Supporting Information

Ligand Content-Dependent Exocytosis Governs the Blood-Brain Barrier Transcytosis of Nanocarriers

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MATERIALS AND METHODS

Materials. Chloroauric acid ($HAuCl_4$), sodium borohydride ($NaBH_4$), cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTAC), cytochalasin D (CytoD), FITC-labeled Dextrans (10 kDa), dimethyl sulfoxide (DMSO) and L-ascorbic acid were obtained from Aladdin Reagent Co., Ltd (Shanghai, China). Soybean lecithin, cholesterol and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from AVT Pharmaceutical Tech Co., Ltd (Shanghai, China). Nystatin and chlorpromazine (CPZ) were purchased from Macklin Biochemical Co., Ltd (Shanghai, China). CellMask™ Orange stains were purchased from Thermo Scientific (USA). Z-Phe-Phe-Phe-OH was purchased from Shanghai Hongye Biotechnology Co., Ltd. Hoechst 33342 and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Beyotime Biotechnology (Shanghai, China). Sodium fluorescein was purchased from Wuhan Yuanye Biotechnology Co., Ltd (Wuhan, China). Sucrose and mannose were obtained from Solarbio Science & Technology Co., Ltd. Anti-GLUT 1 was purchased from Sanying Biotechnology Co., Ltd. SH-PEG_{2000}-Cy5 and DSPE-PEG_{2000}-ANG (1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol)-2000-Angiopep-2) were purchased from Taisei Biotechnology Co., Ltd. SH-PEG_{2000}-MAN was purchased from Pengshuo Biotechnology Co., Ltd (Shanghai, China). SH-PEG_{2000}-MAN-FITC was obtained from Weihua Biotechnology Co., Ltd (Guangzhou, China). Chloroform was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All reagents were analytical or better grade and used without further purification. All aqueous solutions were prepared using ultrapure water (Milli-Q, Millipore).

Cell culture and animals. The hCMEC/D3 cells, which are immortalized human brain microvascular endothelial cells, were procured from Procell Life Science & Technology Co., Ltd (Hubei, China). The complete culture medium for the hCMEC/D3 cells was obtained from Aoruisai Biotechnology Co., Ltd. (Shanghai, China). Trypsin was sourced from Shanghai Xiaopeng Biotechnology Co., Ltd. The cells were incubated at 37 °C in a humidified incubator with an atmosphere of 5 % CO_2.

Female BALB/c nude mice, aged 6-7 weeks, were obtained from the Wuhan Experimental Animal Center. All animals were cared for in accordance with the guidelines set forth in the

Guide for the Care and Use of Laboratory Animals. The procedures conducted in this study received approval from the Animal Care and Use Committee of Wuhan University (SYXK 2015-0027, Wuhan, China).

Synthesis of the core nanoparticles (MAN-Au 0-5). Gold nanoparticles (AuNPs) were synthesized through an optimized three-step seed-mediated growth protocol. In the initial step, a gold cluster solution was prepared by combining $HAuCl_4$ aqueous solution (0.01 M, 0.25 mL) and $NaBH_4$ aqueous solution (0.01 M, 0.60 mL) in CTAB aqueous solution (0.1 M, 9.75 mL), with subsequent reaction at 30 °C for 3 h. Next, 0.12 mL of the synthesized gold cluster solution was introduced into a mixture of CTAB (0.1 M, 9.76 mL), $HAuCl_4$ (0.01 M, 4 mL), and L-ascorbic acid (0.1 M, 15 mL) in aqueous medium. The reaction was allowed to proceed under continuous stirring at 30 °C for 3 h, after which the product was collected by centrifugation at 11490 rcf for 40 min. The resulting precipitate was redispersed in 50 mL deionized water to obtain the gold seed solution.

In the final step, 9 mL of the gold seed solution was added to a blended aqueous system containing CTAC (0.025 M, 180 mL), L-ascorbic acid (0.1 M, 4.5 mL), and $HAuCl_4$ (0.01 M, 9 mL). The reaction mixture was maintained at 30 °C for 3 h, followed by centrifugation at 1750 rcf for 1 h. The collected solid was redispersed in 750 mL deionized water. To this dispersion, CTAB (5.5 g) and $HAuCl_4$ aqueous solution (0.01 M, 2.7 mL) were added, and the system was incubated at 45 °C for 2 h. The final product was isolated through a two-step centrifugation process (1750 rcf for 45 min and 1215 rcf for 45 min), and the resulting AuNPs were redispersed in 50 mL deionized water.

A solution of gold nanoparticles (1 mL, 0.05 mg/mL) was mixed with SH-PEG_{2000}-Cy5 (1 μL, 5 mg/mL) and incubated under shaking conditions at 37 °C for 12 h. The resulting mixture was centrifuged at 5000 rpm for 15 min, and the precipitate was resuspended in 1 mL of deionized water. To prepare six MAN-Au formulations with varying mannose (MAN) contents (designated MAN-Au 0-5), the following steps were performed: MAN-Au 0 (the control group without MAN modification) was prepared without the addition of SH-PEG_{2000}-MAN. Subsequently, for the other five groups (MAN-Au 1-5), 1 μL, 3 μL, 4 μL, 5 μL, or 20 μL of SH-PEG_{2000}-MAN aqueous solution (1 mg/mL) was added separately, followed by incubation at 37 °C with shaking for 12 h. The mixture was then centrifuged again at 5000 rpm

for 15 min, and the precipitate was redispersed in 1 mL of deionized water to yield MAN-Au 0-5 with varying MAN contents.

Synthesis of ANG-Lip. Angiopep-2 (ANG)-modified membrane-fusogenic liposomes (designated as ANG-Lip) were prepared by the thin-film hydration method following established literature procedures. A mixture of DOTAP, lecithin, and cholesterol at a molar ratio of 50:8:10, along with 7.5 mg of DSPE-PEG_{2000}-ANG, was dissolved in chloroform (30 mL). The organic solvent was subsequently removed by rotary evaporation under reduced pressure at 45 °C for 30 min to form a thin lipid film. Hydration of the film was carried out with deionized water (48 mL) under sonication, yielding the final ANG-Lip formulation.

Synthesis of six liposome-coated nanocarriers. Six nanocarriers (designated as ANG-Lip-Au and ANG-Lip-MAN-Au 1-5) were prepared by coating different MAN-modified gold nanoparticles with ANG-Lip liposomes. The preparation of ANG-Lip-MAN-Au 1 was used as an illustrative example to describe the detailed synthesis procedure. The mass ratio of MAN-modified gold cores to ANG-modified liposomes was confirmed to be 1:1.5. Specifically, MAN-modified gold cores aqueous solutions was mixed with an ANG-Lip aqueous solution and sonicated in an ice bath for 10 min. The mixture was then centrifuged twice at 5000 rpm for 10 min. At this speed, "empty" liposomes remained in the supernatant and were removed, whereas both "coated" nanoparticles and "uncoated" gold cores were pelleted and collected. The precipitate was redispersed in water and then mixed with 0.8 M sucrose solution. The mixture was left undisturbed for 1 h to allow aggregation of the "uncoated" gold cores, followed by centrifugation at 3000 rpm for 20 min. The "uncoated" gold cores tended to aggregate in sucrose solutions due to the residual small amount of cetyltrimethylammonium bromide (CTAB) as a stabilizer. In contrast, "coated" nanoparticles did not aggregate in sucrose solution due to the protective liposomal shell that prevented direct contact between the gold core and sucrose. The aggregated "uncoated" gold cores formed a precipitate at the bottom, while the supernatant contained the purified "coated" nanoparticles.

Quantification of MAN content in nanocarriers. Fluorescence method was used to determine the MAN content in nanocarriers. FITC-labeled SH-PEG_{2000}-MAN was employed as a substitute for unlabeled SH-PEG_{2000}-MAN to prepare fluorescently labeled core nanoparticles with varying MAN contents following the same synthesis procedure used for the

unlabeled counterparts. Following synthesis, the samples were centrifuged at 5000 rpm for 15 min, and the fluorescence intensity of the resulting supernatants was measured. The amount of unbound FITC-labeled SH-PEG_{2000}-MAN was calculated using a pre-established standard curve correlating its concentration with fluorescence intensity, with the conjugated MAN content obtained by subtracting the unbound amount from the total initial amount added. These FITC-labeled core nanoparticles were then coated with liposomes to generate six FITC-labeled liposome-coated nanocarriers. After centrifugation at 5000 rpm for 10 min, the supernatant showed negligible fluorescence intensity, confirming that MAN did not detach during liposome coating. The MAN content in the liposome-coated nanocarriers was calculated based on that of the corresponding core nanoparticles.

Cytotoxicity assay. The hCMEC/D3 cells were seeded in a 96-well plate at a density of 1×10^5 cells per well and allowed to adhere overnight. Then the cells were treated separately with six PEGylated gold nanoparticle cores (MAN-Au 0-5) and their corresponding liposome-coated derivatives (ANG-Lip-Au and ANG-Lip-MAN-Au 1-5) for 48 h. The gold nanoparticles concentration ranged from 6.25μg/mL to 800 μg/mL. After treatment, 20 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for an additional 4 h. The supernatant was then carefully removed, and the resulting formazan crystals were solubilized in 150 μL of dimethyl sulfoxide (DMSO). Absorbance was measured at 490 nm using a Bio-Rad 550 microplate reader. Cell viability was calculated according to the following formula:

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_0}{A_{\text{control}} - A_0} \times 100$$

The absorbance of the experimental group (A_{sample}) was measured from cells treated with both nanocarriers and MTT. The control absorbance ($A_{control}$) was obtained from cells treated with MTT only, and the blank absorbance (A_0) was measured from untreated cells.

Investigation of the cell internalization pathways of the nanocarriers. To track the dissociation between the liposomal shell and the nanocarrier core, the hCMEC/D3 cells were seeded onto glass-bottom culture dishes (1×10^5 cells/dish) and incubated for 24 h. The liposomal shell of ANG-Lip-MAN-Au 2 was specifically labeled with CellMask™ Orange

plasma membrane stain. Then, the labeled nanocarriers (C_{AuNPs} = 0.05 mg/mL) were added to the cells and cultured for 2 h. Subsequently, the cells were washed three times with PBS to remove nanocarriers not internalized by cells. Then, 1 μL of 5 μg/mL Hoechst 33342 staining solution was added to cells to stain cell nuclei for 10 min, and the cells were washed three times with PBS again. A Carl Zeiss LSM880 CLSM was used to observe intracellular fluorescence signals, and Z-stack scanning (continuous scanning of different cell sections) was performed to obtain spatial distribution images of the nanocarrier core (Cy5-labeled, red fluorescence) and liposomal shell (CellMask™ Orange-labeled, green fluorescence). Image J software was applied to analyze the gray value profiles of the green (liposomal shell) and red (nanocarrier core) fluorescence channels along the white arrows marked in the merged CLSM images.

To study the colocalization of the nanocarrier core and lysosomes, ANG-Lip-MAN-Au 2 without labeled liposomal shells were directly added to cells and cultured for 2 h. The concentration of gold nanoparticles was 0.05 mg/mL. After incubation, the cells were washed with PBS to remove uninternalized nanocarriers. Subsequently, 1 μL of 5 μg/mL Hoechst 33342 staining solution (for nuclear staining, incubated for 10 min) and 1 μL of 1 mM Lyso-Tracker Green staining solution (for lysosome staining, incubated for 15 min) were added to cells. After staining, the cells were washed three times with PBS again. The CLSM was used for Z-stack scanning to obtain spatial distribution images of the nanocarrier core (Cy5-labeled, red fluorescence) and lysosomes (Lyso-Tracker Green-labeled, green fluorescence).

To investigate the cellular uptake pathways of liposome-coated nanocarriers, hCMEC/D3 cells were subjected to pretreatment with various inhibitors targeting distinct delivery pathways. The hCMEC/D3 cells were seeded in six-well plates (2×10^5 cells/ per well) and cultured for 24 h. These inhibitors included chlorpromazine (30 μM), nystatin (30 μM), cytochalasin D (10 μM), and Z-Phe-Phe-Phe-OH (50 μg/mL). Following the pretreatment period for 1 h, the cells were washed three times with PBS to remove residual inhibitors. Subsequently, the nanocarriers (C_{AuNPs} = 0.05 mg/mL) were individually added to cells and cultured for 2 h. Additionally, the cells treated with exclusively nanocarriers for 2 h at 4 °C or 37 °C were also used for comparative analysis. The intracellular fluorescence intensity was quantified via flow cytometry (FCM, BD Accuri C6).

In vitro BBB crossing efficiency. To evaluate in vitro BBB crossing efficiency of nanocarriers, the hCMEC/D3 cells were seeded in 12-well transwell plates at a density of 5.0 × 10^5 cells/per well. The transwell plates were equipped with Labselect Transwell polyether membrane cell culture inserts, which featured an average pore size of 0.4 μm, a diameter of 12 mm, and a growth area of 1.1 cm^2.

The permeability of FITC-labeled Dextran (10 kDa) and sodium fluorescein were measured to verify the integrity of the in vitro BBB model. FITC-labeled Dextran solution (0.5 μg/mL, 1 mL) and sodium fluorescein solution (10 μg/mL, 1 mL) were added separately to the apical chamber of the in vitro BBB model and incubated with the cells for 1 h. After incubation, the culture medium in the basolateral chamber was collected, and fluorescence detection was performed using fluorometer (RF-6000, Shimadzu). The detection parameters for FITC-labeled Dextran were set as excitation wavelength of 490 nm and emission wavelength of 523 nm. For sodium fluorescein, the detection parameters were set as excitation wavelength of 490 nm and emission wavelength of 520 nm. The standard concentration curves for FITC-labeled Dextran and sodium fluorescein were constructed. The amount of FITC-labeled Dextran or sodium fluorescein that permeated the monolayer was calculated using the corresponding calibration curves. The apparent permeability coefficient (P_{app}) was calculated as:

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

Q represented the total amount of FITC-labeled Dextran or sodium fluorescein that have permeated through the apical chamber (μg). A denoted the surface area of the transwell filter (cm^2). C was the initial concentration of the FITC-labeled Dextran or sodium fluorescein in the apical chamber. t referred to the duration of the experiment (s). In this experiment, the value of A was 1.1 cm^2. The incubation time with the FITC-labeled Dextrans was 1 h, which converted to 3600 s.

To study the impact of nanocarriers on the barrier integrity, six liposome coated nanocarriers (ANG-Lip-Au, ANG-Lip-Au 1, ANG-Lip-MAN-Au 2, ANG-Lip-MAN-Au 3, ANG-Lip-MAN-Au 4, ANG-Lip-MAN-Au 5) with varying mannose (MAN) contents were added to the apical chamber, followed by incubation in an incubator for 24 h. The concentration of gold nanoparticles was 0.05 mg/mL. After incubation, TEER values and the permeability of FITC-Dextran (10 kDa) and sodium fluorescein across the BBB monolayer were measured.

To evaluate the in vitro BBB crossing efficiency of nanocarriers, hCMEC/D3 cells were seeded in 12-well transwell plates at a density of 5.0×10^5 cells/per well.Various nanocarriers (C_{AuNPs} = 0.05 mg/mL) were introduced into the apical chamber and incubated for 24 h. Subsequently, the culture medium from the basolateral chamber was collected and its fluorescence intensity was measured using a fluorometer (RF-6000, Shimadzu), with the excitation (λ_{ex}) set at 640 nm and the emission (λ_{em}) set at 670 nm. The BBB penetration efficiencies of the nanocarriers were then determined using their respective standard concentration curves.

To further assess whether MAN enhanced the trans-BBB delivery of nanocarriers into the brain parenchyma, ANG-Lip-MAN-Au 1-5 (C_{AuNPs} = 0.05 mg/mL) were added separately to the apical chambers of transwell plates and incubated at 37 °C for 12 h. After incubation, the medium in both the apical and basolateral chambers was carefully removed, and the cell layers were gently washed three times with PBS buffer to thoroughly eliminate nanocarriers not taken up by the cells. Subsequently, an equal volume of fresh complete medium was added to both the apical and basolateral chambers, and the model was placed again in a 37 °C incubator for an additional 12 h. Following this, the medium from the apical and basolateral chambers of each well was collected. The fluorescence intensity of the apical chamber medium (I_{Api}) and that of the basolateral chamber medium (I_{Bas}) were measured using a fluorescence spectrophotometer.

In vivo fluorescence imaging. Healthy BALB/c nude mice (n = 3) received tail vein injections of different nanocarriers (C_{AuNPs} = 13 mg kg^{-1}). The fluorescence signals of brain were acquired using an IVIS Spectrum (PerkinElmer) in vivo imaging system. At 10 h post-injection, mice were euthanized to collect the main organs (heart, liver, spleen, lung, kidney and brain) for ex vivo fluorescence imaging.

Biosafety evaluation. To assess the in vivo biosafety of different nanocarriers, different nanocarriers (C_{AuNPs} = 13 mg kg^{-1}) were administered to healthy BALB/c mice (n = 3) via intravenous injection, respectively. Meanwhile, a control group was established in which mice were treated with PBS. Mice were euthanized at 24 h and 30 days post-injection to harvest major organs (heart, liver, spleen, lungs, kidneys, and brain) and blood samples. The major organs were fixed in 4% paraformaldehyde, paraffin embedded, sectioned at 4 μm, stained with

hematoxylin and eosin (H&E), and finally imaged under an optical microscope. Additionally, white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were detected.

Statistical analysis. Statistical significance was performed by using the software Graphpad Prism. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****) are considered statistically significant, while ns means no statistical difference.

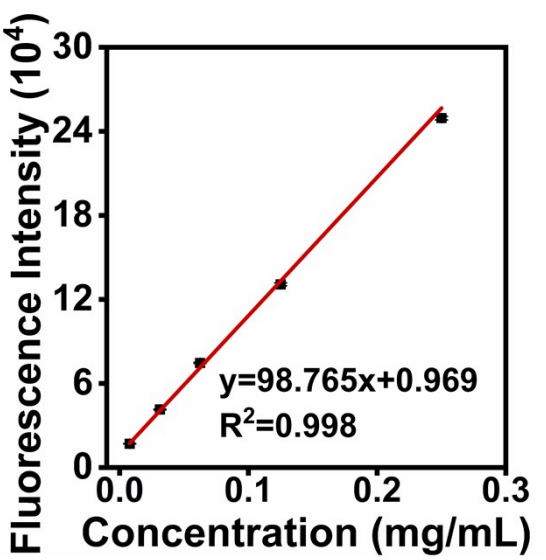

Fig. S1 Standard concentration curve of FITC-labeled SH-PEG_{2000}-MAN.

Table S1 MAN content in six core nanoparticles and liposome-coated nanocarriers.

Sample	Quantified MAN content (wt%) by fluorescence method	Theoretical MAN content (wt%)
MAN-Au 0	0	0
MAN-Au 1	1.76 ± 0.01	1.79
MAN-Au 2	5.12 ±0.06	5.17
MAN-Au 3	6.56 ± 0.20	6.78
MAN-Au 4	8.22 ± 0.13	8.33
MAN-Au 5	26.31 ± 0.19	26.67
ANG-Lip-Au	0	0
ANG-Lip-MAN-Au 1	0.72 ± 0.03	0.76
ANG-Lip-MAN-Au 2	2.17 ± 0.07	2.26
ANG-Lip-MAN-Au 3	2.78 ± 0.06	2.99
ANG-Lip-MAN-Au 4	3.51 ± 0.05	3.70
ANG-Lip-MAN-Au 5	12.71 ± 0.18	13.33

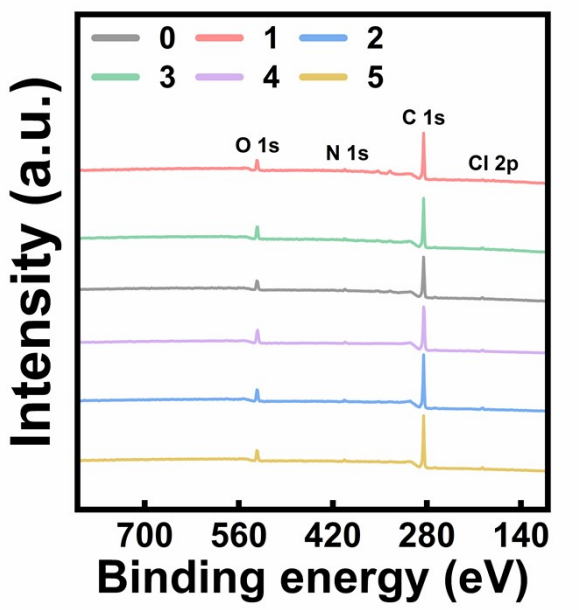

Fig. S2 X-ray photoelectron spectroscopy (XPS) survey spectra of nanocarriers. 0: ANG-Lip-Au, 1: ANG-Lip-MAN-Au 1, 2: ANG-Lip-MAN-Au 2, 3: ANG-Lip-MAN-Au 3, 4: ANG-Lip-MAN-Au 4, 5: ANG-Lip-MAN-Au 5.

Table S2 Carbon and oxygen contents (at%) of six liposome-coated nanocarriers measured by XPS.

Sample	Carbon (at%)	Oxygen (at%)
ANG-Lip-Au	83.30	10.51
ANG-Lip-MAN-Au 1	82.64	11.12
ANG-Lip-MAN-Au 2	83.71	10.97
ANG-Lip-MAN-Au 3	82.40	11.60
ANG-Lip-MAN-Au 4	83.48	11.46
ANG-Lip-MAN-Au 5	84.41	10.23

Table S3 The description, sizes, and zeta potentials of six core nanoparticles and six liposome-coated nanocarriers.

Sample	Description	Size (nm)	Zeta potential (mV)
MAN-Au 0	gold nanoparticles modified by Cy5 and MAN (0 wt%)	56.9 ± 0.7	+29.2 ± 0.8
MAN-Au 1	gold nanoparticles modified by Cy5 and MAN (1.76 wt%)	57.1 ± 0.3	+29.0 ± 0.6
MAN-Au 2	gold nanoparticles modified by Cy5 and MAN (5.12 wt%)	57.8 ± 0.9	+28.9 ± 0.5
MAN-Au 3	gold nanoparticles modified by Cy5 and MAN (6.56 wt%)	58.0 ± 1.1	+28.5 ± 0.7
MAN-Au 4	gold nanoparticles modified by Cy5 and MAN (8.22 wt%)	57.9 ± 0.5	+27.8 ± 0.6
MAN-Au 5	gold nanoparticles modified by Cy5 and MAN (26.31 wt%)	58.0 ± 0.9	+26.0 ± 0.8
ANG-Lip-Au	MAN-Au 0 coated by liposome	76.4 ± 1.4	+41.5 ± 1.1
ANG-Lip-MAN-Au 1	MAN-Au 1 coated by liposome	76.7 ± 1.4	+42.1 ± 1.2
ANG-Lip-MAN-Au 2	MAN-Au 2 coated by liposome	77.3 ± 1.3	+42.6 ± 1.3
ANG-Lip-MAN-Au 3	MAN-Au 3 coated by liposome	77.8 ± 1.1	+42.5 ± 1.1
ANG-Lip-MAN-Au 4	MAN-Au 4 coated by liposome	76.9 ± 1.2	+43.1 ± 1.5
ANG-Lip-MAN-Au 5	MAN-Au 5 coated by liposome	77.1 ± 1.5	+42.6 ± 1.3

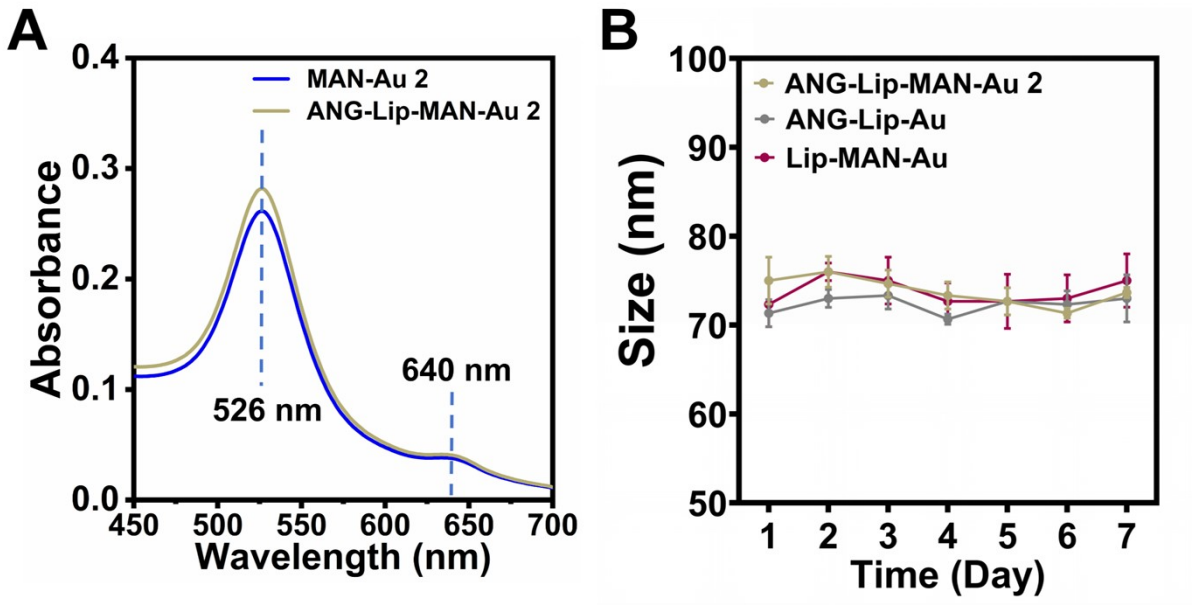

Fig. S3 (A) UV absorption spectroscopy of MAN-Au 2 and ANG-Lip-MAN-Au 2. **(B)** Hydrodynamic size stability of nanocarriers over 7 days.

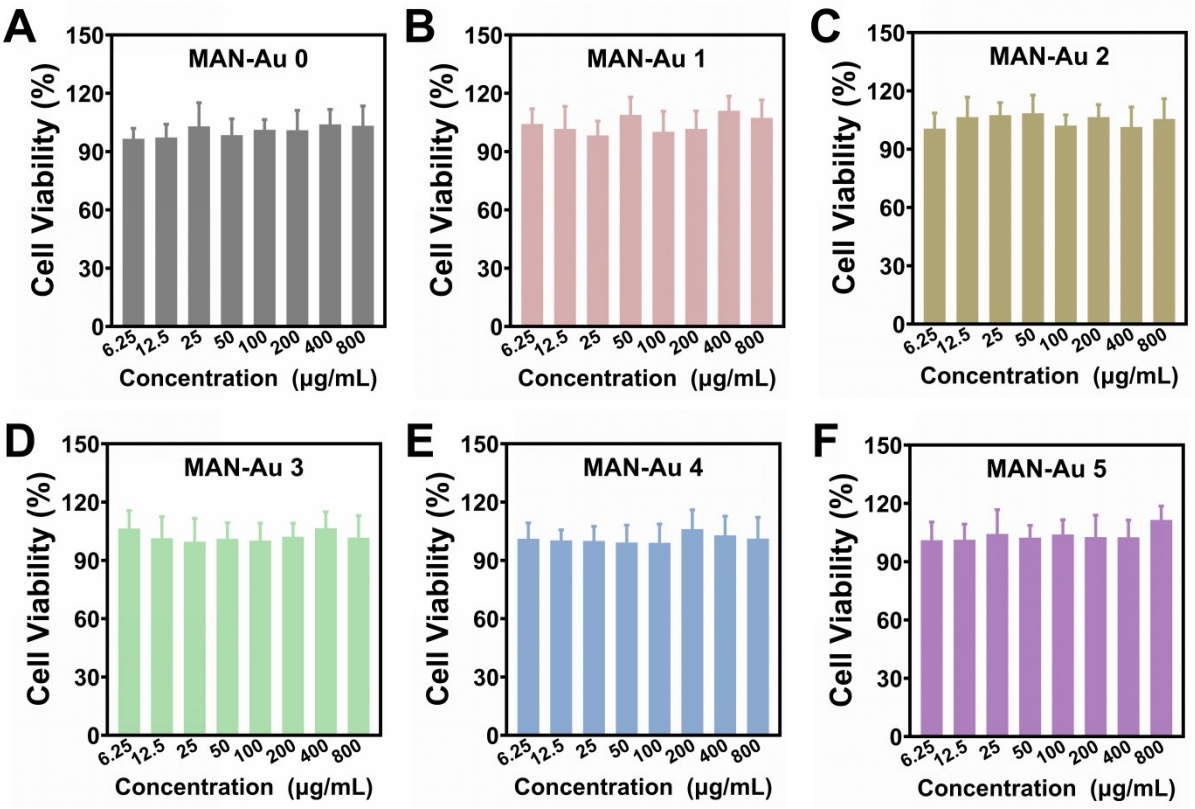

Fig. S4 Cytotoxicity of six core nanoparticles against hCMEC/D3 cells after 48 h incubation.

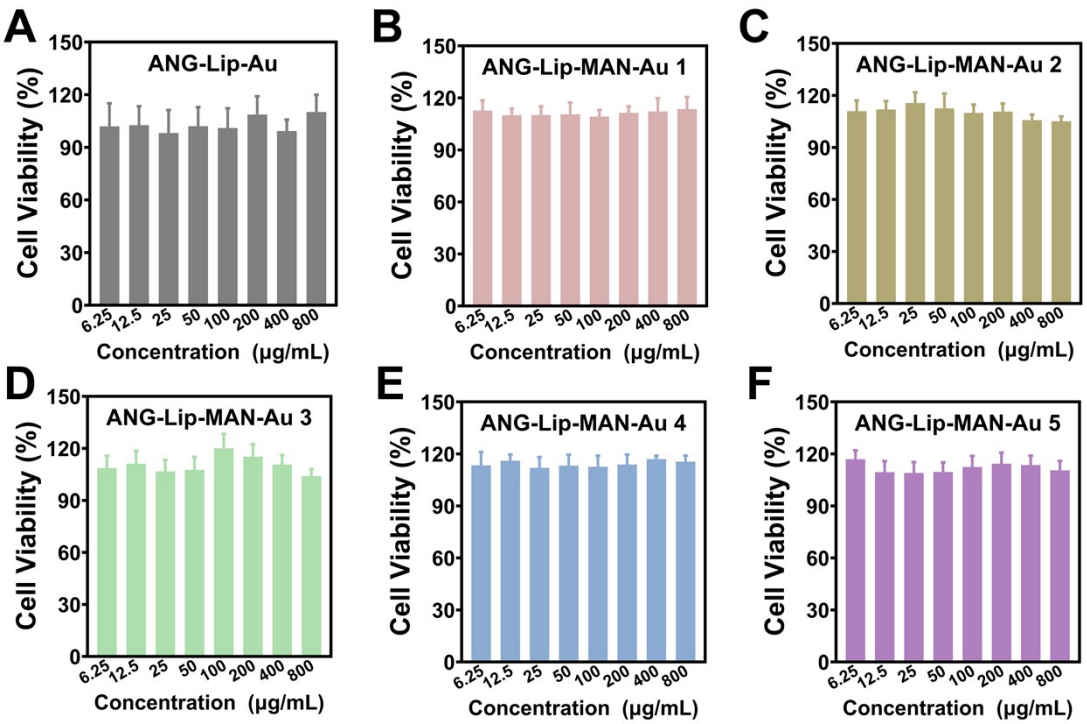

Fig. S5 Cytotoxicity of six liposome-coated nanocarriers against hCMEC/D3 cells after 24 h incubation.

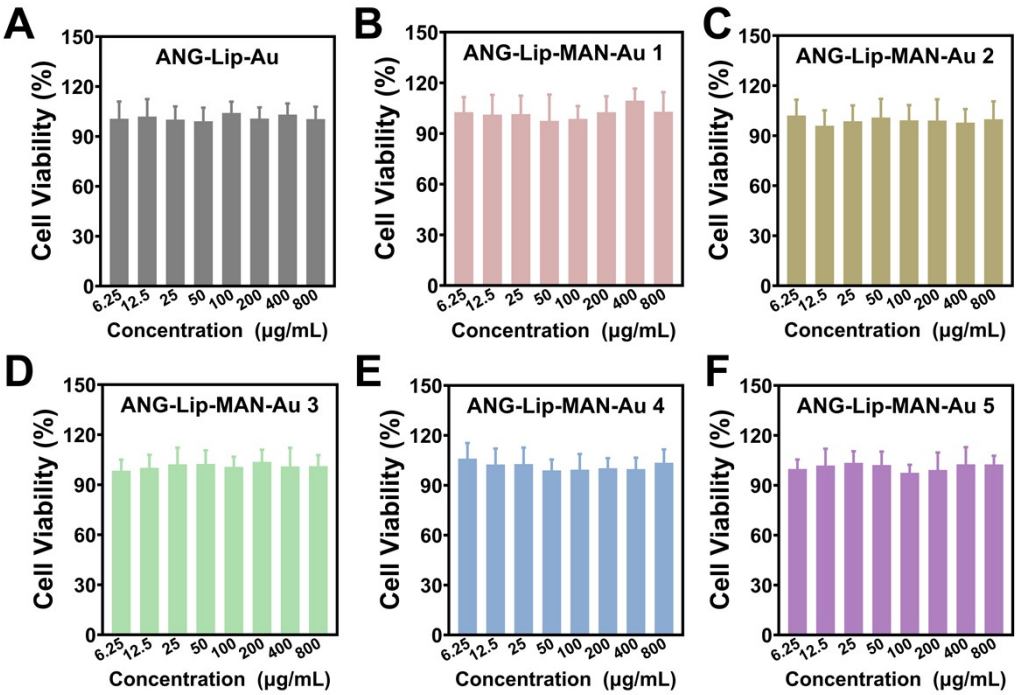

Fig. S6 Cytotoxicity of six liposome-coated nanocarriers against hCMEC/D3 cells after 48 h incubation.

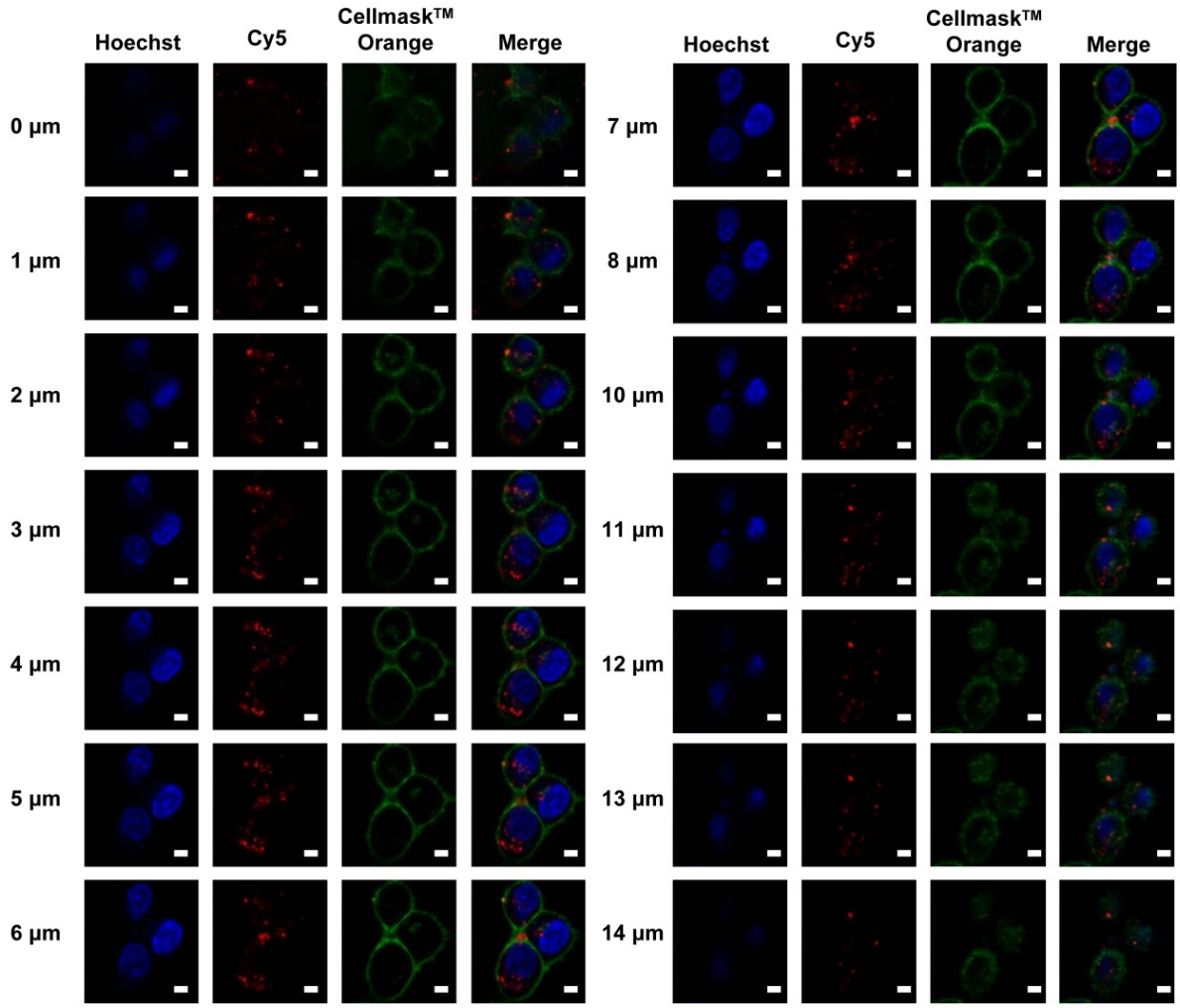

Fig. S7 Serial Z-stack confocal images of hCMEC/D3 cells treated with ANG-Lip-MAN-Au 2 for 2 h (at 1 µm depth intervals). The liposomal shell of nanocarriers was stained with CellMask™ Orange plasma membrane stain (green fluorescence), and the nanocarrier core was labeled with Cy5 (red fluorescence). Cell nuclei were stained with Hoechst 33342 (blue fluorescence). Scale bar: 5 µm.

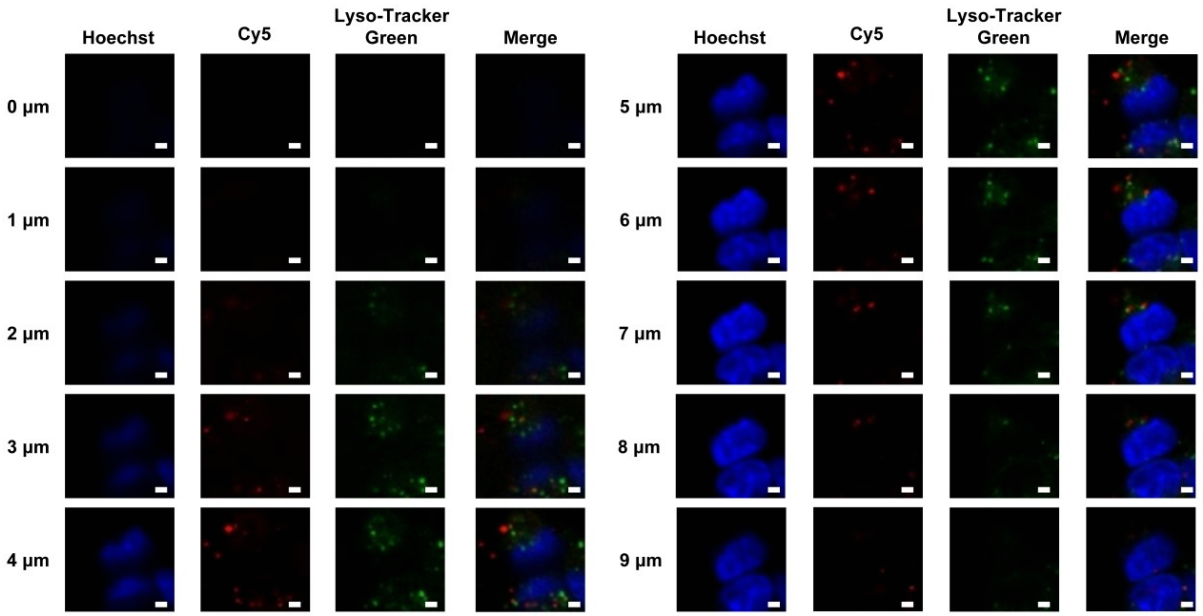

Fig. S8 Serial Z-stack CLSM images of hCMEC/D3 cells treated with ANG-Lip-MAN-Au 2 nanocarriers for 2 h (at 1 µm depth intervals). Lysosomes were stained with Lyso-Tracker Green (green fluorescence), and the nanocarrier core was labeled with Cy5 (red fluorescence). Cell nuclei were stained with Hoechst 33342 (blue fluorescence). Scale bar: 2 µm.

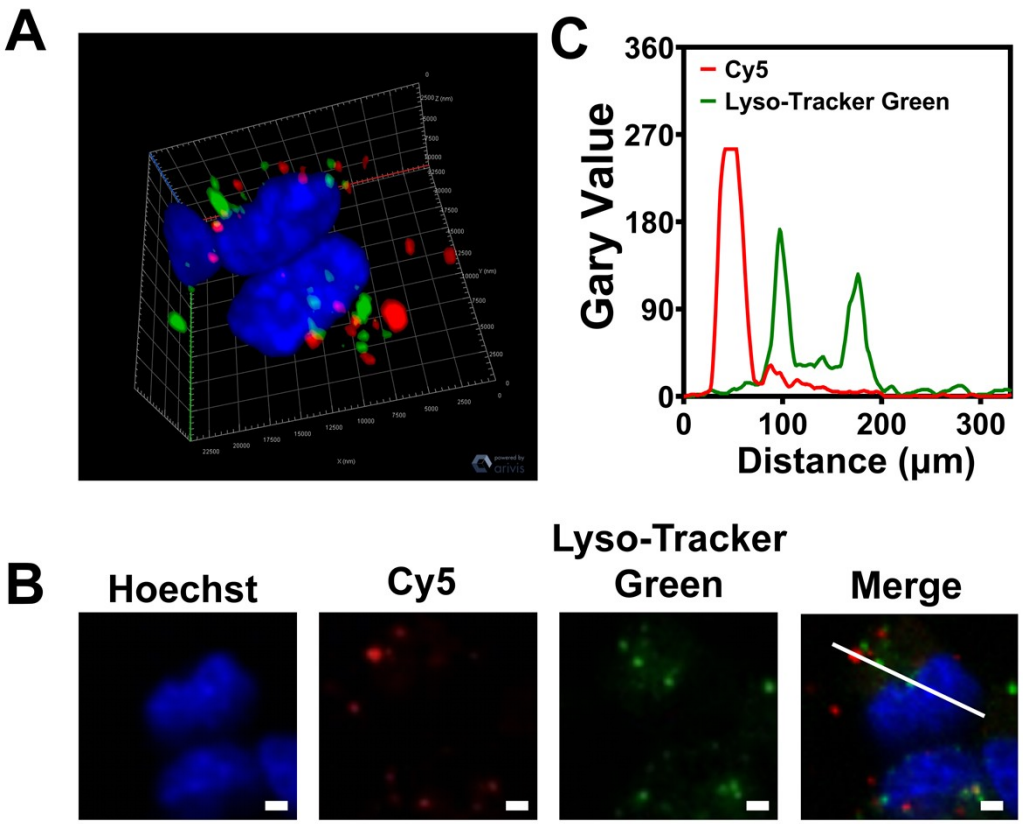

Fig. S9 (A) Confocal 3D reconstruction image of hCMEC/D3 cells treated with ANG-Lip-MAN-Au 2 for 2 h. Lysosomes were stained with Lyso-Tracker Green (green fluorescence), and the nanocarrier core was labeled with Cy5 (red fluorescence). Cell nuclei were stained with Hoechst 33342 (blue fluorescence). **(B)** A single optical section from confocal z-stack imaging. Scale bar: 2 μm. **(C)** The gray values of the red (the core of nanocarriers) and green (lysosomes) channels along the corresponding white arrows in the "Merge" images were analyzed using Image J software.

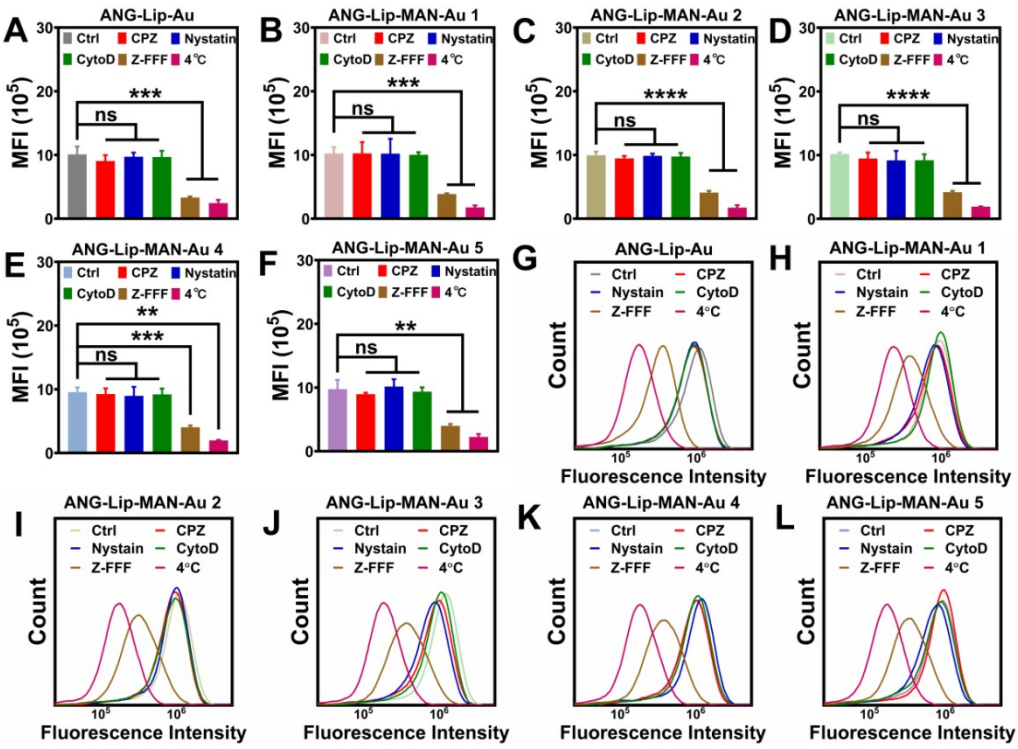

Fig. S10 Flow cytometry analysis and mean fluorescence intensity (MFI) of hCMEC/D3 cells with different nanocarriers for 2 h at 4 °C or in the presence of endocytosis inhibitors at 37 °C. These nanocarriers included ANG-Lip-Au **(A** and **G)**, ANG-Lip-MAN-Au 1 **(B** and **H)**, ANG-Lip-MAN-Au 2 **(C** and **I)**, ANG-Lip-MAN-Au 3 **(D** and **J)**, ANG-Lip-MAN-Au 4 **(E** and **K)**, and ANG-Lip-MAN-Au 5 **(F** and **L)**. The control group was the cells incubated with nanocarriers at 37 °C. Data are presented as mean ± SD ($n \geq 3$). $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****) are considered statistically significant, while ns means no statistical difference.

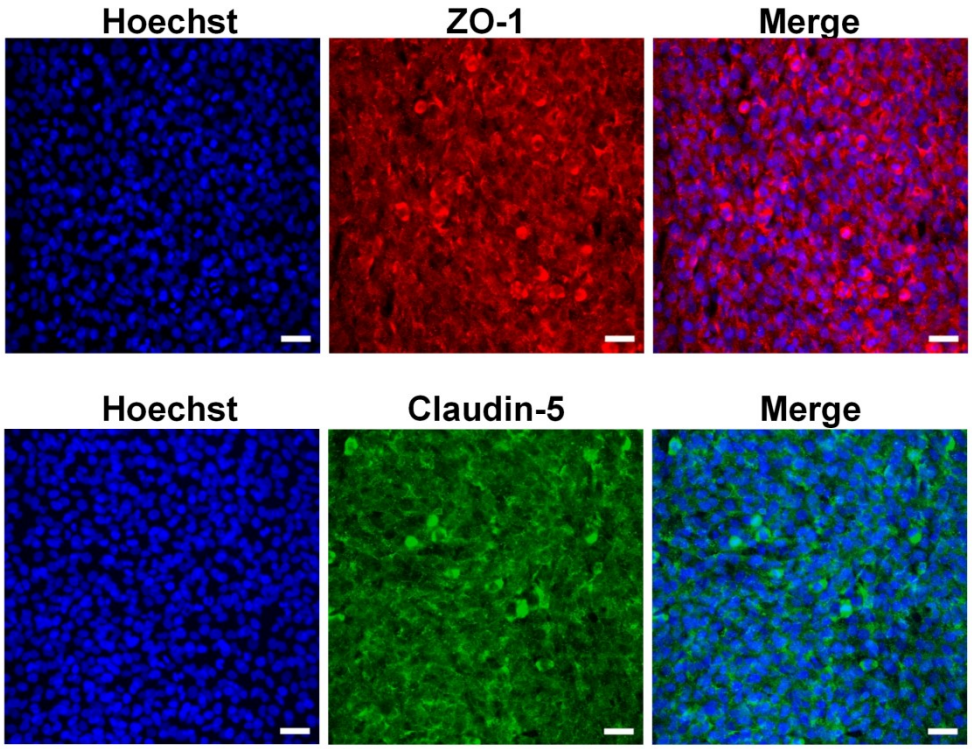

Fig. S11 Immunofluorescence images of ZO-1 (red) and Claudin-5 (green) in BBB monolayer. Nuclei were stained by Hoechst 33342 (blue). Scale bar: 20 μm.

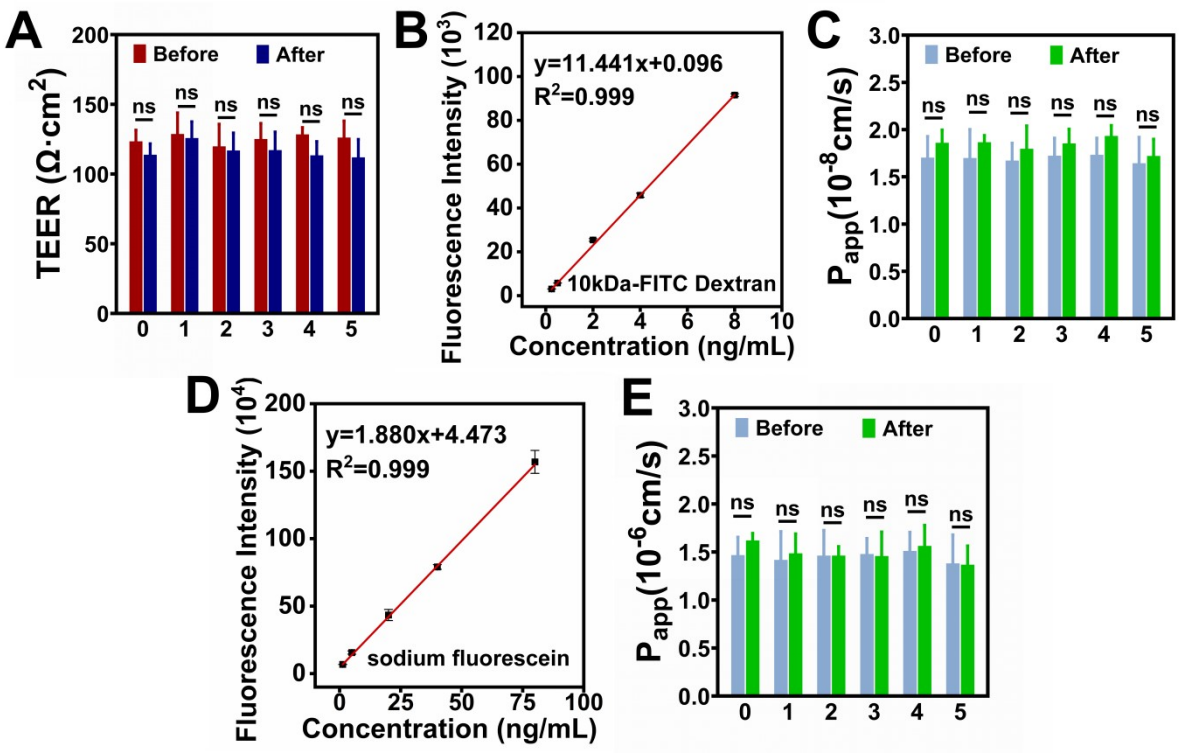

Fig. S12 (A) Transendothelial electrical resistance (TEER) values of the BBB monolayer before and after treatment with six liposome coated nanocarriers with varying mannose (MAN) contents (C_{AuNPs} = 0.05 mg/mL) for 24 h. **(B)** Standard concentration curve of 10 kDa FITC-Dextran. **(C)** Permeability of 10 kDa FITC-Dextran across the BBB monolayer before and after treatment with nanocarriers (C_{AuNPs} = 0.05 mg/mL) for 24 h. **(D)** Standard concentration curve of sodium fluorescein. **(E)** Permeability of sodium fluorescein across the BBB monolayer before and after treatment with nanocarriers (C_{AuNPs} = 0.05 mg/mL) for 24 h. 0: ANG-Lip-Au, 1: ANG-Lip-MAN-Au 1, 2:ANG-Lip-MAN-Au 2, 3: ANG-Lip-MAN-Au 3, 4: ANG-Lip-MAN-Au 4, 5: ANG-Lip-MAN-Au 5.

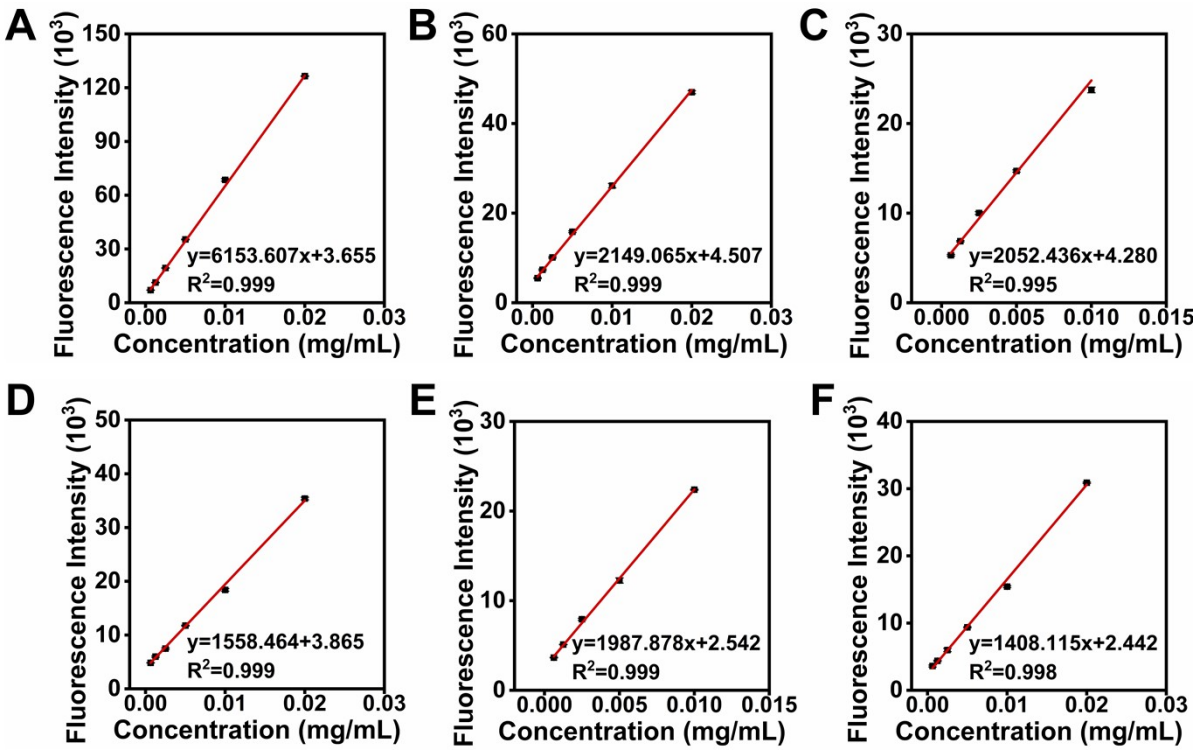

Fig. S13 Calibrated standard concentration curves of the cores (MAN-Au 0-5) of six liposome coated nanocarriers. (λ_{ex} = 640 nm, λ_{em} = 670 nm). A: MAN-Au 0; B: MAN-Au 1; C: MAN-Au 2; D: MAN-Au 3; E: MAN-Au 4; F: MAN-Au 5.

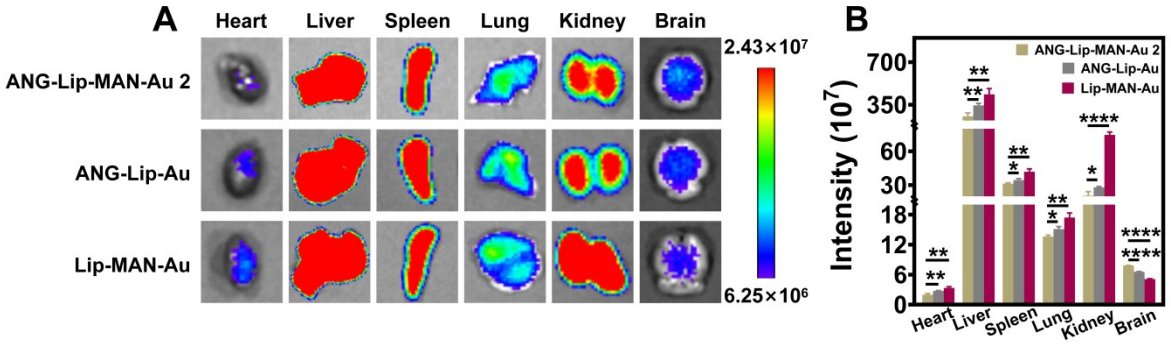

Fig. S14 (A) Ex vivo fluorescence images and **(B)** corresponding quantitative fluorescence analysis of various organs in normal mice treated with different nanocarriers (ANG-Lip-MAN-Au 2, ANG-Lip-Au and Lip-MAN-Au) at 10 h post-injection. C_{AuNPs} = 13 mg kg^{-1}.

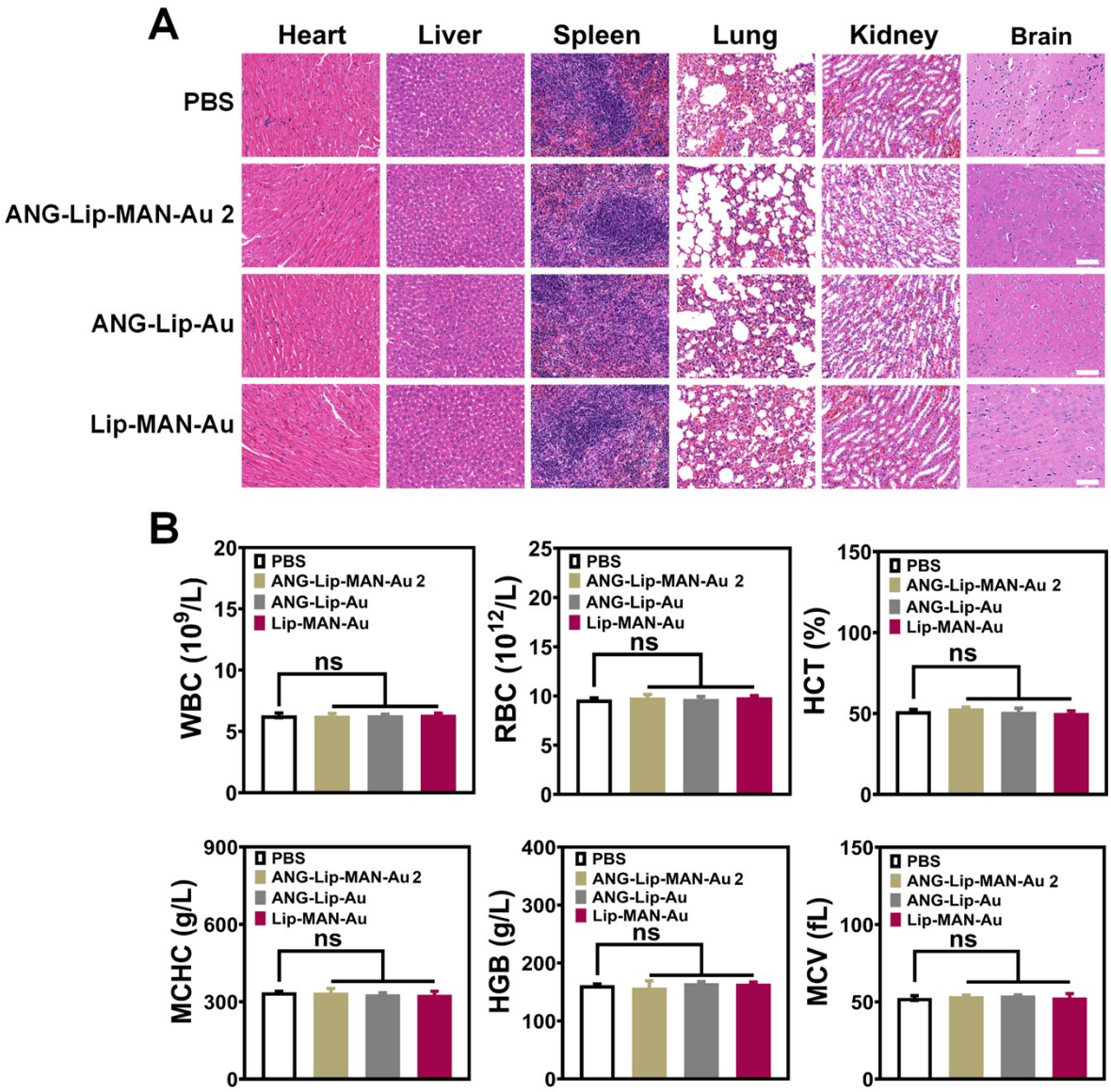

Fig. S15 (A) H&E staining images of major organs (heart, liver, spleen, lung, kidney and brain) of mice after different treatments at 24 h. Scale bar: 60 μm. **(B)** Blood routine analysis for BALB/c mice with different treatments at 24 h. Data are presented as mean ± standard deviation (SD) (n = 3). The ns means no statistical difference.

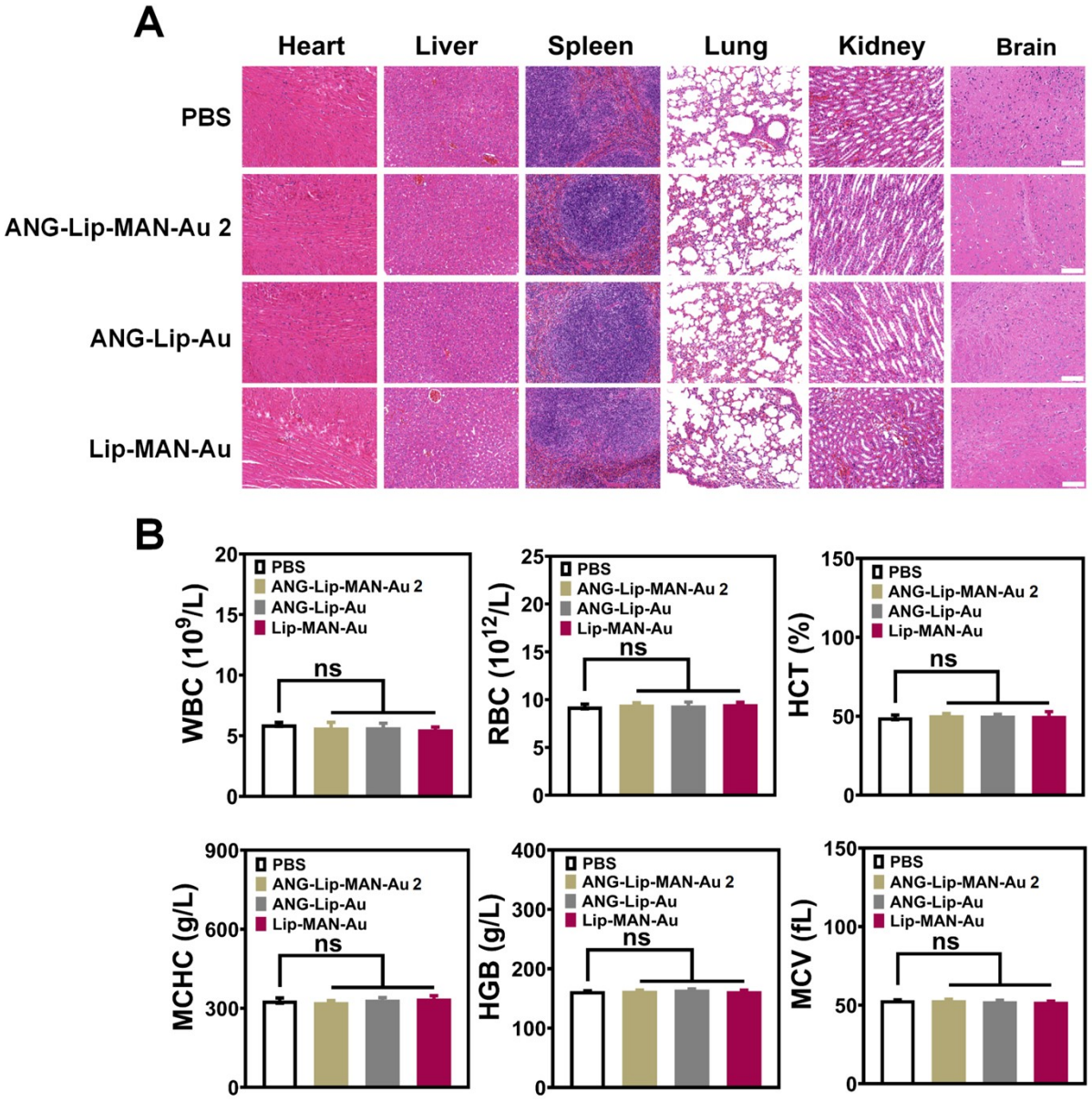

Fig. S16 (A) H&E staining images of major organs (heart, liver, spleen, lung, kidney and brain) of mice after different treatments at 30th day. Scale bar: 60 μm. **(B)** Blood routine analysis for BALB/c mice with different treatments at 30th day. Data are presented as mean ± standard deviation (SD) (n = 3). The ns means no statistical difference.