTX. Finally, the collected conjugate LPTX-DR was dispersed in water to form spherical nucleic acid (PLDR) through self-assembly. Other spherical nucleic acids (SNAs), including PLNR, PLDN, PLD and PLN, were also fabricated using the same methodology as PLDR, serving as control groups. PLDN comprised PTX, ASO and a non-specific siRNA sequence. PLNR comprised PTX, a non-specific DNA sequence and siRab7. PLD comprised PTX and ASO. PLN comprised PTX and a non-specific DNA sequence.

The hydrodynamic size of spherical nucleic acids was determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd UK). The morphology of spherical nucleic acids was observed by transmission electron microscope (JEM-100CXII).

2.4 Critical micelle concentration (CMC)

The CMC of LPTX-DR was detected by using pyrene as a fluorescence probe [20]. The pyrene acetone solutions (2 mg/mL, 50 μL) were prepared in a series of sample bottles respectively, which were placed in an oven in the dark and allowed to stand at a constant temperature of 30 °C overnight. After the acetone was volatilized, varying concentrations of LPTX-DR (0.0023, 0.0047, 0.0094, 0.0188, 0.0375, 0.056, 0.075, 0.1125, 0.15, 0.225, 0.3, 0.45, 0.9, 1.2,

1.8, 2.4, 3.6 and 4.8 μM) aqueous solutions were added to the above sample bottles. Then, these sample bottles were kept away from light and allowed to stand at room temperature for 12 h. Subsequently, the fluorescence emission spectrum of pyrene in each sample bottle was determined by a fluorescence spectrometer. The excitation wavelength was set to 334 nm, and the emission wavelength range was set to 350 nm to 500 nm. After the test, a relationship curve was drawn based on the ratio of the fluorescence intensity at 394 nm and the fluorescence intensity at 378 nm of pyrene to the logarithm of the concentration in the sample solution. The critical micelle concentration value of LPTX-DR was determined by obtaining the intersection point of the tangents on both sides of the curve.

2.5 Drug release kinetics

To assess the drug release behavior, PLDR (containing 1 mg PTX) solution was transferred into a dialysis bag with a cutoff molecular weight of 3 kDa. This setup was then immersed in 5 mL of PBS (pH 7.4) supplemented with 10 mM DTT and subjected to agitation at 37 °C on a shaking table set to 100 rpm. Meanwhile, PLDR was immersed in the PBS solution without DTT as the control group. At specific time intervals, the amount of PTX released outside the dialysis bag was determined by high-performance liquid chromatography (HPLC). The HPLC system included a C18 column and a UV detector. The mobile phase was composed of HPLC grade acetonitrile and deionized water (5:45, v/v). The flow rate was set at 1.0 mL/min. PTX in the eluent was detected at the wavelength of 227 nm. The drug release kinetics were determined by calculating the cumulative release amounts at various time intervals.

2.6 Cell uptake assay

To evaluate cellular internalization of SNAs, the bEnd.3 cells or U87MG cells were seeded in glass-bottom dishes (1×10^6 cells/dish) and incubated for 24 h. Cells were incubated with Cy5-labeled spherical nucleic acids and Cy5-labeled nucleic acids ($C_{\text{Cy5}} = 0.15 \mu\text{M}$) for different times. Subsequently, the cells underwent three washes with PBS, after which the cell nuclei were stained with Hoechst 33342 (5 $\mu\text{g/mL}$, 10 min). The intracellular fluorescence was observed using confocal laser scanning microscopy (CLSM, Carl Zeiss LSM880), exciting at 633 nm and collecting emission in the range of 650-750 nm.

For flow cytometry analysis, the bEnd.3 cells or U87MG cells were seeded in six-well plates (2×10^5 cells/ per well) and cultured for 24 h. Next, Cy5-labeled spherical nucleic acids and Cy5-labeled nucleic acids ($C_{\text{Cy5}} = 0.15 \mu\text{M}$) were added in six-well plates and cultured with cells for different times. After incubation, cells were washed with PBS, digested with trypsin, and re-suspended in PBS to prepare single-cell suspensions. For quantitative fluorescence analysis, 10,000 viable single cells were collected per sample. Detection was performed on a BD Accuri C6 flow cytometer using the APC channel (excitation: 633 nm; emission: 660/20 nm bandpass filter).

To explore a role for scavenger receptors (SRs) in mediating the endocytosis of SNAs, the inhibitor blocking

assay was performed by introducing polyinosinic acid (poly I). The bEnd.3 cells or U87MG cells were treated with Cy5-labeled spherical nucleic acids ($C_{Cy5} = 0.15 \mu\text{M}$) in the presence of poly I (0.2 mg/mL) for 8 h. The cells treated with Cy5-labeled spherical nucleic acids in the absence of poly I for 8 h were used as a control group for comparative analysis. Subsequently, the cells were washed with PBS and digested into a cell suspension using trypsin. The intracellular fluorescence intensity was quantified via flow cytometry.

2.7 Cytotoxicity assay

The bEnd.3 cells were seeded at a 96-well plate (1×10^5 cells/well) and incubated overnight. Then, different SNAs (PLDR, PLDN and PLNR) at various concentrations (25, 37.5, 50, 75, 100, 150 and 300 nM) were added to each well. After 48 h incubation, MTT solution (5 mg/mL , $20 \mu\text{L}$) was added into wells and incubated for another 4 h. Finally, the solution in the well plate was removed and replaced with DMSO ($150 \mu\text{L}$). The absorbance of each well at 490 nm was measured on the microplate reader (Bio-Rad 550). Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_0}{A_{\text{control}} - A_0}$$

A_{sample} was measured from the cells treated with SNAs and MTT. A_{control} was measured from the cells treated with MTT only. A_0 was measured from untreated cells.

The in vitro anti-tumor effect of SNAs was evaluated in U87MG cells by an MTT assay and calcein AM/PI co-staining assay. The U87MG cells were treated with the different SNAs (PLDR, PLDN, PLD, PLNR and PLN) with various concentrations (100, 150 and 300 nM). After 48 h incubation, the cell viability was detected by the aforementioned method. For calcein AM/PI co-staining assay, the U87MG cells were treated with different SNAs (PLDR, PLNR and PLN) at a concentration of $0.3 \mu\text{M}$ for 48 h. After washing three times with PBS, the cells were stained with Hoechst 33342 ($5 \mu\text{g/mL}$, 10 min) to visualize nuclei, with calcein-AM ($1 \mu\text{M}$, 30 min) to identify live cells, and with propidium iodide ($1 \mu\text{M}$, 30 min) to assess cell death. The fluorescence signals in cells were observed using confocal laser scanning microscopy (CLSM, Carl Zeiss LSM880).

2.8 Lysosome reduction assay

The bEnd.3 cells were seeded in glass-bottom dishes (1×10^5 cells/dish) and incubated for 24 h. Then, cells were cultured with the medium containing different SNAs ($0.3 \mu\text{M}$) for different time. After washing three times with PBS, the medium containing Hoechst 33342 ($5 \mu\text{g/mL}$) and lyso-tracker green ($1 \mu\text{M}$) was added and incubated for 10 min. After that, the medium was aspirated and washed three times with PBS for confocal laser scanning and flow cytometry analysis. Cells that were not subjected to any treatment were set as the control group.

2.9 Intracellular transport pathway assay

To investigate whether a reduction in lysosome quantity can modulate the intracellular trafficking pathways of spherical nucleic acids, bEnd.3 cells were initially pre-incubated with PLDR ($0.3 \mu\text{M}$) for 24 h, followed by treatment with Cy5-

PLDR ($0.15 \mu\text{M}$) for an additional 6 h. After washing three times with PBS, the cells were incubated overnight with specific primary antibodies, followed by fluorescently labeled secondary antibodies to detect the primary antibodies, enabling visualization of the early endosome marker EEA1, the recycling endosome marker VAMP 3, and SNARE complexes. Cy3-labeled anti-EEA1 antibody, Cy3-labeled anti-VAMP-3 antibody, and FITC-labeled anti-SNAP-23 antibody were used for labeling EEA1, VAMP-3, and SNARE complexes, respectively. Lysosomes were stained with LysoTracker Green. Subsequently, the co-localization of Cy5-PLDR and fluorescently labeled transport vesicles was examined using confocal laser scanning microscopy. The Pearson's correlation coefficient was calculated using ImageJ software.

2.10 BBB crossing efficiency in vitro

In order to evaluate the in vitro BBB crossing efficiency of different SNAs, the BBB model in vitro was conducted using LabScler Transwell polyester membrane cell culture inserts having a mean pore size of $0.4 \mu\text{m}$, a diameter of 12 mm, and an area of 1.1 cm^2 . The bEnd.3 cells (1×10^6 cells/well) were seeded into the apical chamber to form a dense cell monolayer. The structure integrity and barrier function of the BBB model in vitro were evaluated through the expression of tight junction proteins and the permeability of FITC-labeled dextrans with different molecular weights (4, 10 and 70 kDa). FITC-labeled dextrans ($500 \mu\text{g/mL}$) were added to the apical chamber and incubated with cells for 2 h, respectively. Following the incubation period, the culture medium in the basolateral chamber was collected for fluorescence measurement and the amount of FITC-labeled dextran was calculated based on the standard concentration curves for each FITC-labeled dextran. Then, the apparent permeability coefficient (P_{app}) was calculated as [21]:

$$P_{\text{app}} (\text{cm/s}) = (Q/t) \times (1/A) \times (1/C)$$

Q represented the total amount of FITC-labeled dextrans that had permeated through the apical chamber (μg), A was the surface area of the transwell filter (cm^2), C was the initial concentration of the FITC-labeled dextrans in the apical chamber ($\mu\text{g/mL}$), and t referred to the duration of the experiment (s).

After the successful establishment of the in vitro BBB model, U87MG cells were seeded into the basolateral chamber for the BBB permeability experiment. Different SNAs (PLDR or PLDN, $0.3 \mu\text{M}$) were added to the apical chamber for a 24 h pre-incubation. Subsequently, Cy5-PLDR or Cy5-PLDN ($0.3 \mu\text{M}$) were added to the apical chamber, followed by another 12 h incubation, respectively. After the completion of incubation, U87MG cells were collected for flow cytometry analysis to assess the BBB crossing efficiency of SNAs. The group in which only Cy5-PLDR was added into the in vitro BBB model was designated as the control group.

2.11 In vivo fluorescence imaging in healthy mice and orthotopic glioblastoma mice model

The orthotopic glioblastoma mice model was conducted using female BALB/c nude mice ($20 \pm 2 \text{ g}$). The mice were anesthetized with isoflurane gas, and their brains were

immobilized in a stereotaxic instrument. Subsequently, the external skin on the brains of mice was incised with surgical scissors. Then, a small hole in the right striatum (right lateral: 2.0 mm, bregma: 1.0 mm, depth: 2.5 mm) was drilled using a cranial drill. After that, U87-Luc cells were injected into the small hole using a mouse adaptor. Finally, the wound was sutured with bone glue, and tumor growth was monitored through bioluminescent signals after the mice were intraperitoneally injected with the luciferase substrate D-luciferin potassium (7.5 mg/mL in PBS, 125 μ L) at 47 mg/kg for 5 min.

To investigate the enrichment of SNAs and free nucleic acids in the brains of mice (healthy BALB/c nude mice and orthotopic glioblastoma mice), Cy5-DR and Cy5-PLDR (10 μ M, 200 μ L) were injected into the mice via the tail vein ($n = 3$), respectively. The fluorescence signals in the brain were monitored using an in vivo imaging system (IVIS Spectrum, PerkinElmer).

To further validate the effect of siRab7 on the BBB transcytosis of subsequently added SNAs in vivo, PLDR and PLDN (6 μ M, 200 μ L) were injected into the mice via the tail vein ($n = 3$). After 24 h post injection, Cy5-PLDR and Cy5-PLDN (6 μ M, 200 μ L) were injected into the mice via the tail vein. Mice without pre-injection were set as control group. The fluorescence signals in the brain were monitored using an in vivo imaging system (IVIS Spectrum, PerkinElmer).

2.12 In vivo antitumor assay.

Different SNAs, including PLDR, PLDN and PLNR (25 μ M, 200 μ L), were administered to orthotopic glioblastoma mice via tail vein injection, with a second dose delivered 24 h later. PBS was injected into mice as the control group. Then, tumor growth was monitored using bioluminescence imaging, with images recorded every other day during the 16 days treatment period. On the 16th day of treatment, mice were sacrificed to collect the brains for hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase uridine triphosphate nick end labeling (TUNEL) staining.

2.13 Biosafety evaluation

Different SNAs, including PLDR, PLDN and PLNR (25 μ M, 200 μ L), were intravenously injected into the healthy BALB/c mice ($n = 3$). PBS-treated mice were set as control group. After the 30th day post-injection, the mice were euthanized to obtain major organs (heart, liver, spleen, lung, kidney, and brain) and the blood samples. The obtained major organs were fixed in 4 % paraformaldehyde, paraffin embedded, sectioned at 4 μ m and stained with hematoxylin and eosin (H&E). Moreover, the multiple indicators of blood routine examination including red blood count (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were analyzed. Furthermore, the levels of liver function indicators (alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) as well as kidney function indicators (creatinine kinase (CK), blood urea nitrogen (BUN) and creatinine (CREA)) were detected by a blood biochemistry analyzer.

In order to explore the impact of SNAs on the BBB integrity, the Evans blue (EB) assay was conducted. Mice

were administered PLDR for varying durations (1, 2, 3, 4, 7, 10 and 20 days), while the control group received PBS treatment. All mice then received an intraperitoneal injection of 100 μ L of 2% EB solution prepared in PBS. After 30 min, mice were euthanized via cervical dislocation following retro-orbital bleeding, and whole brains were harvested. Brain tissues were homogenized in PBS, and the homogenate was centrifuged (3000 rpm for 10 min). The resulting supernatant was collected for absorbance at 620 nm using a microplate reader (Bio-Rad 550).

2.14 Statistical analysis

Statistical significance was determined using Student's t-test (two-tailed) in Excel, in which $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****) are considered statistically significant, while ns means no statistical difference.

3 Results and discussion

3.1 Preparation and characterization of spherical nucleic acids

An amphiphilic conjugate (LPTX-DR) was synthesized through the following three sequential steps, as outlined in Scheme 1: (i) the conjugation of ASO and siRab7 via a DNA bridge to form a nucleic acid complex (DR), (ii) the synthesis of a redox-responsive paclitaxel prodrug (LPTX), and (iii) the subsequent attachment of LPTX to DR. First, the DNA bridge was employed to conjugate ASO with siRab7 via complementary base pairing to generate the DR, following previously reported literature procedures [22]. Nucleic acid sequences used in this study were presented in Table S1 in the ESM. Polyacrylamide gel electrophoresis (PAGE) was utilized to validate the successful preparation of DR, as evidenced by the presence of high molecular weight products following co-incubation with ASO, siRab7, and DNA bridge (lane 6, Fig. 1(a)). Subsequently, the redox-responsive paclitaxel prodrug (LPTX) was synthesized through an esterification reaction between paclitaxel (PTX), which contained hydroxyl groups, and a disulfide linker bearing carboxylic acid groups. The successful preparation of the disulfide linker and LPTX was verified by $^1\text{H-NMR}$ (Figs. S1 and S2 in the ESM). Ultimately, the benzyl bromide group of LPTX reacted with the phosphorothioate group at the ASO terminus of DR, leading to the formation of the amphiphilic conjugate LPTX-DR (Fig. S3 in the ESM).

Notably, the amphiphilic conjugate LPTX-DR was capable of self-assembling into spherical nucleic acids-based micellar nanocarriers (PLDR) in aqueous solution, attributed to the presence of hydrophilic components (DR) and hydrophobic components (LPTX). Further, the amphipathicity and self-assembly property of LPTX-DR were examined by measuring the critical micelle concentration (CMC) in H_2O using pyrene as a fluorescence probe (Fig. 1(b)). The LPTX-DR conjugate exhibited a CMC value of 2.8×10^{-7} M, indicating its amphiphilic property and its capacity to maintain an aggregated state even at a low concentration. Analysis by 1% agarose gel electrophoresis (AGE) demonstrated a reduced migration rate of PLDR compared to DR (Fig. 1(c)), indicating the successful assembly of PLDR. The hydrodynamic diameter of PLDR

nanocarriers, as measured by dynamic light scattering (DLS), was approximately 60 nm (Fig. 1(d)), corroborating the findings obtained from transmission electron microscopy (TEM). Furthermore, TEM analysis revealed the distinct spherical morphology of the PLDR nanocarriers (Fig. 1(e)). Additionally, the stability of the PLDR was substantiated, as evidenced by the relatively stable particle size observed after storage in water at 4 °C for a duration of seven days (Fig. 1(f)). The Cy5 fluorophore-labeled ASO (Cy5-ASO) and Cy3 fluorophore-labeled siRab7 (Cy3-siRab7) were employed to assess the loading capacity of ASO and siRab7 on the PLDR nanocarriers. Based on the standard curves for Cy5-ASO and Cy3-siRab7, the encapsulation efficiencies for ASO and siRab7 were determined to be 76.7% and 50%, respectively (Fig. S4 in the ESM). Furthermore, the ratio of ASO to siRab7 within the PLDR was found to be 23:15. To investigate the redox-responsive drug release behavior of PLDR, they were incubated with 10 mM dithiothreitol (DTT) to simulate the intracellular redox environment of tumor cells. As shown in Fig. 1(g), nearly complete release of PTX

from PLDR was observed after 48 hours of exposure to the reducing conditions, whereas only minimal PTX release occurred under non-reducing conditions. These findings were attributed to the cleavage of disulfide bonds within PLDR under reducing conditions. The redox-responsive nature of PLDR was further supported by 1% agarose gel electrophoresis (AGE) analysis. Notably, incubation of PLDR with DTT for 24 hours led to the appearance of new bands with lower molecular weight (Fig. 1(h)). Other spherical nucleic acids (SNA)-based nanocarriers were also fabricated using the same methodology as PLDR, serving as control groups. These included PLDN (comprising ASO and a non-specific siRNA sequence), PLNR (comprising a non-specific DNA sequence and siRab7), PLD (comprising ASO only), and PLN (comprising a non-specific DNA sequence only). The descriptions of these nanocarriers were given in the list of abbreviations (Table S2 in the ESM). All control SNAs displayed comparable particle sizes and maintained excellent colloidal stability (Figs. 1(d) and 1(f)).

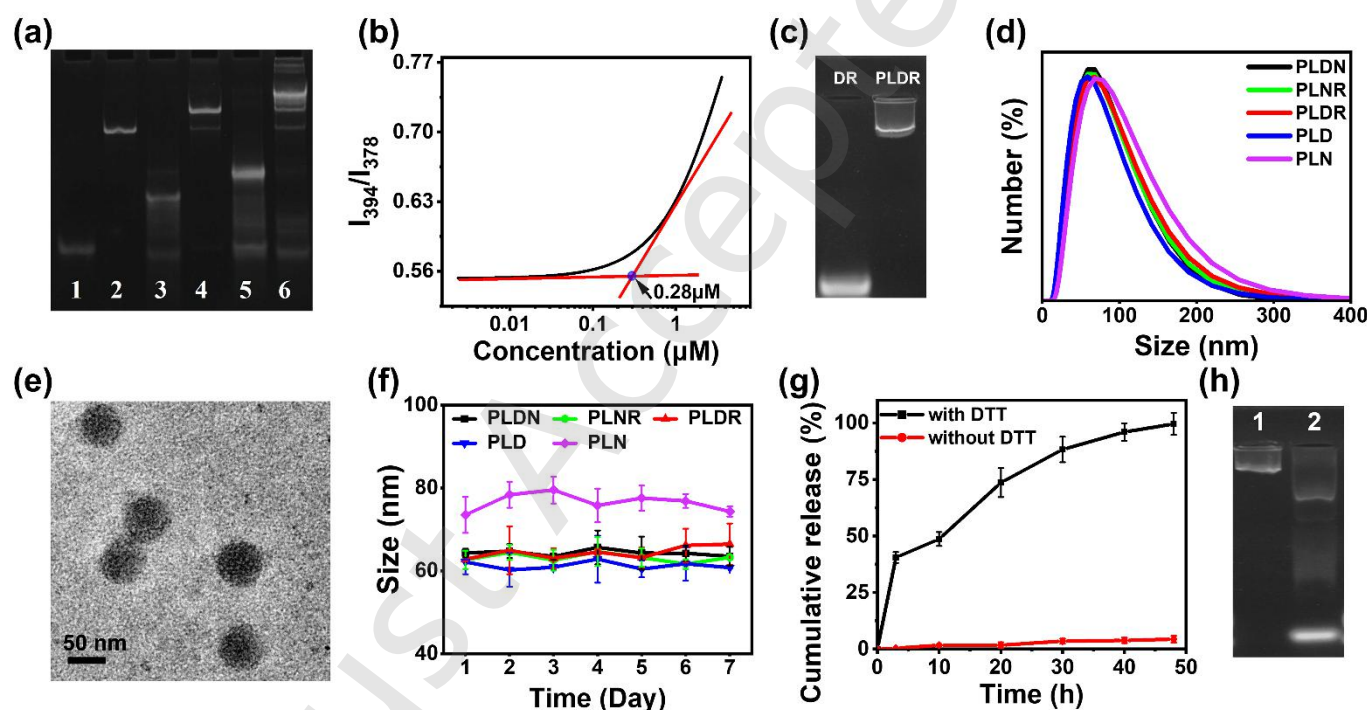


Figure 1 Preparation and characterization of SNAs. (a) Polyacrylamide gel electrophoresis (PAGE) analysis of DNA bridge (1), ASO (2), siRab7 (3), nucleic acid complex composed of ASO and DNA bridge (4), nucleic acid complex composed of DNA bridge and siRab7 (5), nucleic acid complex composed of ASO, DNA bridge and siRab7 (6). (b) Critical micelle concentration (CMC) of PLDR. (c) Agarose gel electrophoresis (AGE) analysis of DR and PLDR. (d) Hydrodynamic size distribution of SNAs. (e) TEM image of PLDR. Scale bar: 50 nm. (f) The size changes of SNAs in deionized water during the course of 7 days. (g) The release curves of PTX from PLDR in the presence or absence of DTT. (h) Agarose gel electrophoresis (AGE) analysis of PLDR before (lane 1) and after (lane 2) 24-hour incubation with DTT.

3.2 Study on the cellular uptake of spherical nucleic acids by BMECs

Brain microvascular endothelial cells (BMECs) constitute the core structural component of the blood-brain barrier (BBB). The bEnd.3 cell line, an immortalized murine brain endothelial cell line, has been widely employed in BBB delivery studies. Accordingly, in this study, we first evaluated the cytotoxicity of spherical nucleic acids (PLDR, PLDN, and PLNR) against bEnd.3 cells using the MTT assay. Even at a concentration of 300 nM, these SNAs exhibited negligible cytotoxicity against the cells following a 48-h incubation (Fig. S5 in the ESM), indicating favorable

biocompatibility. This indicated that in brain microvascular endothelial cells, the low concentration of GSH was insufficient to trigger disulfide bond cleavage in PLDR, thereby preventing the subsequent release of the anticancer agent (PTX and ASO) [23]. This aligned with our expectation that these SNAs cannot induce damage to the BMECs during their translocation across the BBB, thereby minimizing potential toxic and side effects.

The cellular internalization of PLDR by bEnd.3 cells was examined using confocal laser scanning microscopy (CLSM) and flow cytometry (FCM). For fluorescence imaging analysis, Cy5 fluorophore-labeled ASO was utilized as a

substitute for native ASO to prepare fluorescently labeled PLDR nanocarriers (Cy5-PLDR) and fluorescently labeled nucleic acid complexes (Cy5-DR), following the same synthesis protocol as their unlabeled counterparts. As illustrated in Fig. 2(a), the red fluorescence signals in cells treated with Cy5-PLDR progressively intensified over time, suggesting a time-dependent cellular uptake behavior. Furthermore, the Cy5-PLDR-treated group demonstrated significantly brighter red luminescence compared to the Cy5-DR-treated group, both after 4 hours and 6 hours of incubation. These observations were corroborated by flow cytometry (FCM) analysis. Specifically, the intracellular fluorescence intensity of Cy5-PLDR was found to be approximately twice that of Cy5-DR following a 6-hour incubation with bEnd.3 cells (Figs. 2(b) and 2(c)), indicating the higher cell uptake of Cy5-PLDR (spherical nucleic acids) than DR (nucleic acid complexes). This phenomenon may be attributed to the unique three-dimensional (3D) structure of spherical nucleic acids (SNAs). In contrast to free nucleic acids and conventional nucleic acid complexes, the densely packed and highly oriented arrangement of nucleic acids on

the surface of SNAs allows them to exhibit the SNA effect [24]. Specifically, SNAs possess the capability to selectively bind to scavenger receptors (SRs) expressed on the cell membrane, thereby enhancing cellular uptake [25]. It has been reported that brain microvascular endothelial cells, which constitute the primary component of the BBB, highly express scavenger receptors [25]. Based on this observation, it is hypothesized that Cy5-PLDR can specifically bind to these receptors, thereby facilitating cellular uptake.

To validate this hypothesis, we performed competitive experiments. Specifically, bEnd.3 cells were co-cultured with Cy5-PLDR and polyinosinic acid (poly I), a known competitive inhibitor of scavenger receptors (SRs), for a duration of six hours. During this process, poly I was able to compete with Cy5-PLDR for binding to scavenger receptor sites. FCM analysis indicated that the intracellular fluorescence intensity of Cy5-PLDR was significantly reduced by 94% in the presence of poly I, demonstrating that the endocytosis of Cy5-PLDR was mediated by scavenger receptors (SRs) (Figs. 2(d) and 2(e)).

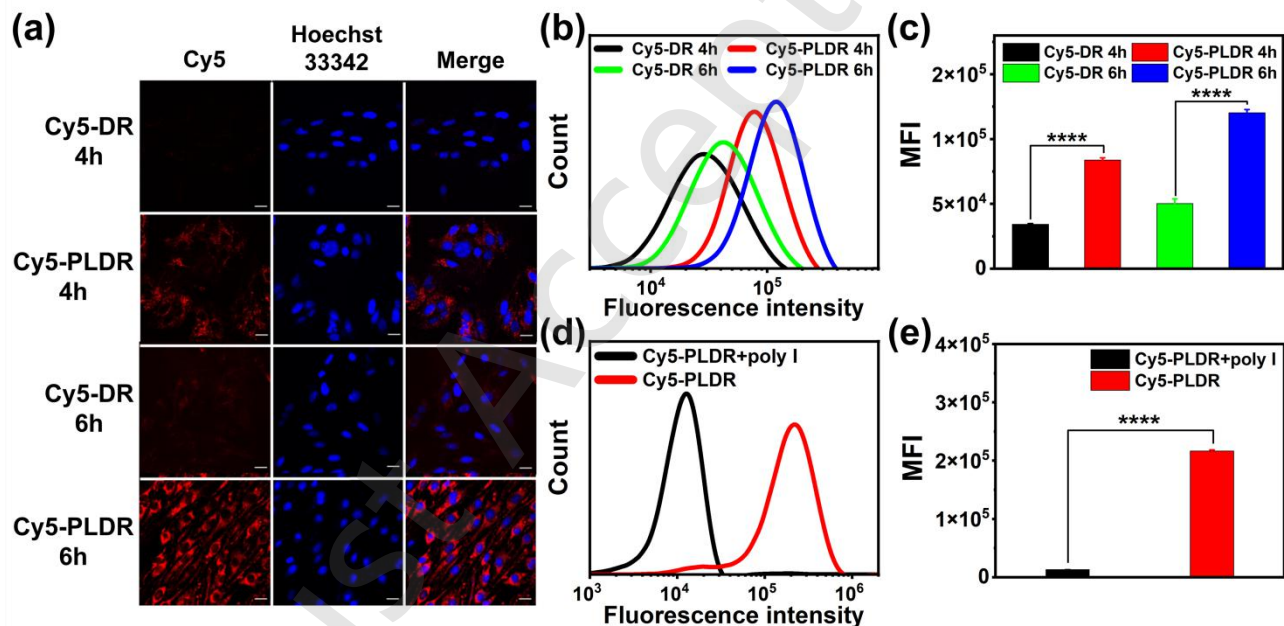


Figure 2 Cellular uptake of SNAs by BMECs. (a) CLSM images, (b) flow cytometry analysis and (c) mean fluorescence intensity (MFI) of bEnd.3 cells at 4 hours or 6 hours post-incubation with Cy5-DR and Cy5-PLDR with a concentration of 0.15 μ M. Cell nuclei were stained with Hoechst 33342 (blue). Scale bar: 20 μ m. (d) Flow cytometry analysis and (e) mean fluorescence intensity (MFI) of bEnd.3 cells treated with Cy5-PLDR (0.15 μ M) in the presence or absence of poly I (0.2 mg/mL) for 6 hours. Data are presented as mean $\pm$ standard deviation (SD) ($n \geq 3$). **** $P < 0.0001$ is considered statistically significant.

3.3 Regulating intracellular transport pathway of spherical nucleic acids in BMECs

To investigate whether siRab7 modified on the surface of spherical nucleic acids (SNAs) could influence the transport pathways of SNAs in BMECs, the regulatory effect of siRab7 on Rab7 protein expression was first assessed. Western blot (WB) analysis demonstrated that the Rab7 protein expression level in bEnd.3 cells was significantly reduced following a 24-hour co-incubation with PLDR (Figs. 3(a) and 3(b)). These findings suggested that the siRNA duplex can be released from SNAs by the ribonuclease Dicer in the cytoplasm and subsequently suppress Rab7 protein expression via the classical RNA interference (RNAi) mechanism. It is reported that the Rab7 protein is involved

in the maturation of the late endosome [15]. And the lysosome is formed from the fusion of the primary lysosome and the late endosome [26]. Therefore, we hypothesized that the down-regulation of Rab7 protein expression might influence lysosome formation. To validate this hypothesis, lysosomes were stained with Lyso-Tracker Green to evaluate the impact of siRab7 in PLDR on lysosome quantity. As illustrated in Fig. 3(c), the green fluorescence intensity of lysosomes in cells exposed to PLDR was markedly diminished compared to those treated with PLDR containing a control random RNA sequence and the untreated control group, irrespective of whether the incubation duration was 12 or 24 hours. Furthermore, prolonging the incubation period resulted in an even greater decrease in lysosome

numbers. This finding indicated that PLDR may lead to a reduction in lysosome numbers. In contrast, no significant difference was observed in the green fluorescence intensity of lysosomes between cells treated with PLDR and the untreated control cells. These results substantiated that siRab7 in PLDR indeed suppressed lysosome biogenesis by down-regulating Rab7 protein expression. The FCM analysis results were consistent with those obtained from CLSM

imaging (Figs. 3(d) and 3(e)). Following a 24-hour co-incubation of PLDR with cells, the lysosome numbers exhibited the most significant reduction, as evidenced by the lowest lysosomal fluorescence intensity. Therefore, in subsequent experiments, a 24-hour incubation period was employed to modulate the intracellular transport pathway of PLDR and to evaluate its impact on the efficiency of PLDR crossing the BBB.

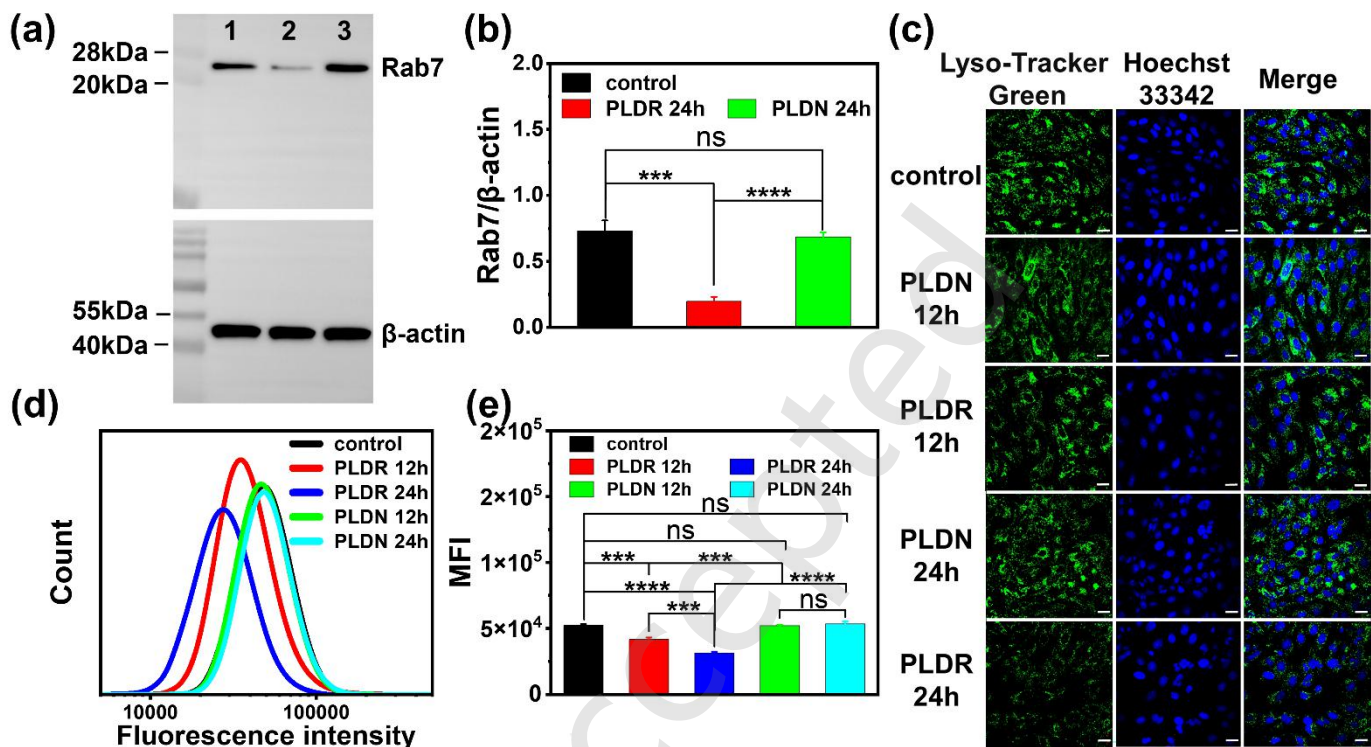


Figure 3 SNAs influenced lysosome formation of BMECs. (a) Western blot analysis of the expression of Rab7 in bEnd.3 cells co-incubated with PLDR (2) and PLDN (3) for 24 hours. The bEnd.3 cells without any treatment served as control group (1). (b) The ratio of Rab7 and β-actin expression in bEnd.3 cells following a 24 hours co-incubation with PLDR or PLDN. (c) CLSM images, (d) flow cytometry analysis and (e) mean fluorescence intensity (MFI) of lysosomes stained with Lyso-Tracker Green in bEnd.3 cells incubated with PLDR and PLDN for 12 hours or 24 hours. The bEnd.3 cells without any treatment served as control group. Scale bar: 20 μm. Data are presented as mean ± SD (n ≥ 3). ***P < 0.001 and ****P < 0.0001 are considered statistically significant, while ns means no statistical difference.

To investigate whether a reduction in lysosome quantity can modulate the intracellular trafficking pathways of spherical nucleic acids, bEnd.3 cells were initially pre-incubated with unlabeled spherical nucleic acids (PLDR) for 24 hours to act on lysosomes, followed by treatment with fluorescently labeled spherical nucleic acids (Cy5-PLDR) for an additional 6 hours. A control group was established by incubating cells solely with Cy5-PLDR for 6 hours without prior PLDR pre-incubation. Early endosomes and recycling endosomes were labeled with Cy3-labeled anti-EEA1 antibody and Cy3-labeled anti-VAMP 3 antibody, respectively. EEA1 and VAMP 3 served as markers for early endosomes and recycling endosomes, respectively. Lysosomes were stained with Lyso-Tracker Green. Subsequently, the colocalization of Cy5-PLDR with these vesicular markers was analyzed by CLSM to investigate the intracellular transport of Cy5-PLDR in bEnd.3 cells. In unpretreated bEnd.3 cells, Cy5-PLDR colocalized with early endosomes, where they were sorted to different routes (Fig. 4(a)). Cy5-PLDR demonstrated strong colocalization with lysosomes but limited colocalization with recycling endosomes, suggesting that Cy5-PLDR primarily follows the degradation route (Figs. 4(b) and 4(c)). By contrast,

following pretreatment of bEnd.3 cells with PLDR for 24 hours, the numbers of lysosomes were significantly reduced, whereas the numbers of early endosomes and recycling endosomes increased (Figs. 4(b) and 4(c)). Furthermore, western blot analysis also confirmed that the expression levels of the early endosomal marker EEA1 and the recycling endosomal marker Rab11 were elevated in PLDR-pretreated bEnd.3 cells compared to those in untreated control cells (Figs. 4(d)-4(f)), which may affect the intracellular transport of SNAs. Interestingly, following a 24-hour pretreatment of bEnd.3 cells with PLDR, a significant change was observed in the colocalization of Cy5-PLDR with transport vesicles within the cells. Specifically, there was a reduction in its colocalization with lysosomes and an increase in its association with recycling endosomes, which was also verified by the Pearson's R value. Pearson's R value was used to quantitatively analyze the co-localization effect of Cy5-PLDR with these vesicular markers. A larger Pearson's R value indicated a better co-localization effect. In the unpretreated group (black column), Pearson's R value between Cy5-PLDR and lysosomes was higher than that between Cy5-PLDR and recycling endosomes (Fig. 4(g)). In contrast, following a 24-hour pretreatment of bEnd.3 cells

with PLDR (red column), the Pearson's R value between Cy5-PLDR and lysosomes significantly decreased, whereas the correlation with recycling endosomes increased (Fig. 4(g)). These findings suggested that PLDR pretreatment

modulated the intracellular trafficking pathway of Cy5-PLDR, favoring the recycling route over the degradation route by decreasing the lysosome numbers and enhancing the recycling endosome numbers.

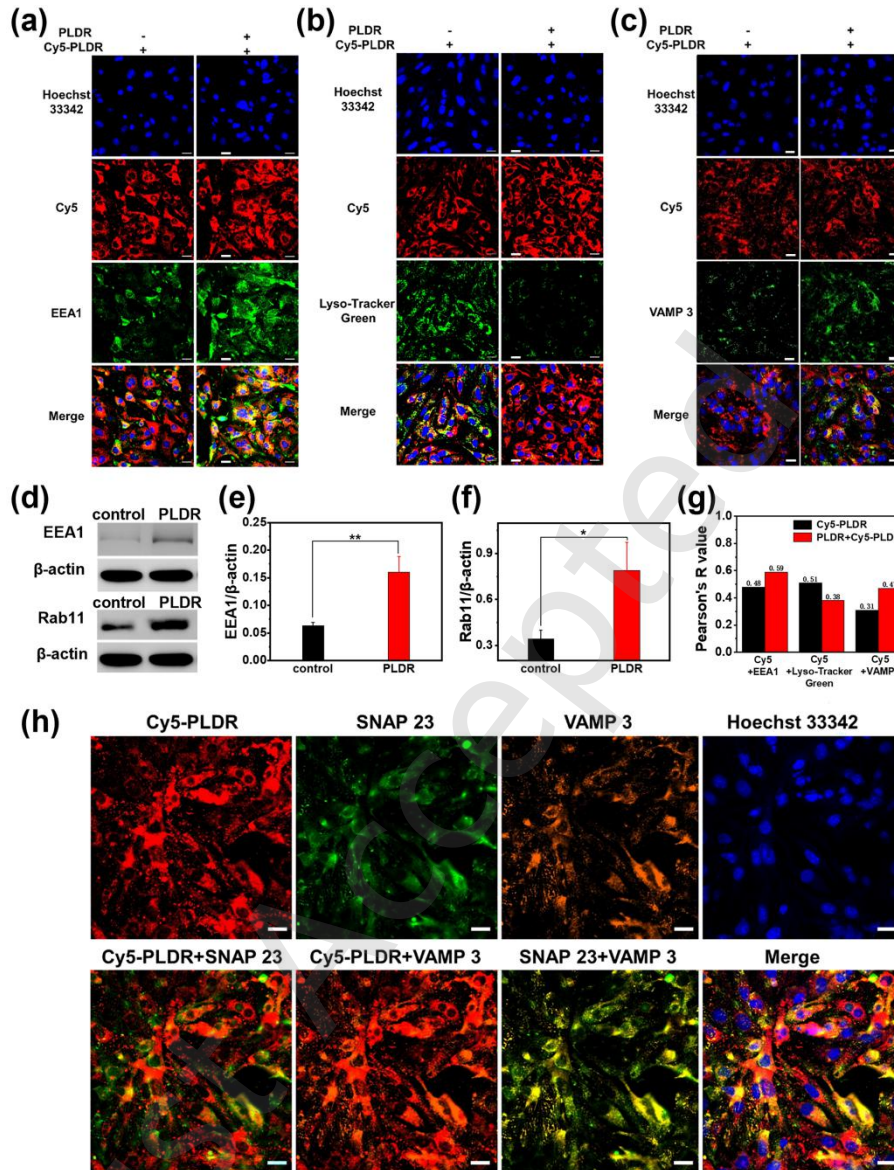


Figure 4 Study on the intracellular transport pathway of SNAs in BMECs. (a-c) CLSM images of the localization of different vesicles with Cy5-PLDR (red) in non-pretreated or PLDR pre-cultured bEnd.3 cells. bEnd.3 cells were initially pre-incubated with PLDR for 24 hours, followed by treatment with Cy5-PLDR for an additional 6 hours. Early endosomes and recycling endosomes were labeled with Cy3-labeled anti-EEA1 antibody and Cy3-labeled anti-VAMP 3 antibody, respectively. Lysosomes were stained with Lyso-Tracker Green. Scale bar: 20 μ m. (d) Western blot analysis of the expression of EEA1 and Rab11 proteins in non-treated or PLDR-treated bEnd.3 cells. (e) The ratio of EEA1 and β -actin expression as well as (f) the ratio of Rab11 and β -actin expression in non-treated or PLDR-treated bEnd.3 cells. (g) Pearson's R value between Cy5-PLDR and early endosomes, lysosomes and recycling endosomes in non-pretreated or PLDR pre-cultured bEnd.3 cells. (h) CLSM images of the colocalization of Cy5-PLDR (red) with plasma membrane (labeled with FITC-labeled anti-SNAP 23 antibody, green) and recycling endosomes (labeled with Cy3-labeled anti-VAMP 3 antibody, orange) in PLDR-pretreated bEnd.3 cells. Scale bar: 20 μ m. Data are presented as mean $\pm$ SD ($n \geq 3$). * $P < 0.05$ and ** $P < 0.01$ are considered statistically significant.

Recycling endosomes can transport their cargo to the basolateral membrane to facilitate transcytosis [13]. The fusion of recycling endosomes with the basolateral plasma membrane is a critical step in completing the transcytosis of intracellular molecules, which is mediated by the interaction between VAMP 3 localized on the recycling endosomes and the t-SNARE complex (SNAP 23/syntaxin 4) located on the basolateral membrane [13]. To assess the final step of Cy5-

PLDR transcytosis, namely the release of Cy5-PLDR on the abluminal side, we investigated the interaction between Cy5-PLDR and the basolateral membrane. Recycling endosomes were labeled using a Cy3-labeled anti-VAMP 3 antibody, while the plasma membrane was labeled with a FITC-labeled anti-SNAP 23 antibody. As illustrated in Fig. 4(h), a significant colocalization of VAMP 3 with SNAP 23 was observed in PLDR-pretreated bEnd.3 cells, indicating

the involvement of these proteins in the fusion of recycling endosomes with the basolateral membrane. Furthermore, Cy5-PLDR exhibited colocalization with both VAMP 3 and SNAP 23, suggesting that Cy5-PLDR, once sorted into the recycling endosomes of cells following PLDR pretreatment, can be released at the basolateral membrane via the VAMP 3/SNAP 23/syntaxin 4 pathway. Furthermore, we examined the potential influence of PLDR pretreatment on the uptake of Cy5-PLDR by bEnd.3 cells. As illustrated in Fig. S6, no significant difference in the fluorescence intensity of Cy5-PLDR was observed between pretreated and untreated cells. This finding indicated that siRab7 in PLDR affected only the intracellular trafficking pathway of Cy5-PLDR, without altering its cellular uptake.

In conclusion, the introduction of siRab7 in PLDR can effectively reduce Rab7 protein expression in brain microvascular endothelial cells, thereby decreasing lysosome numbers. Consequently, when Cy5-PLDR was introduced to cells pre-cultured with PLDR, it preferentially followed the recycling pathway rather than the lysosomal degradation pathway and can be released from the basement membrane, which will be conducive to its BBB transcytosis.

3.4 BBB crossing efficiency in vitro and in vivo

At the cellular level, our study has shown that pre-treatment with PLDR can reduce the number of lysosomes and facilitate the trafficking of spherical nucleic acids along the recycling pathway instead of the lysosomal degradation pathway. Our subsequent research will investigate whether these alterations in the intracellular transport route within BMECs influence the efficiency of spherical nucleic acids in traversing the BBB. An in vitro BBB model was established through a transwell system (Fig. 5(a)). The bEnd.3 cells were seeded into the apical chamber and cultured to form a dense cell monolayer with barrier function. The structural integrity and barrier function of the cell monolayer were validated through the expression of tight junction proteins and the permeability of FITC-labeled dextrans with varying molecular weights (4 kDa, 10 kDa and 70 kDa) [27-29]. Initially, immunofluorescence staining was conducted to detect the presence of the tight junction protein Claudin-5 in the cell monolayer within the upper chamber. Claudin-5 is essential for preserving the barrier function of the blood-brain barrier [30]. The observation of intense green fluorescence within the cells indicated a pronounced expression of tight junction proteins, thereby confirming the structural integrity of the cell monolayer (Fig. S7(a) in the ESM). Following the addition of the FITC-labeled dextrans with varying molecular weights (4 kDa, 10 kDa, and 70 kDa) to the apical chamber and subsequent incubation with the cells for 2 hours, the corresponding apparent permeability coefficients (P_{app}) were determined to assess their permeability across the cell monolayer. The P_{app} values of FITC-labeled dextrans, calculated based on their standard curves, were significantly lower in the presence of a cell monolayer compared to the absence of cells (Figs. S7(b)-S7(e) in the ESM). Furthermore, the permeability of FITC-labeled dextrans decreased markedly as the molecular weight increased from 4 kDa to 70 kDa. These findings

indicated that the bEnd.3 cells in the apical chamber formed a dense monolayer with barrier properties that effectively restricted the penetration of FITC-labeled dextrans, thereby demonstrating the successful establishment of an in vitro BBB model.

To evaluate the BBB-crossing efficiency of spherical nucleic acids, U87MG cells (human glioblastoma cells) were seeded into the basolateral chamber of the in vitro BBB model. This is based on the theoretical premise that spherical nucleic acids, after traversing the cell monolayer of the in vitro BBB model, can be subsequently internalized by U87MG cells located in the basolateral chamber. The uptake efficiency of spherical nucleic acids by U87MG cells in this chamber serves as a direct indicator of their ability to cross the BBB. Subsequently, the bEnd.3 cells were pre-incubated with PLDR (containing siRab7) for 24 hours, followed by the addition of Cy5-labeled PLDR (Cy5-PLDR) to the apical chamber for a 12-hour incubation. Following this incubation period, U87MG cells from the basolateral chamber were harvested for FCM analysis. The control group consisted of bEnd.3 cells that were not pre-incubated with PLDR but were treated with Cy5-PLDR for 12 hours. Another control group included bEnd.3 cells pre-incubated with PLDN (containing a control random RNA sequence) for 24 hours, followed by treatment with Cy5-labeled PLDN (Cy5-PLDN) for 12 hours. FCM analysis revealed that when bEnd.3 cells were not pre-treated but exposed to Cy5-PLDR, the fluorescence signals of Cy5-PLDR were detected in U87MG cells located in the basolateral chamber (Figs. 5(b) and 5(c)), indicating that Cy5-PLDR was capable of crossing the BBB and reaching the U87MG cells. This phenomenon may be attributed to the ability of Cy5-PLDR to specifically target and recognize BMECs via binding to scavenger receptors on their surface, thereby traversing the BBB through receptor-mediated transcytosis (RMT). Furthermore, Cy5-PLDR can also be efficiently internalized by tumor cells through interaction with scavenger receptors that are highly expressed on the tumor cell surface. In contrast, pre-incubation with PLDR resulted in significantly higher intracellular fluorescence intensity in U87MG cells compared to the group without PLDR incubation (Figs. 5(b) and 5(c)). This suggested that PLDR pre-incubation may enhance the BBB permeability of spherical nucleic acids by altering their intracellular transport pathways within brain microvascular endothelial cells (BMECs). Moreover, the intracellular fluorescence intensity observed in U87MG cells within the PLDN pre-treatment group was comparable to that of the group not subjected to PLDR incubation. Both of these groups exhibited lower fluorescence intensity levels compared to the PLDR pre-treatment group. This observed difference could be attributed to the role of siRab7. Following the pre-incubation of BMECs with PLDR, siRab7 was released from PLDR and subsequently suppressed Rab7 protein expression in BMECs, thereby reducing lysosome formation. The subsequently introduced Cy5-PLDR were preferentially transported via the recycling pathway rather than the lysosomal degradation pathway, thereby increasing the likelihood of these spherical nucleic acids crossing the BBB and reaching U87MG cells. Conversely, as PLDN lacked functional siRNA, pre-incubation of BMECs with PLDN did

not modulate the intracellular transport pathways of spherical nucleic acids. Consequently, the Cy5-PLDN added in the subsequent step was predominantly transported via

the lysosomal degradation pathway, which would result in significant degradation of Cy5-PLDN and ultimately lead to a reduction in their BBB permeability.

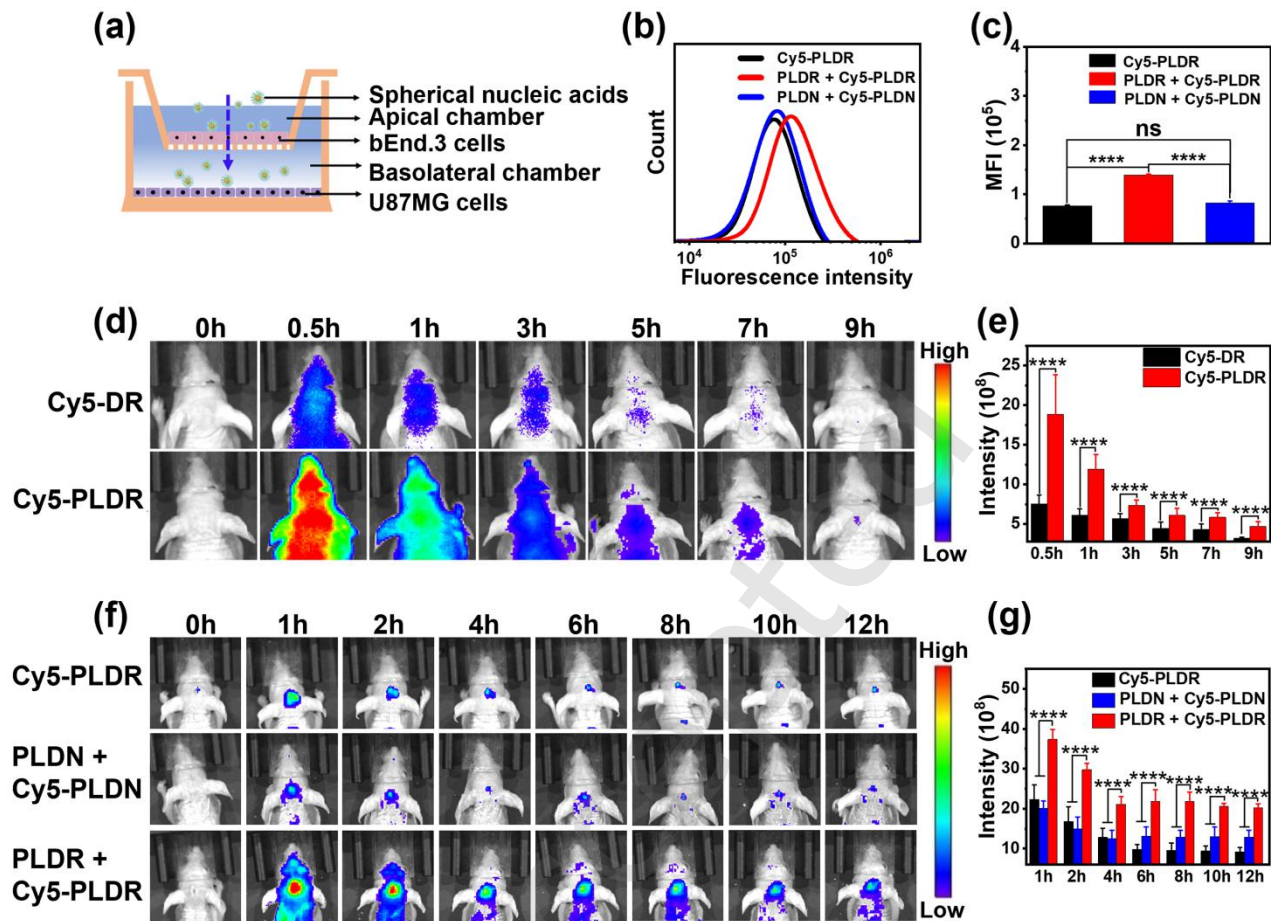


Figure 5 BBB crossing efficiency of SNAs. (a) Illustration of in vitro BBB model. (b) Flow cytometry analysis and (c) mean fluorescence intensity (MFI) of U87MG cells collected from the basolateral chamber of the in vitro BBB model. Cy5-PLDR group represented that bEnd.3 cells were not pre-incubated with PLDR but were treated Cy5-PLDR for 12 hours. PLDR+Cy5-PLDR group represented that bEnd.3 cells were pre-incubated with PLDR for 24 hours prior to being treated with Cy5-PLDR for an additional 12 hours. PLDN+Cy5-PLDN group represented that bEnd.3 cells were pre-incubated with PLDN for 24 hours prior to being treated with Cy5-PLDN for an additional 12 hours. (d) Real-time fluorescence imaging and (e) quantitative fluorescence analysis of brain tissues from healthy mice administered Cy5-PLDR and Cy5-DR at various time points. (f) Real-time fluorescence imaging and (g) quantitative fluorescence analysis of the brain of mice bearing U87MG-Luc orthotopic glioma tumor treated with different nanocarriers at various time points. Cy5-PLDR group referred to mice that received only a single injection of Cy5-PLDR. PLDR+Cy5-PLDR referred to mice that received an initial injection of PLDR 24 hours before being administered a subsequent injection of Cy5-PLDR. PLDN+Cy5-PLDN referred to mice that received an initial injection of PLDN 24 hours before being administered a subsequent injection of Cy5-PLDN. Data are presented as mean $\pm$ SD ($n \geq 3$). **** $P < 0.0001$ is considered statistically significant, while ns means no statistical difference.

We conducted a further investigation into the impact of reduced lysosome numbers induced by spherical nucleic acids on alterations in BBB integrity. Following spherical nucleic acids treatment of bEnd.3 cells, the permeability of FITC-labeled dextrans with varying molecular weights in the in vitro BBB model, as well as the expression level of the tight junction protein Claudin-5 in endothelial cells, remained unchanged (Fig. S8 in the ESM). This suggested that the strategy of modulating intracellular transport pathways by inhibiting lysosome formation via siRNA of spherical nucleic acids did not compromise the structural integrity of the BBB, thereby confirming the safety of this approach.

In an in vitro BBB model, it has been demonstrated that the efficiency of spherical nucleic acids in traversing the BBB

can be significantly improved by modulating their intracellular transport pathways. Building on this foundation, we conducted further evaluations to determine the applicability and efficacy of this strategy in a living system. Initially, we independently assessed the potential of Cy5-PLDR to cross the BBB in vivo, establishing a control basis for subsequent experimental investigations. The Cy5-labeled PLDR nanocarriers (Cy5-PLDR) and Cy5-labeled nucleic acid complexes (Cy5-DR) were administered intravenously to healthy mice with an intact BBB, respectively. Subsequently, the fluorescence signals within the mice's brains were monitored utilizing an in vivo fluorescence imaging system. As shown in Figs. 5(d) and 5(e), the fluorescence signals in the brains of mice treated with Cy5-PLDR were significantly stronger than those in

mice treated with Cy5-DR at all time points, indicating that a greater amount of spherical nucleic acids traversed the BBB and reached the brain parenchyma. This observation could be elucidated from two aspects. On one hand, compared with the nucleic acid complex (Cy5-DR), spherical nucleic acids exhibit a stable three-dimensional structure, characterized by the dense and highly ordered arrangement of nucleic acids on their surface [31]. This structural stability protected the spherical nucleic acids from nuclease degradation and prolonged their circulation time in the bloodstream, significantly improving their ability to cross the BBB [32]. On the other hand, Cy5-PLDR exhibited higher cellular uptake by BMECs compared to Cy5-DR, owing to its binding affinity to scavenger receptors on the BMEC surface, as demonstrated in Figs. 2(a)-2(c). This characteristic further created favorable conditions for traversing the BBB. This finding confirmed that the spherical nucleic acids, Cy5-PLDR, were capable of traversing an intact BBB.

To evaluate the *in vivo* glioma-targeting capability of spherical nucleic acids, we established an orthotopic mouse model bearing luciferase-expressing human glioblastoma tumors (U87MG-Luc). Seven days post-inoculation, Cy5-DR and Cy5-PLDR were administered to glioma-bearing mice through intravenous injection via the tail vein. As illustrated in Fig. S9 in the ESM, at various time intervals post-injection, the fluorescence signals in the brains of mice treated with Cy5-PLDR were more pronounced compared to those in mice treated with Cy5-DR. This observation aligned with the enrichment patterns observed in the brains of normal mice. This phenomenon was not only attributed to the high BBB crossing efficiency of spherical nucleic acids, but also to their specific targeting effect on glioma cells.

To investigate whether the efficiency of spherical nucleic acids crossing the BBB *in vivo* can be enhanced by modulating intracellular transport pathways through the introduction of siRab7, we first administered PLDR (containing functional siRab7) to tumor-bearing mice via tail vein injection. Twenty-four hours after the initial injection, the mice received a subsequent tail vein injection of Cy5-PLDR. Mice receiving only a single injection of Cy5-PLDR served as the control group. Another control group consisted of mice injected with PLDN (containing a control random RNA sequence) for 24 hours, followed by an injection of Cy5-labeled PLDN (Cy5-PLDN). Notably, at various time points, the experimental group pretreated with PLDR demonstrated significantly stronger fluorescence signals in the tumor region compared to the two control groups (the un-pretreated group and the PLDN-pretreated group) (Figs. 5(f) and 5(g)). These findings suggested that PLDR pretreatment facilitated the enhanced penetration of the subsequently administered Cy5-PLDR across the BBB, thereby increasing its accumulation at the brain tumor site. This phenomenon can be ascribed to the regulatory effect of siRab7 within PLDR on the intracellular transport pathway of subsequently injected Cy5-PLDR in BMECs. This regulation facilitated the preferential transport of spherical nucleic acids via the recycling pathway, thereby mitigating their lysosomal degradation. Consequently, this enhanced their translocation across the BBB and increased their

accumulation at tumor sites. Furthermore, the un-pretreated group and the PLDN-pretreated group exhibited similar levels of brain accumulation. This outcome can be attributed to the absence of functional siRNA in PLDN, which did not alter the intracellular transport pathways of Cy5-PLDN administered in the subsequent step, and thus did not affect their BBB permeability.

In conclusion, regulating the intracellular transport pathways of spherical nucleic acids to enhance their efficiency in crossing the BBB and their enrichment levels in brain tumors was an effective strategy.

3.5 Glioma therapy *in vitro* and *in vivo*

We have confirmed that PLDR pretreatment enhanced the BBB penetration efficiency and brain tumor accumulation of the subsequently administered Cy5-PLDR. As a result, PTX and antisense oligonucleotides (ASO) can also be effectively delivered to the tumor site. We next evaluated their anti-tumor effects. Pre-injected PLDR was transported through the lysosomal pathway in brain microvascular endothelial cells. After lysosomal escape, siRNA was released by Dicer. The resulting nanocarriers (PLD) may then cross the BBB and reach the tumor. The second dose of spherical nucleic acids primarily used the recycling pathway to cross the BBB and also reached the tumor site. Therefore, both PLDR and PLD are expected to accumulate at the tumor. Accordingly, we first examined their uptake by tumor cells and their effects on inhibiting proliferation.

To investigate the uptake of PLDR by U87MG cells, Cy5 fluorophore-labeled PLDR (Cy5-PLDR) and Cy5 labeled nucleic acid complexes (Cy5-DR) were incubated with U87MG cells for varying durations, respectively. The results from FCM analysis and CLSM imaging consistently demonstrated that cells treated with Cy5-PLDR exhibited a more intense red fluorescence compared to those treated with Cy5-DR (Figs. 6(a) and 6(b)) and Fig. S10 in the ESM). These findings demonstrated that Cy5-PLDR can be efficiently internalized by U87MG cells, with a significantly higher uptake level compared to the nucleic acid complex (Cy5-DR). Moreover, this result provided supporting evidence for the conclusion presented in Figs. 5(b) and 5(c), which illustrated that Cy5-PLDR was capable of reaching U87MG cells following its traversal across the BBB. To explore the cellular uptake of PLD by U87MG cells, we synthesized Cy5 fluorophore-labeled PLD (Cy5-PLD) through the conjugation of Cy5 fluorophore-labeled ASO (Cy5-ASO) and the redox-responsive paclitaxel prodrug (LPTX). Both CLSM images and FCM analysis revealed that a higher degree of cellular internalization of Cy5-PLD compared to Cy5-ASO (Fig. S11 in the ESM). The results suggested that the internalization of spherical nucleic acids by U87MG cells (refer to Figs. 6(a) and 6(b)) and BMECs (as previously validated in Figs. 2(a)-2(c)) surpassed that of free nucleic acids. In addition, scavenger receptors inhibitors blocking experiments confirmed that this enhanced uptake was mediated by the specific binding affinity of spherical nucleic acids to scavenger receptors located on the cell surface, thereby facilitating cellular internalization (Fig. S12 in the ESM).

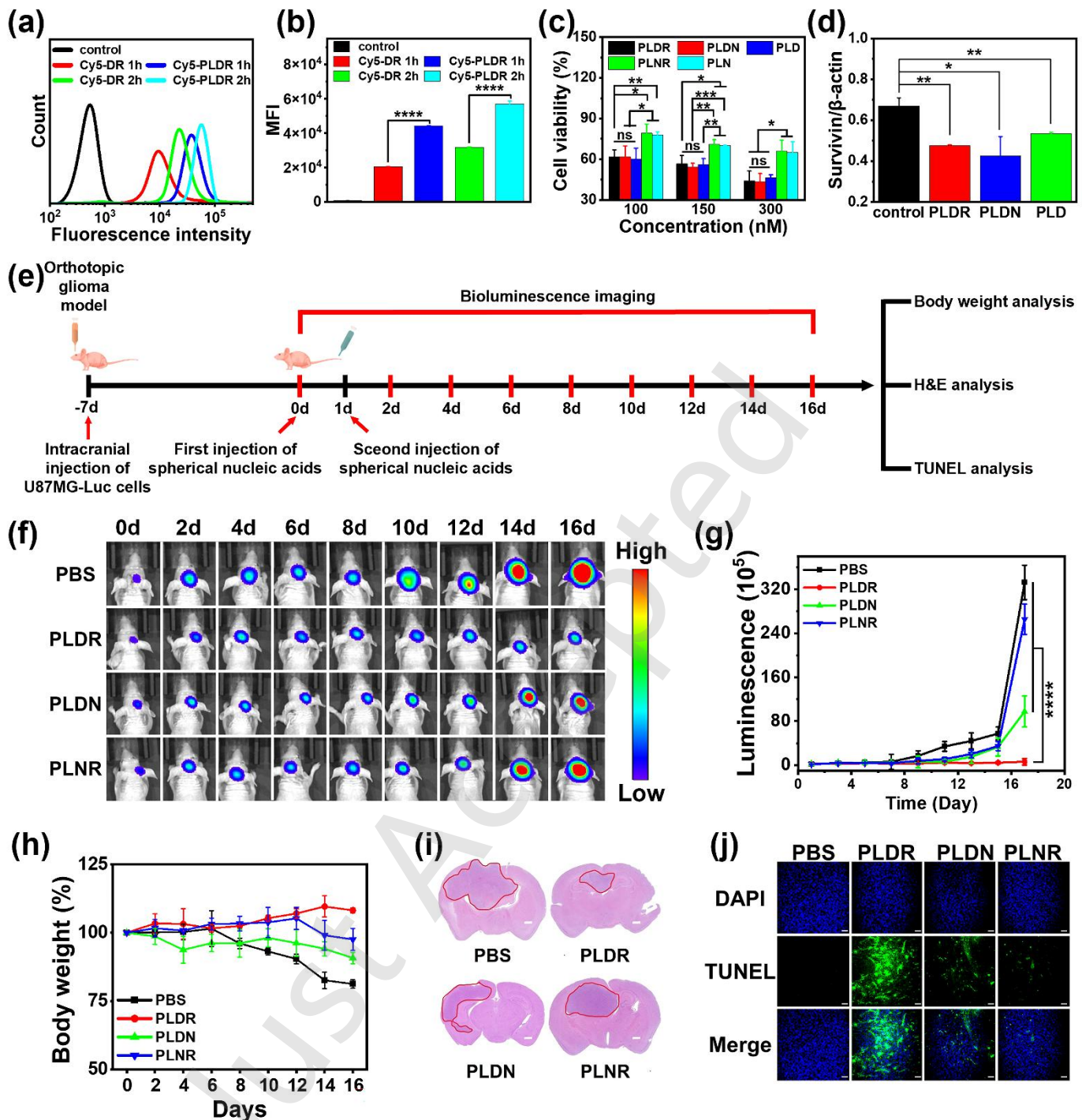


Figure 6 Anti-tumor efficacy of SNAs in vitro and in vivo. (a) FCM analysis and (b) MFI of U87MG cells treated with Cy5-PLDR and Cy5-DR for different times. Untreated U87MG cells served as the control group. (c) Cell viability of U87MG cells treated with different SNAs at varying concentrations (100, 150 and 300 nM) for 48 hours. (d) The ratio of survivin and β -actin expression in U87MG cells incubated with PLDR, PLDN and PLD for 48 hours. The control group referred to U87MG cells without any incubation. (e) Schematic illustration of establishment of orthotopic glioma model and treatment with different SNAs ($n = 3$ per group). (f) Representative bioluminescence images of U87MG-Luc glioma-bearing mice treated with different SNAs. Mice treated with PBS served as control group ($n = 3$ per group). (g) Quantitative bioluminescence signal intensity in the brain of mice during treatment with different SNAs ($n = 3$ per group). (h) Body weight of mice during treatment with different SNAs ($n = 3$ per group). (i) H&E staining of brain sections of orthotopic glioma-bearing mice in all groups on the 16th day of treatment ($n = 3$ per group). Scale bar: 500 μ m. (j) TUNEL staining images of brain sections of orthotopic glioma-bearing mice in all groups on the 16th day of treatment ($n = 3$ per group). Scale bar: 50 μ m. Data are presented as mean $\pm$ SD ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ are considered statistically significant, while ns means no statistical difference.

Subsequently, we investigated the tumor-killing effect of spherical nucleic acids (SNAs) at the cellular level. Paclitaxel (PTX), a broad-spectrum antitumor agent, has been reported to inhibit cancer cell division and induce apoptosis [33].

However, the therapeutic efficacy of PTX is frequently limited by survivin, an inhibitor of apoptosis protein that is commonly overexpressed in gliomas and contributes to chemo-resistance [34]. To overcome this resistance

mechanism, we incorporated an antisense oligonucleotide (ASO) targeting survivin mRNA into our SNA construct. This ASO is designed to downregulate survivin protein expression, thereby sensitizing glioma cells to PTX-induced apoptosis. We therefore hypothesized that co-delivery of PTX and ASO within a single SNA platform would produce a synergistic anti-glioma effect. To test this hypothesis, the cell viability of U87MG cells after incubation with different SNAs for 48 hours was measured through methylthiazolyl-diphenyl-tetrazolium bromide (MTT) assay. These SNAs included PLDR (containing PTX, ASO and siRab7), PLDN (containing PTX, ASO and non-specific siRNA sequence), PLD (containing PTX and ASO), PLNR (containing PTX, non-specific DNA sequence and siRab7) and PLN (containing PTX and non-specific DNA sequence). As shown in Fig. 6(c), the cell viability of U87MG cells was negatively correlated with the concentration of SNAs. Cells treated with SNAs loaded with PTX and ASO (including PLDR, PLDN and PLD) showed much lower viabilities compared to those incubated with SNAs loaded only with PTX (PLNR and PLN), suggesting that the introduction of the ASO could improve anti-tumor efficacy. Western blot (WB) analysis demonstrated that the expression levels of survivin in cells treated with SNAs containing ASO (including PLDR, PLDN, and PLD) were significantly down-regulated (Fig. 6(d) and Fig. S13 in the ESM), thereby confirming the gene silencing efficacy of the antisense sequences. As a result, spherical nucleic acids were efficiently internalized by U87MG cells through binding to scavenger receptors, followed by the release of ASO and PTX in response to elevated glutathione (GSH) levels. The ASO specifically targeted and silenced the mRNA of survivin protein, thereby reducing its intracellular expression. This reduction in the expression level of survivin synergized with PTX to induce effective U87MG cell death. Moreover, the viability of U87MG cells remained comparable among PLDR (containing PTX, ASO, and siRab7), PLDN (containing PTX, ASO, and a non-specific siRNA sequence), and PLD (containing PTX and ASO), indicating that siRab7 did not affect the tumor-killing effect of SNAs (Fig. 6(c)).

The tumor cell-killing capability of spherical nucleic acids was further validated through calcein acetoxymethyl ester (calcein AM) and propidium iodide (PI) staining assays. Confocal laser scanning microscopy (CLSM) imaging of U87MG cells treated with PLDR (containing PTX, ASO, and siRab7) exhibited the highest intensity of red fluorescence (indicating dead cells) and the lowest intensity of green fluorescence (indicating live cells), compared to cells treated with PLNR (containing PTX, non-specific DNA sequences, and siRab7) or PLN (containing PTX and non-specific DNA sequences) (Fig. S14 in the ESM). This observation indicated that PLDR demonstrated the greatest efficacy in inducing tumor cell death. Notably, no significant difference in fluorescence signals was observed between cells incubated with PLNR and those incubated with PLN, further confirming that siRab7 did not exert a significant effect on the *in vitro* glioma cell-killing activity.

Next, the *in vivo* antitumor efficacy of various spherical nucleic acids (SNAs) was evaluated in an orthotopic mouse model bearing luciferase-expressing human glioblastoma tumors. The SNAs tested included PLDR (containing PTX, ASO and siRab7), PLDN (containing PTX, ASO and non-

specific siRNA sequence) and PLNR (containing PTX, non-specific DNA sequence and siRab7), with PBS administered as the control group. Seven days post-inoculation, these SNAs were administered through the tail vein, with a subsequent injection occurring 24 hours later (Fig. 6(e)). Tumor sizes in the mice were assessed every other day using bioluminescence imaging, and tumor growth curves were subsequently plotted. As illustrated in Figs. 6(f) and 6(g), mice administered with PBS exhibited the most intense bioluminescence signals in the brain, indicative of accelerated tumor growth. In contrast, the bioluminescence signals in the brains of mice injected with PLDR (containing PTX, ASO and siRab7) were comparatively weaker than those in mice injected with PLDN (containing PTX, ASO and non-specific siRNA sequence), suggesting that PLDR more effectively inhibited tumor growth. This outcome can be attributed to the modulation of the BBB transport pathway by PLDR during the initial injection, which facilitated the efficient crossing of the BBB by PLDR in the subsequent injection. Consequently, PLDR was effectively concentrated in the glioma region, thereby maximizing its tumor inhibition efficacy. In addition, the PLNR (containing PTX, non-specific DNA sequences, and siRab7) group exhibited stronger bioluminescence signals in the brains of mice compared to the PLDR (containing PTX, ASO and siRab7) group, demonstrating that the combination of PTX-mediated chemotherapy and ASO-mediated gene therapy significantly enhances therapeutic efficacy. The weight of mice injected with PLDR the group showed no obvious change, while mice in other groups experienced varying degrees of weight loss (Fig. 6(h)), in accordance with the tumor inhibition effect. Following a 16-day treatment period, the brains of the mice were harvested and subjected to hematoxylin and eosin (H&E) staining to evaluate the antitumor efficacy across different groups. As illustrated in Fig. 6(i), the tumor size in the brains of mice treated with PLDR was the smallest, thereby corroborating the superior tumor growth inhibition effect of PLDR. Moreover, terminal deoxynucleotidyl transferase uridine triphosphate nick end labeling (TUNEL) staining was conducted on tumor tissue sections from different experimental groups, where green fluorescence indicates apoptotic cells. As shown in Fig. 6(j) and Fig. S15 in the ESM, the strongest green fluorescence was observed in the PLDR-treated group, implying the most significant apoptosis of tumor cells. The above findings demonstrated that PLDR efficiently inhibited glioma growth, making it a promising candidate for brain tumor therapy.

The biosafety of the SNAs (PLDR, PLDN and PLNR) was evaluated through H&E staining of major organs, blood routine examination and blood biochemistry analysis. Different SNAs were individually injected into healthy BALB/c mice via the tail vein. Mice injected with PBS served as the control group. On day 30 post-injection, major organs (heart, liver, spleen, lung, kidney and brain) and blood samples were collected for analysis. H&E staining images showed no significant pathological changes in major organs of the mice across all groups (Fig. S16 in the ESM), suggesting that these SNAs did not cause organ damage. The blood routine results showed that SNAs had little influence on red blood count (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), and mean corpuscular

hemoglobin concentration (MCHC) (Fig. S17 in the ESM). Additionally, the levels of liver function indicators (alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) as well as kidney function indicators (creatinine kinase (CK), blood urea nitrogen (BUN) and creatinine (CREA)) in the SNAs-treated mice exhibited no difference compared to the PBS-treated mice (Fig. S18 in the ESM), indicating that SNAs possessed biological safety with no side effects. Furthermore, the impact of SNAs on the BBB integrity was assessed using the Evans blue (EB) assay. Under normal conditions, EB is unable to cross the intact BBB. However, when the BBB is impaired, EB can enter the brain parenchyma through the damaged sites. By detecting the content of Evans Blue (EB) in brain tissue, we can assess the integrity of the BBB. Following the injection of PLDR into healthy mice for different days, the mice were subsequently subjected to EB administration. After 30 min, the mice were sacrificed to collect brain tissues for analysis of EB content. As shown in Fig. S19 in the ESM, the content of Evans blue in the brains of mice remained unchanged compared with the control group (without injection of PLDR), indicating that the injection of PLDR would not affect the integrity of the BBB. These results demonstrated that PLDR possessed good biosafety and had the potential for clinical treatment of glioma.

4 Conclusions

In summary, we successfully constructed a spherical nucleic acids (SNAs)-based nanocarrier (PLDR) to enhance BBB transcytosis by modulating intracellular transport pathways. PLDR demonstrated improved internalization by BMECs via scavenger receptor (SR)-mediated endocytosis. Upon entry into BMECs, PLDR released siRab7, which effectively down-regulated Rab7 protein expression and consequently reduced lysosome formation. As a result, subsequently added Cy5-PLDR was preferentially transported through the recycling pathway rather than the lysosomal degradation pathway. Both in vitro BBB models and in vivo experiments confirmed that PLDR pretreatment significantly improved BBB penetration efficiency and facilitated the accumulation of subsequently administered Cy5-PLDR in brain tumors. Moreover, PLDR exhibited glioma-targeting capability, enabling cellular uptake by U87MG cells, followed by the triggered release of antisense oligonucleotides (ASO) and paclitaxel (PTX) in response to elevated glutathione (GSH) levels. The down-regulation of survivin expression by ASO synergized with PTX to effectively induce U87MG cell death. This strategy pioneers a new path to increase the transcytosis of nanocarriers into the brain parenchyma by regulating their transport pathway within brain microvascular endothelial cells, which has the potential to be used in the treatment of other brain-related diseases.

Electronic Supplementary Material: Supplementary material (Figs. S1-S19 and Tables S1-S2) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94909184>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and/or the Electronic

Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

Li Zhang: Data curation, investigation, validation, formal analysis, visualization, software, writing-original draft. Hemei Cheng: Data curation, investigation, validation, visualization, software, writing-original draft. Nian Wang: Formal analysis, investigation, validation. Weibin Li: Formal analysis, investigation, validation. Zhennan Mao : Formal analysis, investigation. Guixi Jin : Investigation. Mengting Xiang: Investigation. Xinyue Zhang: Investigation. Caixia Wang: Review & editing, supervision, project administration. Zhihong Liu: Review & editing, funding acquisition, supervision, project administration. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and the procedures were approved by the Wuhan University of China Animal Care and Use Committee (SYXK 2015-0027, Wuhan, China).

Use of AI statement

None.

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Modulation of intracellular transport pathways to enhance the transcytosis of nanodrugs across the blood-brain barrier

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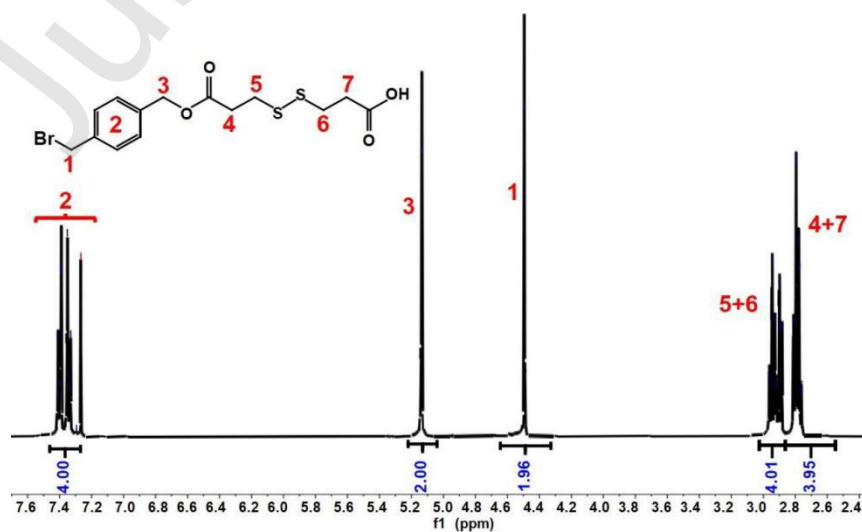
Supporting information to <https://doi.org/10.26599/NR.2026.94909184>

Table S1 Nucleic acid sequences used in this study

Oligonucleotides	Sequences (5' to 3')
ASO	T*T *T CCTTGCGTTACGATCCGTTACGCACC
ASO-Cy5	T*T *T CCTTGCGTTACGATCCGTTACGCACC-Cy5
DNA bridge	GAACTTCAGGGTCAGCTTGCCGGTGCCTAACGGATCGTAACG
siRab7	GGCAAGCTGACCCTGAAGTTCUGCUGUGUUCUGGUGUUUGAU Antisense strand: AUCAAACACCAGAACACAGCA
control DNA	T*T *T TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
control siRNA	GGCAAGCTGACCCTGAAGTTC AUGAUGAUCAUCAUUAACCAGG Antisense strand: CCUGGUAAUGAUGAUCAUCAU

Table S2 The list of abbreviations

Abbreviation	Description
SNAs	Spherical nucleic acids
PTX	Paclitaxel
ASO	An antisense oligodeoxynucleotide to target survivin mRNA and downregulate survivin protein expression
siRab7	A siRNA duplex to downregulate Rab7 protein expression
DR	A nucleic acid complex composed of ASO and siRab7
LPTX	Redox-responsive paclitaxel prodrug
LPTX-DR	Amphiphilic conjugate
PLDR	SNAs comprising PTX, ASO and siRab7
PLDN	SNAs comprising PTX, ASO and a non-specific siRNA sequence
PLNR	SNAs comprising PTX, a non-specific DNA sequence and siRab7
PLD	SNAs comprising PTX and ASO
PLN	SNAs comprising PTX and non-specific DNA sequence
Cy5-PLDR	SNAs comprising PTX, Cy5 fluorophore-labeled ASO and siRab7
Cy5-DR	Cy5 fluorophore-labeled nucleic acid complexes
Cy5-PLD	SNAs comprising PTX and Cy5 fluorophore-labeled ASO
Cy5-PLDN	SNAs comprising PTX, Cy5 fluorophore-labeled ASO and a non-specific siRNA sequence
BMECs	Brain microvessel endothelial cells
RMT	Receptor-mediated transcytosis
GSH	Glutathione
SRs	Scavenger receptors
DTT	Dithiothreitol
CLSM	Confocal laser scanning microscopy
FCM	Flow cytometry
PAGE	Polyacrylamide gel electrophoresis
AGE	Agarose gel electrophoresis
DLS	Dynamic light scattering
TEM	Transmission electron microscopy
CMC	Critical micelle concentration

Figure S1 ¹H-NMR spectrum of the GSH-responsive linker in CDCl₃.

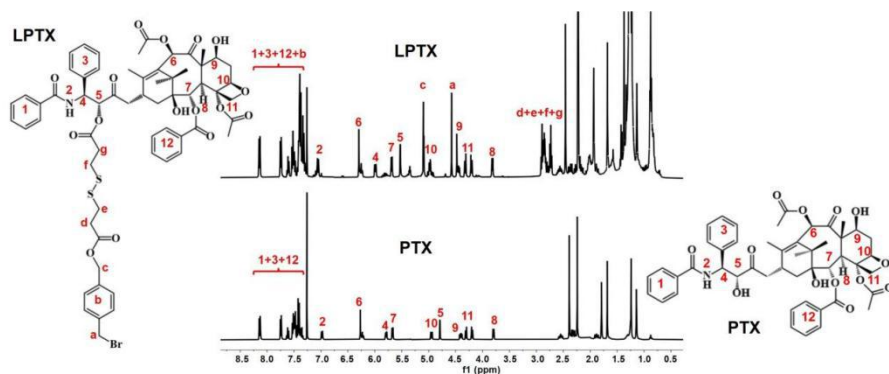


Figure S2 ^1H -NMR spectrum of the LPTX in CDCl_3 .

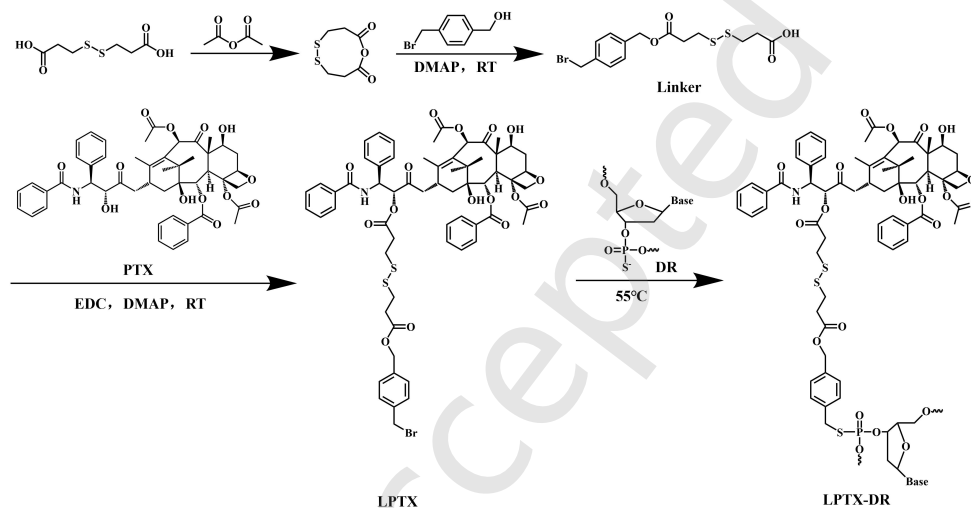


Figure S3 Schematic illustration of the synthesis of LPTX-DR.

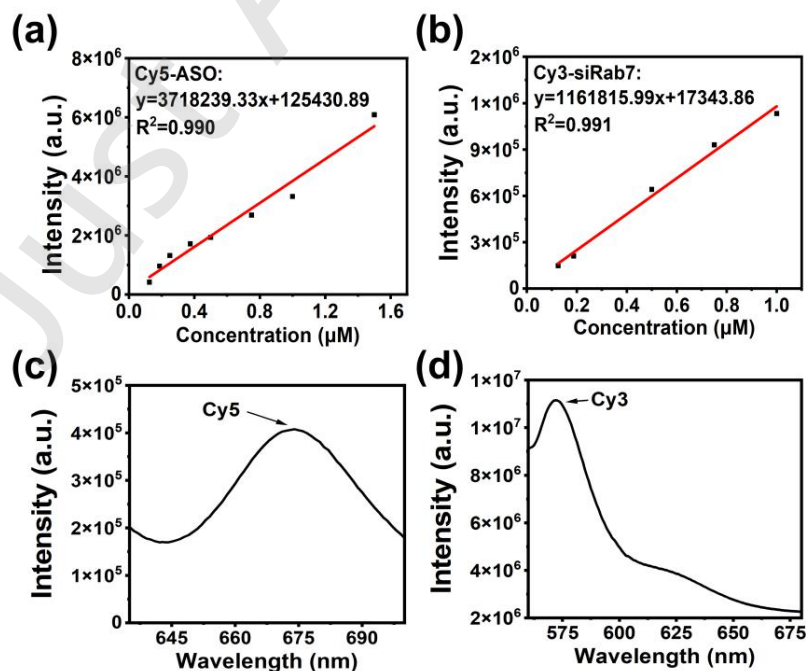


Figure S4 Calibrated standard concentration curves of Cy5-ASO (a) and Cy3-siRab7 (b). (c) Fluorescence emission spectra of PLDR (containing Cy5-ASO). $\lambda_{\text{ex}}=630$ nm. (d) Fluorescence emission spectra of PLDR (containing Cy3-siRab7). $\lambda_{\text{ex}}=550$ nm.

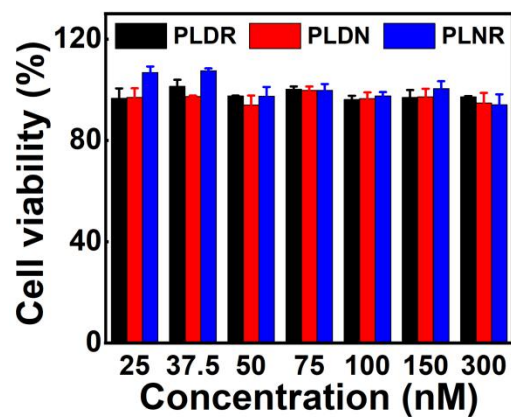


Figure S5 Cell cytotoxicity of PLDR, PLDN and PLNR against bEnd.3 cells after 48 hours incubation.

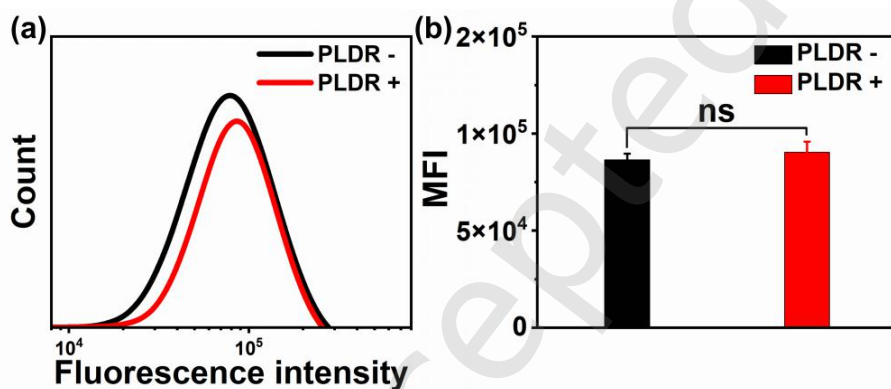


Figure S6 (a) Flow cytometry analysis and (b) mean fluorescence intensity (MFI) of bEnd.3 cells incubated with Cy5-PLDR (0.15 μ M). PLDR- group represented that non-pretreated bEnd.3 cells incubated with Cy5-PLDR for 4 hours. PLDR+ group represented that PLDR-pretreated bEnd.3 cells incubated with Cy5-PLDR. bEnd.3 cells were initially pre-incubated with PLDR for 24 hours, followed by treatment with Cy5-PLDR for an additional 4 hours.

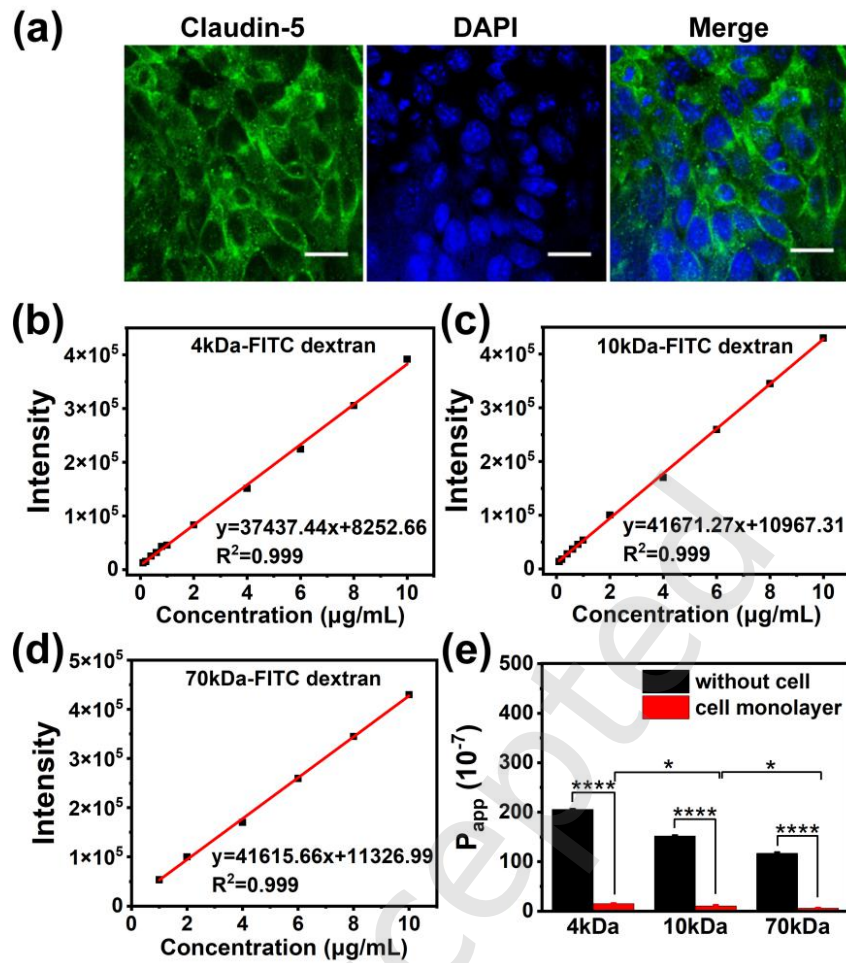


Figure S7 (a) Immunofluorescence images of Claudin-5 (green) in BBB monolayer. Nuclei were stained by Hoechst 33342 (blue). (b-d) Calibrated standard concentration curves of FITC-labeled dextrans with different molecular weights (4 kDa, 10 kDa and 70 kDa) (λ_{ex} =490 nm; λ_{em} =525 nm). (e) The apparent permeability coefficient (P_{app}) of FITC-labeled dextrans in vitro BBB model with cell monolayer or without cell.

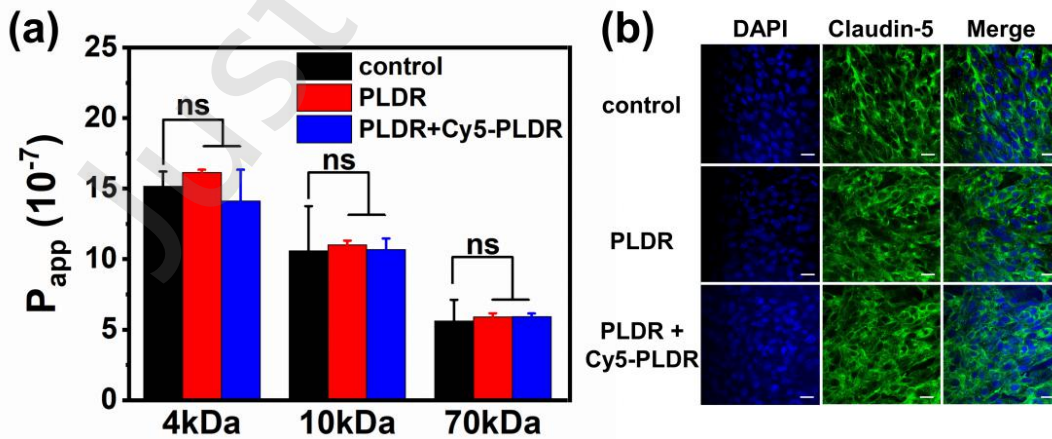


Figure S8 (a) The apparent permeability coefficient (P_{app}) of FITC-labeled dextrans with different molecular weights (4, 10 and 70 kDa) and (b) the immunofluorescence images of Claudin-5 (green) in BBB monolayer after the different treatment to the apical chamber. Scale bar: 20 μ m. Control group represented that the apical chamber without any treatment. PLDR group represented that PLDR was added in the apical chamber and incubated for 24 hours. PLDR + Cy5-PLDR group represented that PLDR was first added to the apical chamber for incubation for 24 hours, and then Cy5-PLDR was added for further incubation for 12 hours. Nuclei were stained by Hoechst 33342 (blue).

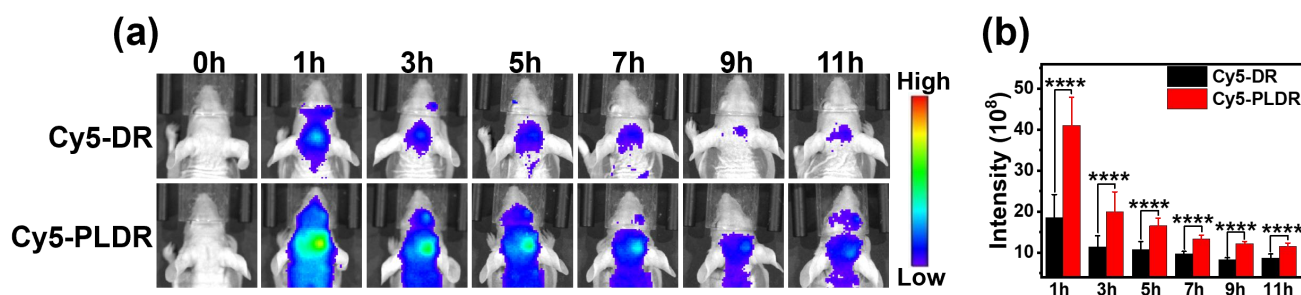


Figure S9 (a) Real-time fluorescence imaging and (b) quantitative fluorescence analysis of the brain of mice bearing U87MG-Luc orthotopic glioma tumor treated with different nanocarriers at various time points. Cy5-DR group referred to mice that received only a single injection of Cy5-DR. Cy5-PLDR group referred to mice that received only a single injection of Cy5-PLDR. Data are presented as mean $\pm$ SD ($n \geq 3$). ****P < 0.0001 is considered statistically significant.

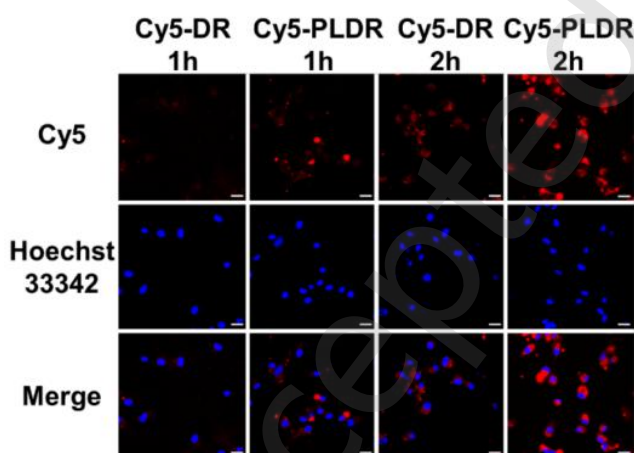


Figure S10 CLSM imaging of U87MG cells treated with Cy5-DR and Cy5-PLDR (0.3 μ M) for different time. Scale bar: 20 μ m.

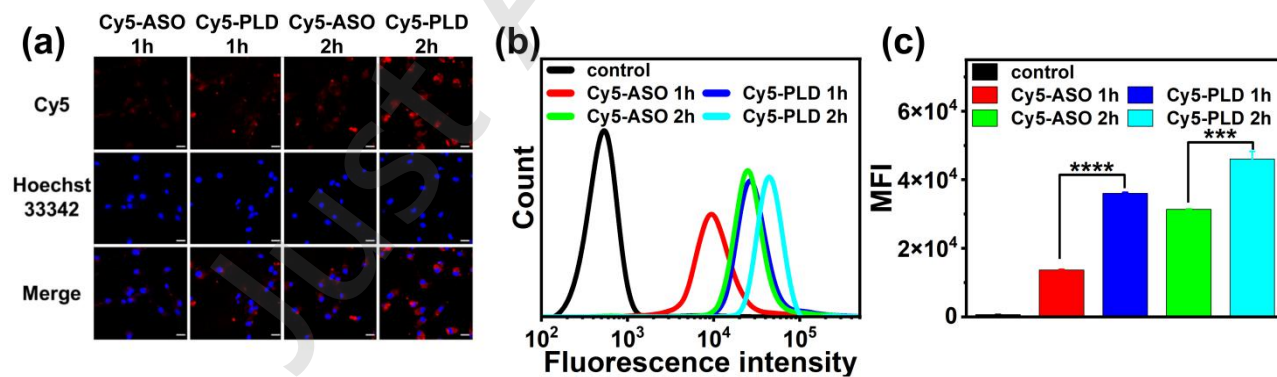


Figure S11 (a) CLSM imaging, (b) FCM analysis and (c) MFI of U87MG cells treated with Cy5-ASO and Cy5-PLD for different time. Scale bar: 20 μ m.

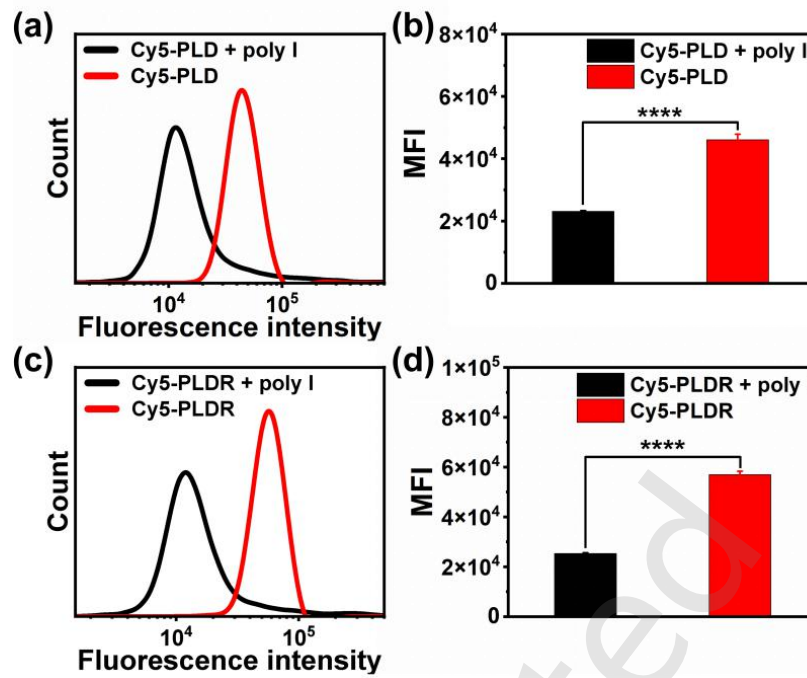


Figure S12 Flow cytometry analysis and mean fluorescence intensity (MFI) of U87MG cells incubated with Cy5-PLD (0.15 μ M) ((a) and (b)) or Cy5-PLDR (0.15 μ M) ((c) and (d)) in the presence or absence of poly I (0.2 mg/mL) for 8 hours.



Figure S13 Western blot analysis of the expression of survivin protein in U87MG cells after incubation with different SNAs (PLDR, PLDN and PLD) for 48 hours. The U87MG cells without any treatment served as control group.

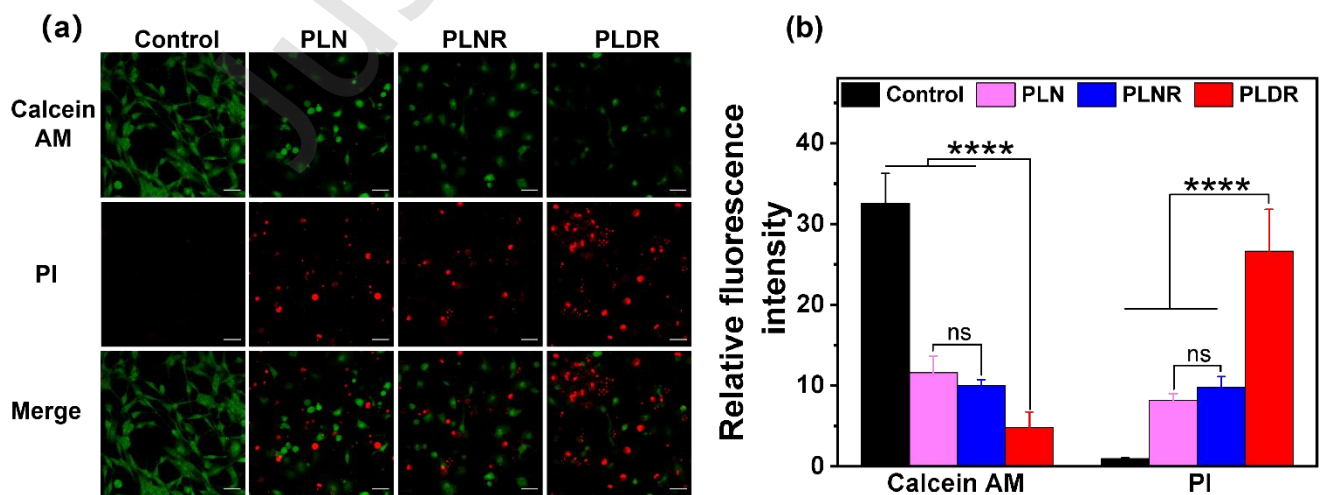


Figure S14 (a) CLSM images and (b) relative fluorescence intensity of calcein AM/PI co-staining in U87MG cells after incubated with different SNAs (PLDR, PLNR and PLN) (0.3 μ M) for 48 hours. The live cells were stained by calcein-AM (green fluorescence) and dead cells were stained by PI (red fluorescence). Scale bar: 20 μ m.

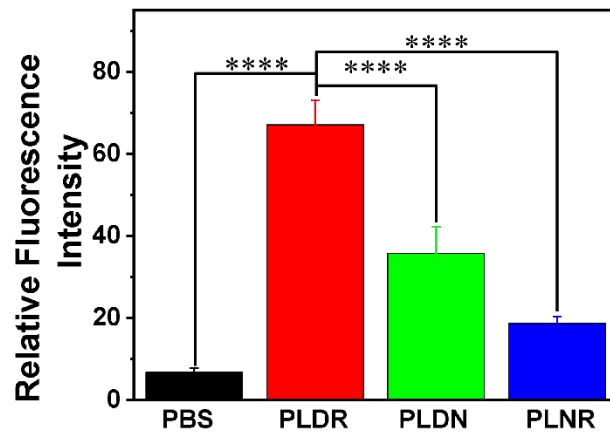


Figure S15 The relative green fluorescence intensities of TUNEL-stained brain sections, obtained from all groups of orthotopic glioma-bearing mice on day 16 of treatment, were quantified using a Zen 3.0 software.

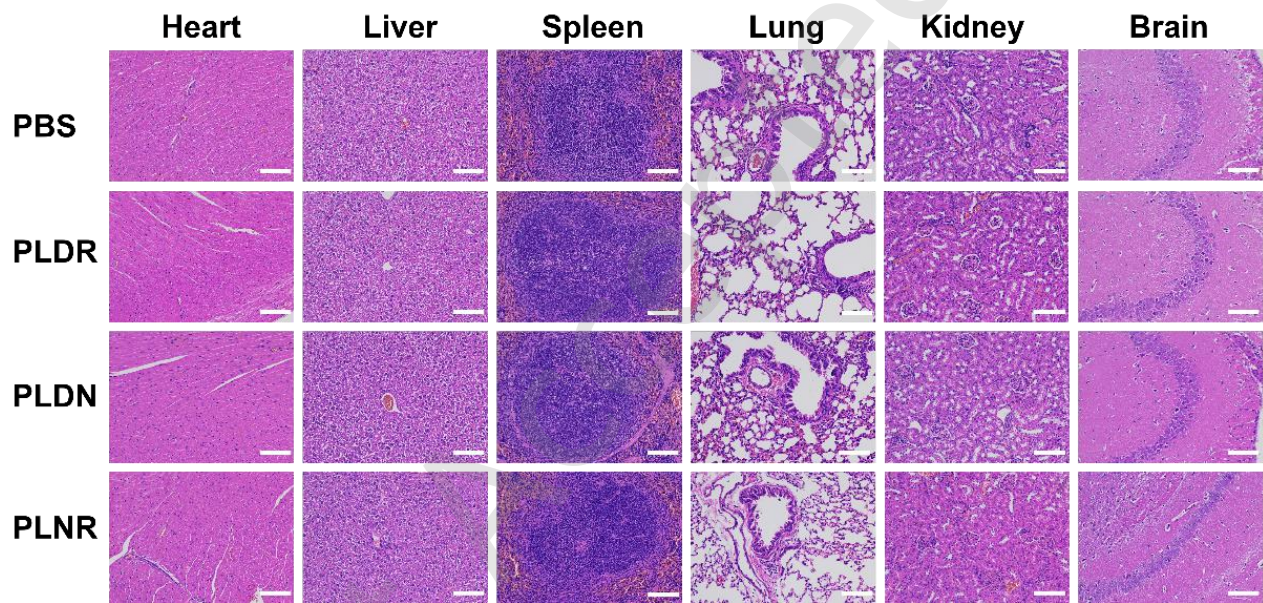


Figure S16 H&E staining images of major organs (heart, liver, spleen, lung, kidney and brain) of mice after different treatment (PBS, PLDR, PLDN and PLNR) at 30th day. Scale bar: 100 μ m.

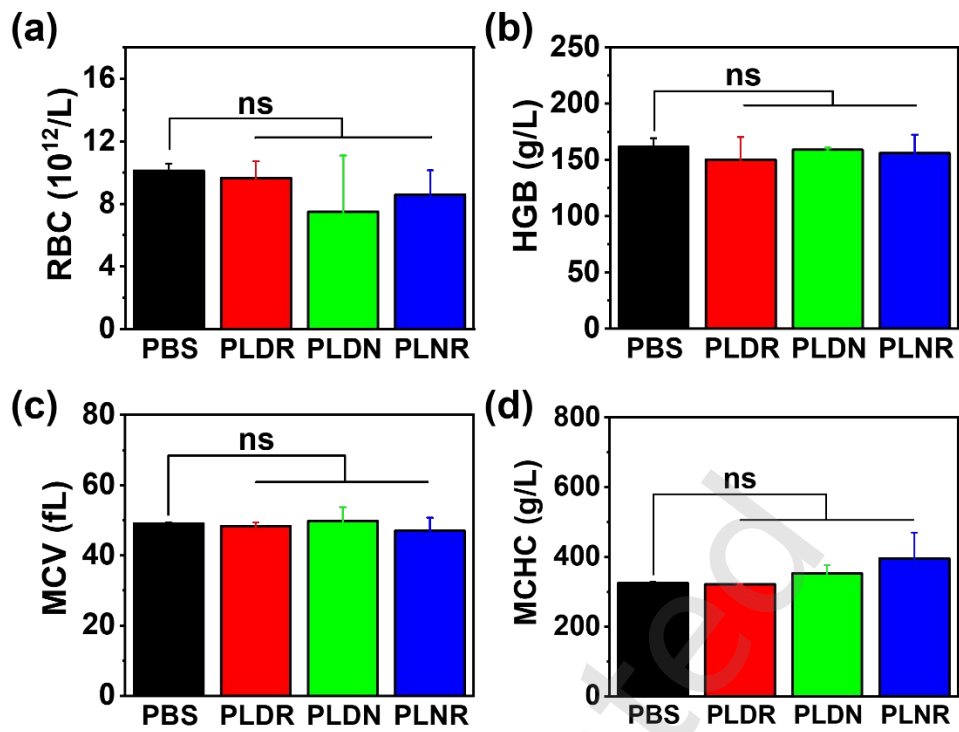


Figure S17 Blood routine analysis of mice after different treatments (PBS, PLDR, PLDN and PLNR) at 30th day. (a) RBC: red blood count, (b) HGB: hemoglobin, (c) MCV: mean corpuscular volume and (d) MCHC: mean corpuscular hemoglobin concentration.

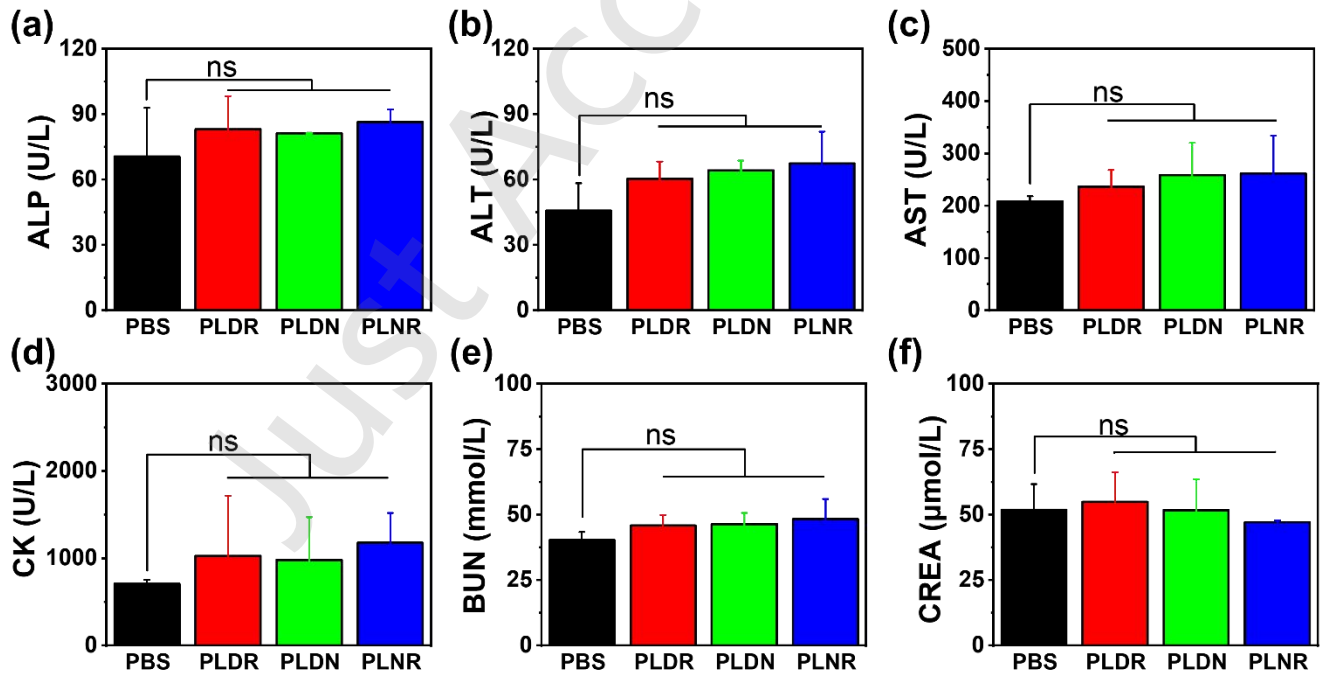


Figure S18 Blood biochemistry test for mice with different treatments (PBS, PLDR, PLDN and PLNR) at 30th day. (a) ALP: alkaline phosphatase; (b) ALT: alanine aminotransferase; (c) AST: aspartate aminotransferase; (d) CK: creatine kinase; (e) BUN: blood urea nitrogen and (f) CREA: creatinine.

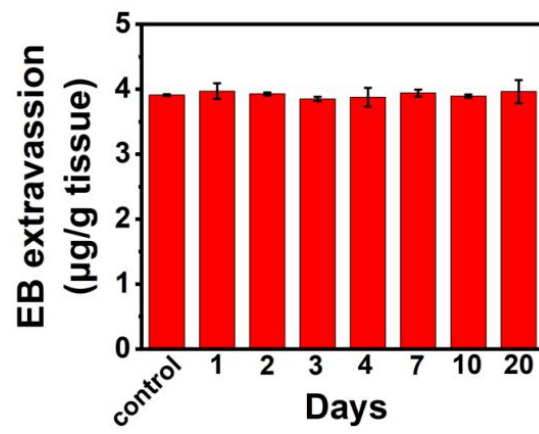


Figure S19 EB extravasation in brain of mice treated with PLDR at different days. Control group represented mice treated with PBS.