Supporting Information

Precise Delivery of Nanomedicines to M2 Macrophages by Combining "Eat Me/Don't Eat Me" Signals and Its Anticancer Application

Yixuan Tang,[†] Zhongjie Tang,[‡] Pingrong Li,[‡] Kaicheng Tang,[‡] Zhongyi Ma,[‡] Yantong Wang,[‡] Xiaoyou Wang,[‡] and Chong Li[‡*]*

.

[†] Institute of MateriaMedica, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, Shandong 250000, PR China.

[‡]Medical Research Institute, College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, PR China.

[*]Corresponding author: Chong Li, email: chongli@swu.edu.cn; Yixuan Tang, email: tangyixuan@sdfmu.edu.cn

Table of Contents

Supporting Information 1
Table of Contents 1
Methods 2
- Materials 2
- Cell lines and Animals 3
- Preparation and Characterization of Liposomes 4
- SPR analysis 5
- Phagocytosis Percentage Assay 6
- Flow Cytometry Analysis of M1 TAMs and M2 TAMs 6

Supporting Figures and Tables 8
- Figure S1 8
- Figure S2 8
- Figure S3 9
- Figure S4 9
- Figure S5 10
- Figure S6 10
- Figure S7 11
- Figure S8 11
- Figure S9 12
- Figure S10 12
- Figure S11 13
- Figure S12 13
- Figure S13 14
- Figure S14 14
- Figure S15 15
- Figure S16 16
- Figure S17 17
- Figure S18 17
- Table S1 18
- Table S2 19
- Table S3 20

References: 21

Methods:

Materials. Egg Lecithin Phosphatidylcholine (EPC) (AVT# N01002), Cholesterol (Cho) (AVT# O01001) and Distearoyl Phosphoethanolamine (DSPE) (AVT# S03004) were provided by A.V.T. Pharmaceutical Ltd. (Shanghai, China). Quantum™ R-PE MESF Medium Level (cat# FCSC827B) was from Bio-Rad Laboratories, Inc. N-(amino (polyethylene glycol)-2000) (PEG2000-NH_2) is a gift provided by Dr. Yang Yu (Southwest University, Chongqing, China). Dithiobis (succinimidyl propionate) (DSP) (Cat# R-S-0005) was supplied by Ruixi Bio. Technology Co. Ltd (Xian, China). Palmitic acid labeled Self-peptide (Pal-self) (palmitic acid-GNYTCEVTELSREGKTVIELK) and palmitic acid labeled 4N1K peptide (Pal-4N1K) (palmitic acid-CSSKRFYVVMWKK) were synthesized by means of solid phase peptide synthesis performed using Fmoc-protected amino acids and purified to homogeneity by reverse-phase high-performance liquid chromatography. DSPE-S-S-PEG2000 and N-oleoyl galactose were syhthesized as described elsewhere.[1-3] Egg Liss Rhod PE (Cat# 810146) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). DAPI (Cat# D4054), DiO (Cat# D4007), DiI (Cat# D4010), DiD (Cat# D4019) and Live & Dead™ Viability/Cytotoxicity Assay Kit (Cat# L6037) were supplied by US Everbright Inc (Suzhou, China). Rabbit Anti-CD80 (Cat# bs-2708R, 1:250 for IHC), Rabbit Anti-THBS1 antibody (Cat# bs-2715R), Rabbit anti-CD86 (Cat# bs-1035R, 1:200 for IF), Rabbit anti-CD206 (Cat# bs-4727R, 1:200 for IF), Rabbit anti-CD31 (Cat# bs-0195R, 1:250 for IHC), PE labeled donkey anti-rabbit IgG (Cat# bs-0295D-PE, 1:200 for IF), and HRP labeled Goat Anti-Rabbit IgG (Cat# bs-40295G-HRP,

1:5000 for WB) were purchased from Bioss biotechnology co. LTD (Beijing, China). mouse IgM (Cat# BF01011) was provided by Biodragon Immunotechnologies Co. Ltd (Beijing, China). Rabbit anti-iNOS (Cat# GB11119, 1:250 for IHC) was supplied by Servicebio Co. Ltd (Wuhan, China). 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (Cat# AK141964) was supplied by Ark Pharm, Inc (Libertyville, USA). DOX (Cat# 41701A), D-(+)-Chondrosamine Hydrochloride (Cat# 31361C) were purchased from Shanghai Titan Scientific Co. Ltd (Shanghai, China). Oleoyl Chloride (Cat# O0053) was provided by TCI Co. Ltd (Shanghai, China). Phosphotyrosine antibody (Cat# BM4986, 1: 500 for WB) was supplied by BOSTER Biological Technology co.LTD (Wuhan, China). CoraLite488 – conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (Cat# SA00013-2, 1:200 for IF), Rabbit anti-SIRPα (Cat# 14482-1-AP, 1:100 for IP and 1:500 for WB) was supplied by Proteintech Group, Inc (Wuhan, China). PerCP anti-mouse F4/80 (Biolegend#123125), PerCP/Cyanine5.5 anti-mouse CD86 (Biolegend#105028), APC anti-mouse/human CD11b (Biolegend#101212), FITC anti-mouse CD206 (Biolegend#141703), PE anti-mouse CD36 (Biolegend#102605), PE anti-mouse CD301 (Biolegend#145703) were purchased from BioLegend, Inc. (San Diego, USA). PE anti-SIRPα(Cat# 80270-R103-P-100) was purchased from Sino Biological Inc.(Beijing, China). LPS (Cat# ST1470), IL-4 (Cat# P5916), IFN-γ (Cat# P6137), M-CSF (Cat# P6015), Protein A+G Agarose (Fast Flow) (Cat# P2055) and TUNEL cell apoptosis kit (Cat# C1098) was from Beyotime Biotechnology (Jiangsu, China). The other chemicals and reagents were of analytical grade.

Cell lines and Animals. RAW264.7 was provided by Cell bank of Chinese

Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) (BI, Israel) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, USA), and detached for passage using 0.25% Trypsin/0.5mM EDTA (BI, Israel). A20 cells was purchased from Procell Life Science & Technology and cultured in RPMI 1640 medium supplemented with 10%FBS. Cells were kept in an incubation chamber at 37 °C and 5% CO_2 with a humidified atmosphere. The female Balb/c mice (4 weeks, 18-22 g) and the male C57BL/6 mice (4 weeks, 18-22 g) were raised in laboratory animal environment of pathogen-free, housed on a 12-hour light/dark cycle at 22-24℃ and 30-50% relative humidity. All animal experiments were conducted under guidelines of Ethical Review Committee of experimental animals at the Southwest University of China.

Preparation and Characterization of Liposomes. Liposomes were prepared by thin-film evaporation method. In brief, EPC and Cho were mixed at a molar ratio of 7:3. Liposomes modified with DSPE-mPEG2000, DSPE-S-S-mPEG2000, palmitic acid modified Self-peptide and N-oleoyl galactose were added at a molar ratio of 3%, 3%, 1% and 1% respectively, or as depicted in the related figures. The Egg Liss Rhod PE EPC, DiI or DiD were added to weighed lipid film at a concentration of 40 μg Egg Liss Rhod PE per 8 mg EPC, 10 μg DiI per 8 mg EPC and 10 μg DiD per 8 mg EPC, respectively. After vacuum drying, the lipid film was hydrated with the PBS at 37°C, and then ultrasonicated under an ice water bath and sequentially extruded through a polycarbonate membrane with pore sizes of 100 nm to prepare liposomes. Liposomes loaded with HPTS or clodronate were also prepared by thin-film evaporation except

that the mixed lipid film was hydrated in HPTS buffer (10 mM HPTS in PBS) or clodronate buffer (0.7M clodronate in PBS) before the formation of liposomes by extrusion.

For liposomes loaded with DOX, the lipid film was hydrated with the 250 mM Ammonium sulfate at 37°C, and prepared as mentioned above. Then liposomes were dialysis (KW CO 8000~14000 Da) with distilled water for 6 h, DOX was added with a ratio of 1:10 (w/w) and incubation at 37℃ for 1 h. After that, liposome/DOX was dialysis (KW CO 8000~14000 Da) with PBS, and store at 4 ℃ before use.

The size distributions and morphologies of various liposomes were determined by a dynamic light scattering method with a Zeta Sizer Nano Series (Nano ZS 90, Malvern, U.K.) and transmission electronic microscopy (TEM) (FEI Tecnai G20, USA), respectively. Every sample was measured in triplicate.

SPR analysis. The affinity between 4N1K peptide and Self-peptide was validated by SPR using an OpenSPR system (Nicoya Lifesciences, Waterloo, Canada). SPR was performed under a constant flow rate of 20 μL/min at 25 ℃. Palmitic acid labeled 4N1K peptide was modified on the liposome at the molar ratio of 1% with thin-film hydration method as depicted in liposome preparation and then fixed on Lip-1 sensor chip (Nicoya Lifesciences, Canada). Afterward, different concentrations of Self-peptides were tested in SPR at a flow rate of 20 μL/min with PBS buffer. All concentrations of peptides were performed three times. After each test, the chip was regenerated with 10 mM HCl. Blank liposome was served as the negative control.

Phagocytosis Percentage Assay. LPS/IFN-γ activated M1 RAW264.7 was cultured in 96-well plate, and HPTS loaded liposomes were added to the wells and incubated with cells for a given period, then the cells were washed with cold PBS. Fluorescence (excitation spectra 400-480 nm; bandwidth, 5 nm) was measured at an emission wavelength of 510 nm (bandwidth, 5 nm) with SYNERGY H1 microplate reader (BioTek, USA). The phagocytosed percent was calculated by the ratio of the fluorescent excitated at 450 nm/413 nm and the equation was as follows:

$$\text{Percent phagocytosed} = \frac{\text{ratio (pH7.4)} - \text{ratio (measured)}}{\text{ratio (pH7.4)} - \text{ratio (pH6.0)}}$$

Ratio (measured) means 450/413 ratio of liposomes treated with cells. Ratio (pH7.4) and ratio (pH6.0) are the fluorescence ratio of liposomes in PBS and acidified buffer (pH=6.0), respectively.[4]

Flow Cytometry Analysis of M1 TAMs and M2 TAMs. Balb/c mice were implanted subcutaneously with A20 B-cell lymphoma cells (5×10^6). When the tumor volumes reached ~50 mm^3 (day 0), the mice were treated with PBS/DOX/DOX-loaded liposomes at days 0, 2 and 4. On day 8, the tumors were cut and placed in ice-cold FACS buffer (pH 7.40, 0.1M PBS, 1mM EDTA, 1% BSA, 50U/mL DNase I). After digest with 0.25% Trypsin at 37°C for 30 minutes. The suspension was filtered with a 200-mesh cell sieve and centrifuged at 1000 rpm for 5-10 minutes. After that, cells were collected and preincubated with 1% BSA for 30 min. And it was then stained with PerCP/Cyanine5.5 anti-mouse CD86, APC anti-mouse/human CD11b, FITC anti-mouse CD206 for 1 hour. Finally, samples were washed in FACS buffer thrice, fixed in

1% paraformaldehyde, and analyzed using BD FACS Calibur.[5]

Supporting Figures and Tables

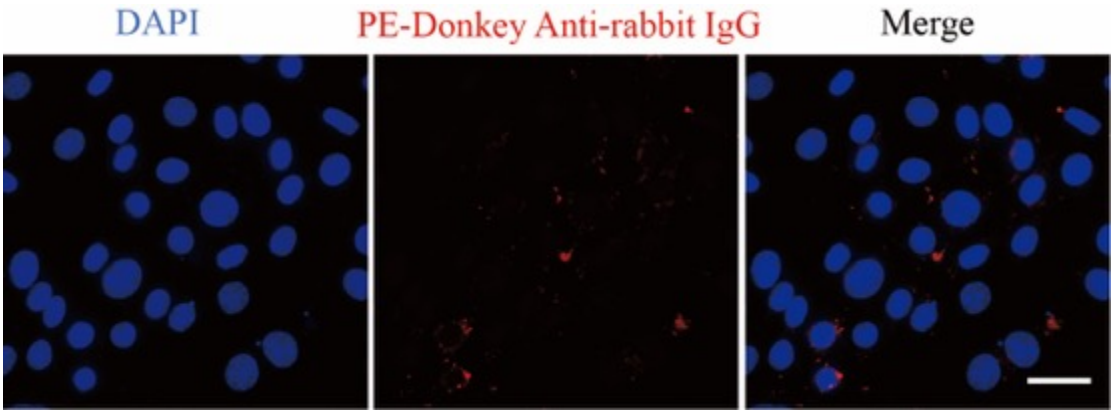

Figure S1. Negative control of immunofluorescence staining of CD86 in M1 RAW264.7, cells were incubated with PBS instead of rabbit anti-CD86, and then incubated with PE-Donkey anti-rabbit IgG, , scale bar= 10μm.

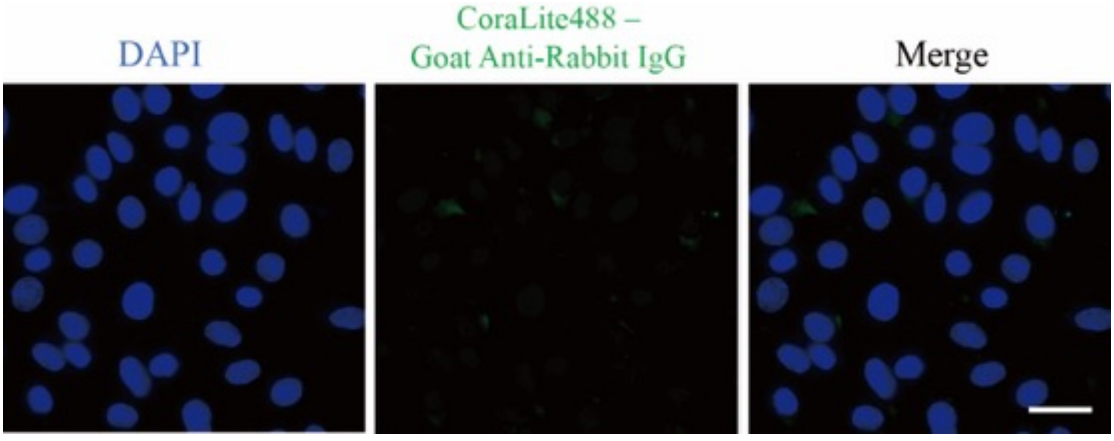

Figure S2. Negative control of immunofluorescence staining of CD206 in M2 RAW264.7, cells were incubated with PBS instead of rabbit anti-CD206, and then incubated with CoraLite488-Goat anti-rabbit IgG, , scale bar= 10μm.

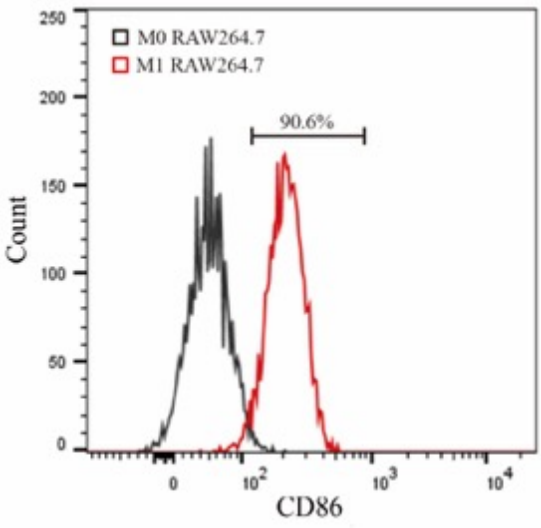

Figure S3. Flow cytometry analysis of cell expression of CD86 on RAW264.7.

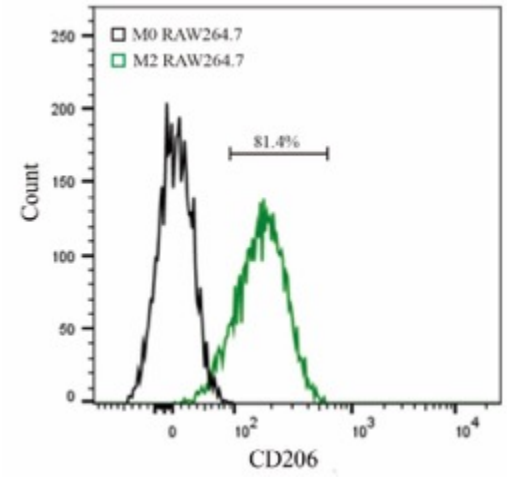

Figure S4. Flow cytometry analysis of cell expression of CD206 on RAW264.7.

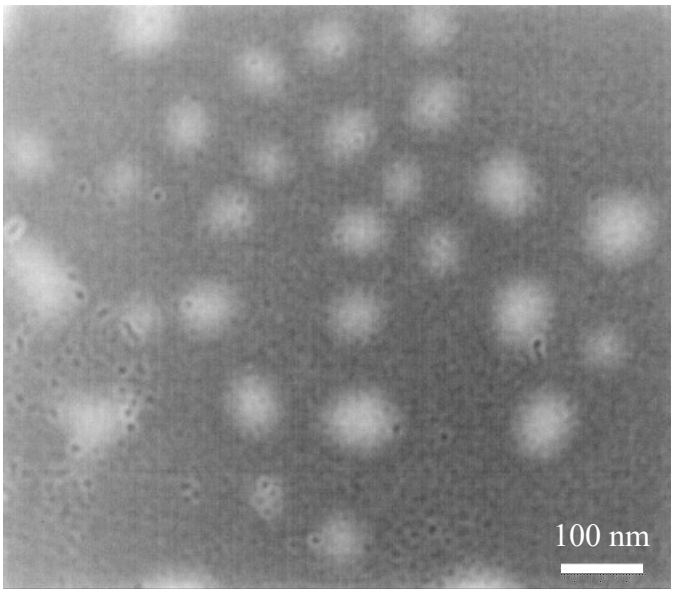

Figure S5. TEM image of GS-Lip.

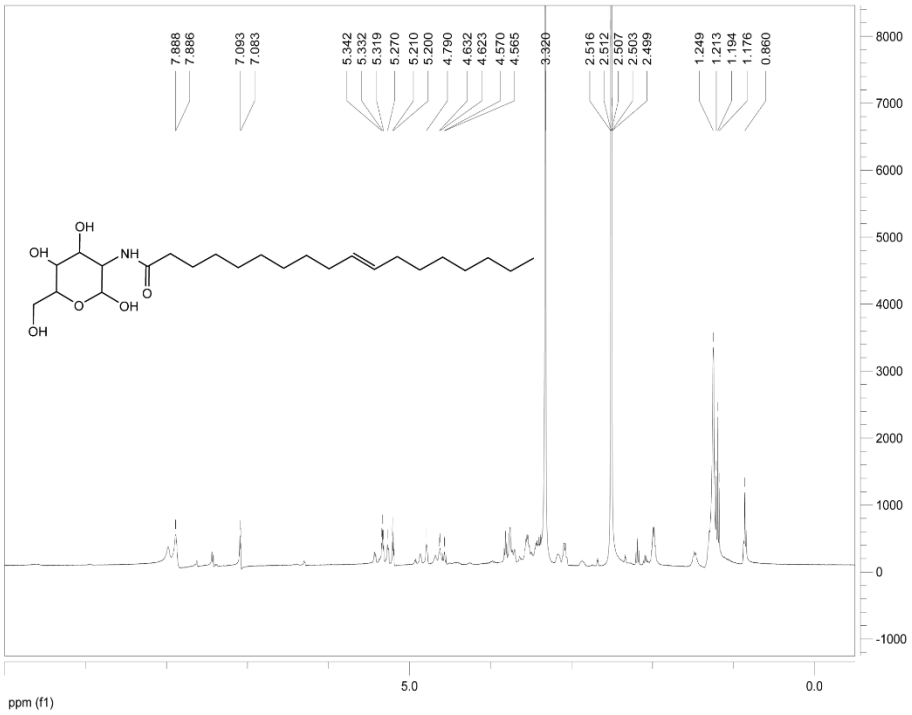

Figure S6. NMR image of N-Oleoyl galactose

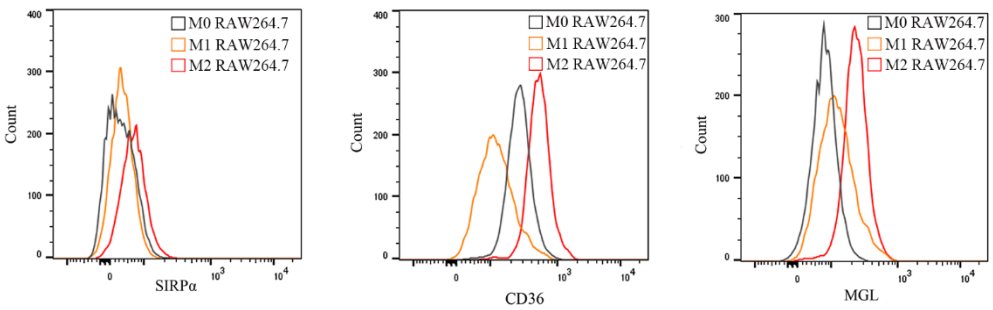

Figure S7. Expression of SIRPα, CD36 and MGL on RAW264.7.

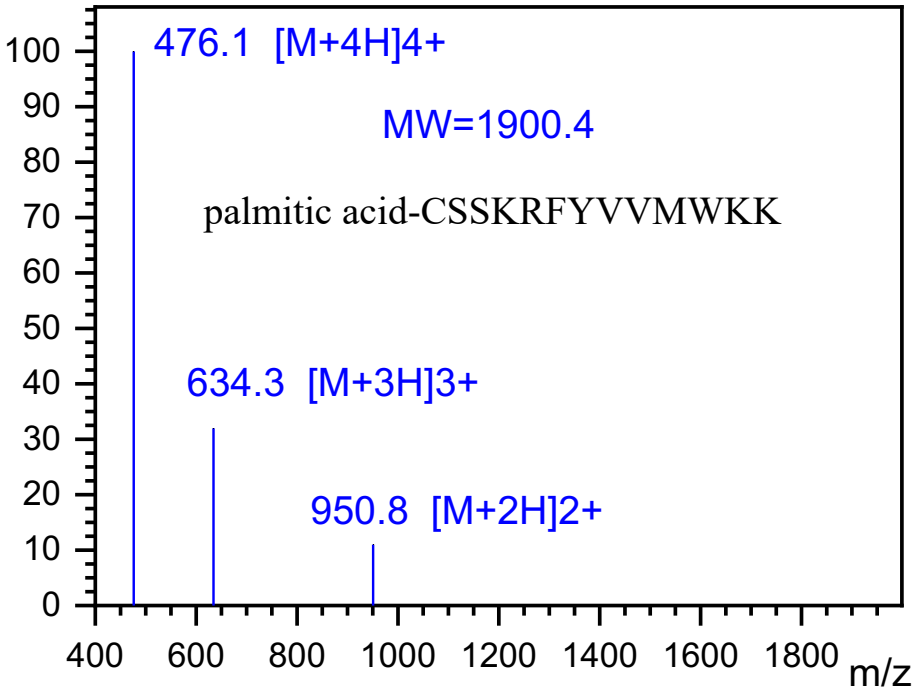

Figure S8. ESI-MS analysis of palmitic acid labeled 4N1K peptide.

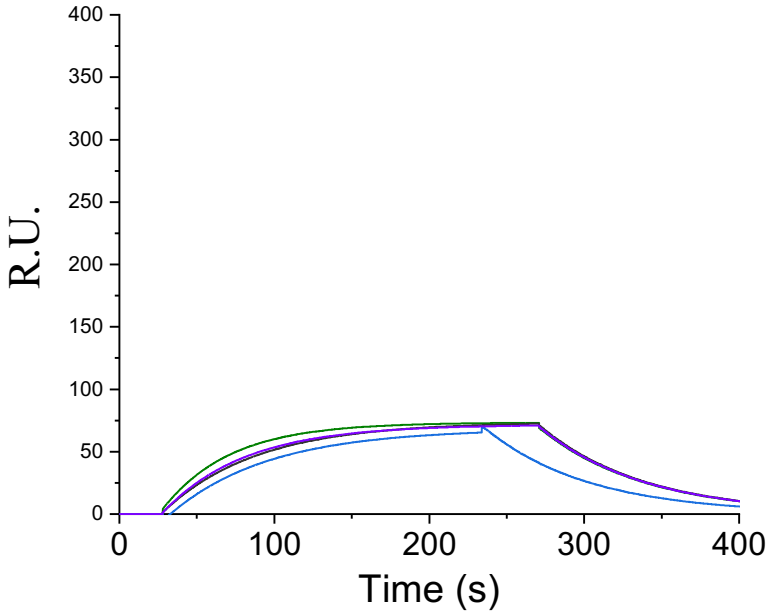

Figure S9. SPR response unit of interaction between self-peptide and blank liposome.

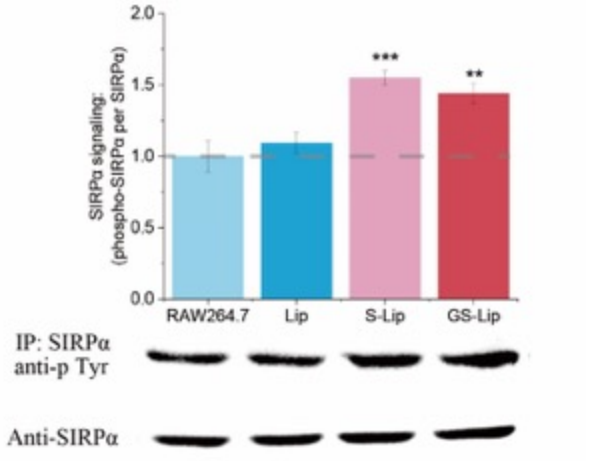

Figure S10. Expression of phosphotyrosine and total SIRPα on M0 RAW264.7 as determined by immunoprecipitation and immunoblotting for normalization. Data are shown as means ± SD (n = 3), *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* M0 RAW264.7.

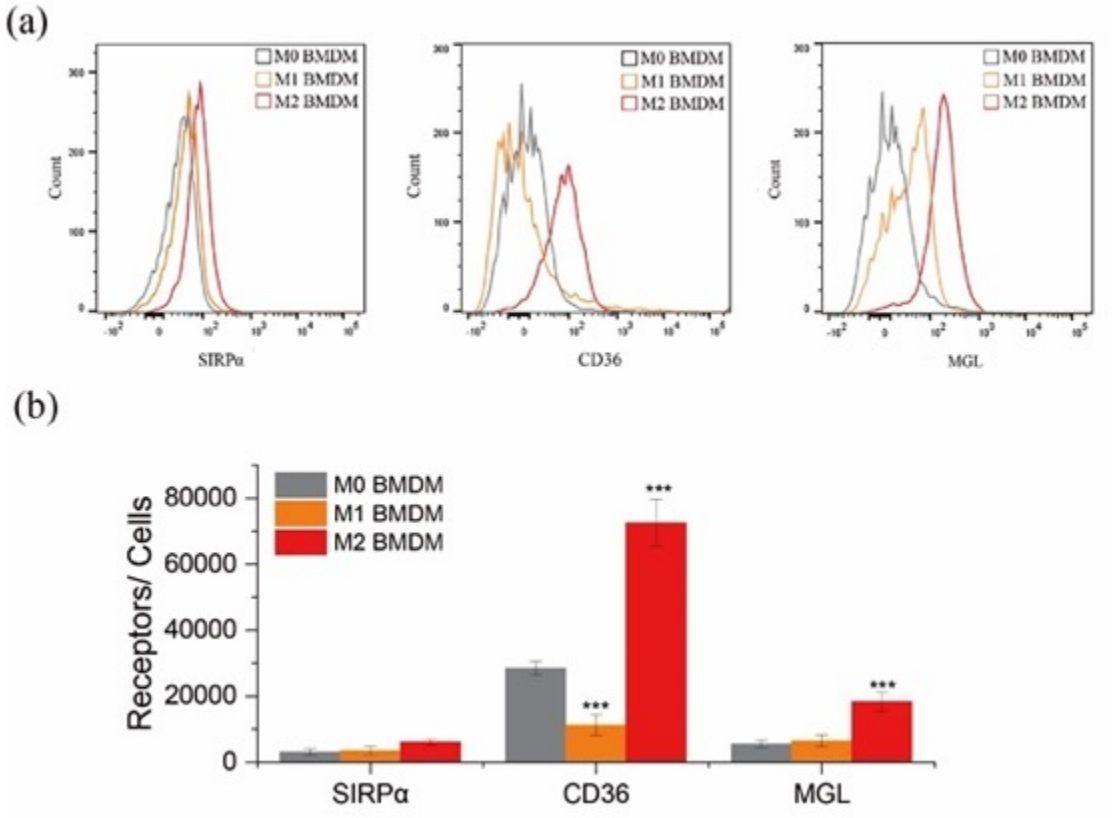

Figure S11. (a) Flow cytometry and (b) quantitative analysis of SIRPα, CD36 and MGL expression on BMDM. Data are shown as means ± SD (n = 3), *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* M0 BMDM.

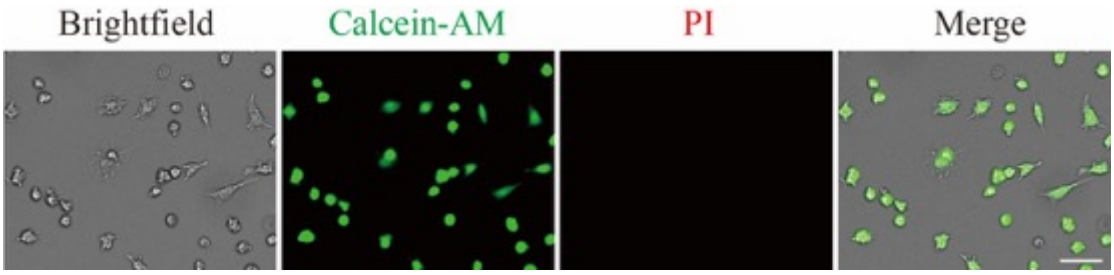

Figure S12. Fluorescence images observation and in PBS treated M1 BMDM, scale bars= 50 μm.

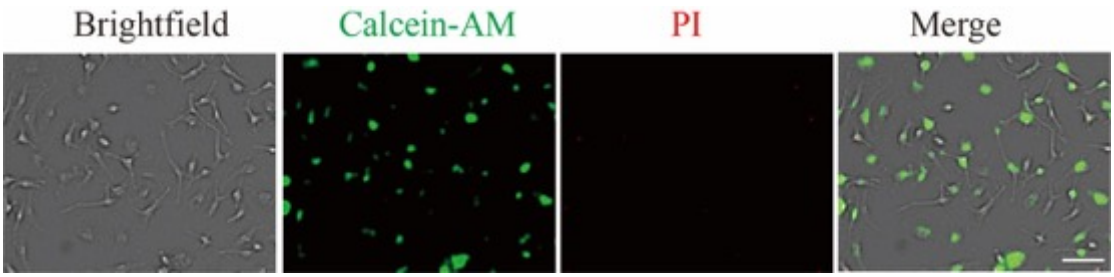

Figure S13. Fluorescence images observation and in PBS treated M2 BMDM, scale bars= 50 μm.

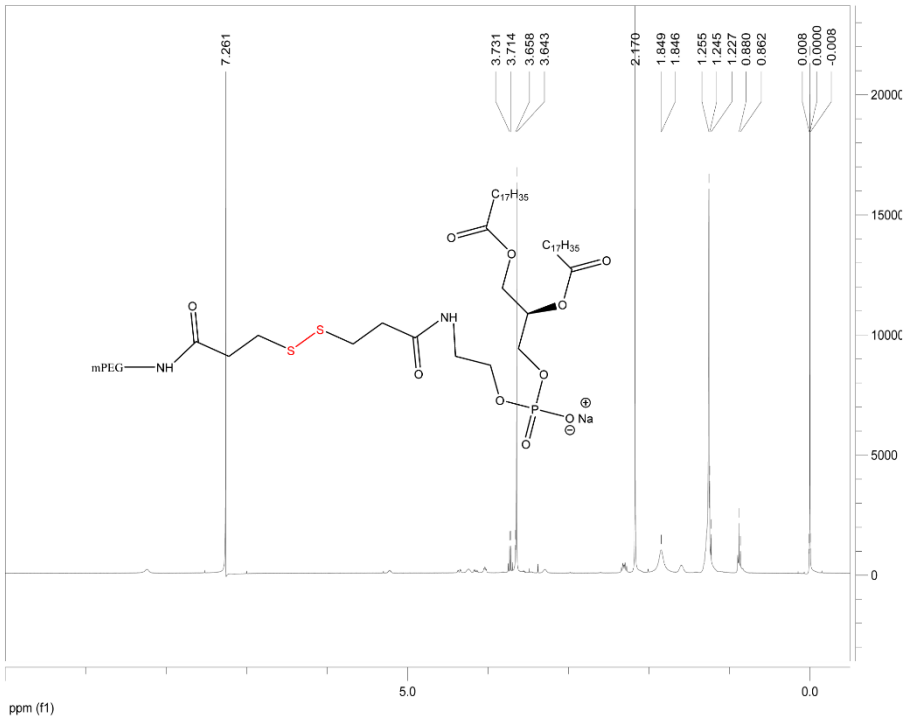

Figure S14. NMR image of DSPE-S-S-PEG2000

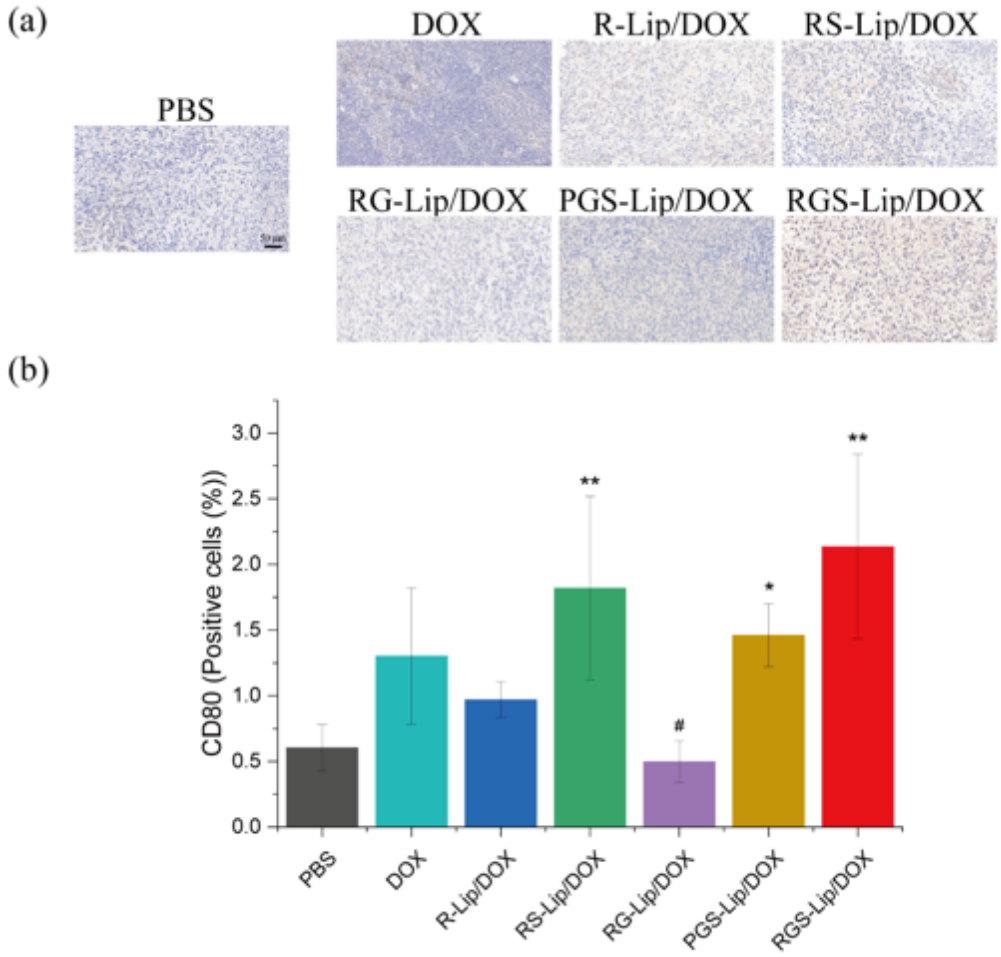

Figure S15. (a) CD80 staining of tumor section and (b) detection of CD80+ cells in tumor section.

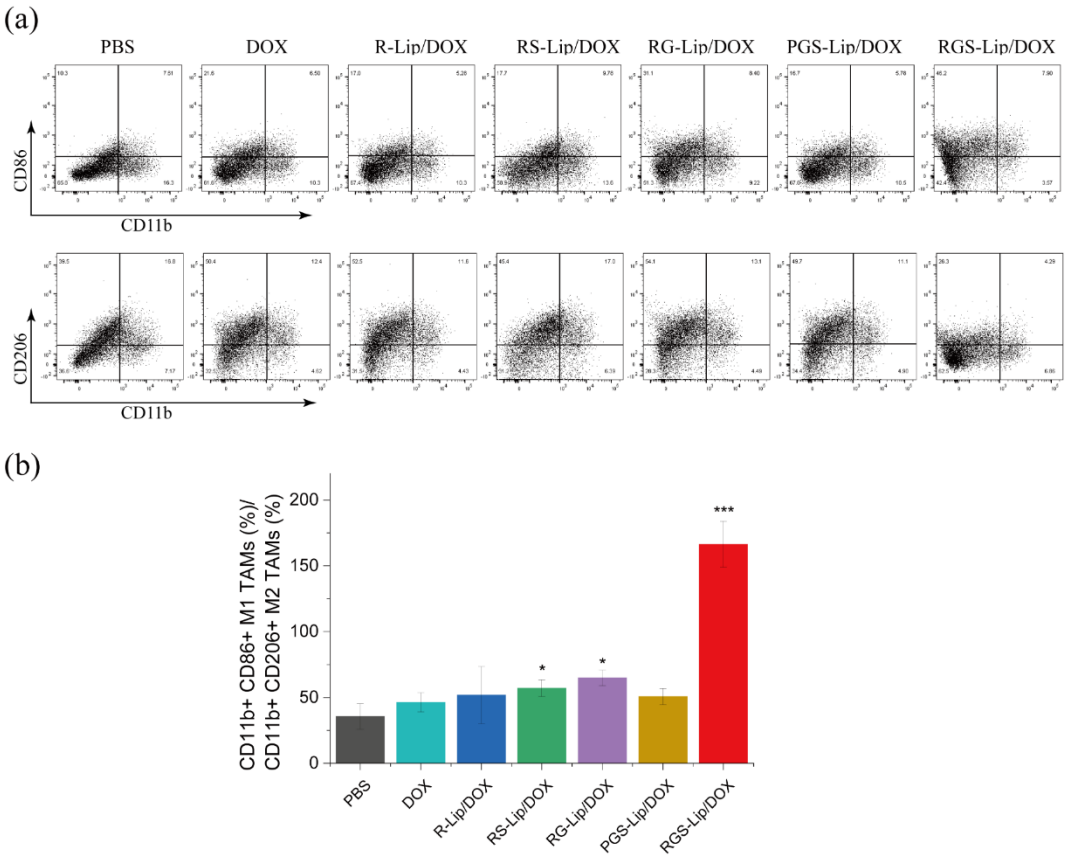

Figure S16. (a) Flow cytometry analysis of CD11b+ CD86+ M1 TAMs and CD11b+ CD206+ M2 TAMs from A20 tumors. Balb/c mice were implanted subcutaneously with A20 B-cell lymphoma cells. When the tumor volumes reached ~50 mm^3 (day 0), the mice were treated with PBS/DOX/DOX-loaded liposomes at days 0, 2 and 4. On day 8 tumors were homogenized for flow cytometric analysis. (b) The ratio between CD11b+ CD86+ M1 TAMs and CD11b+ CD206+ M2 TAMs, Data are shown as the means ± SD (n = 3), *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* PBS treated group.

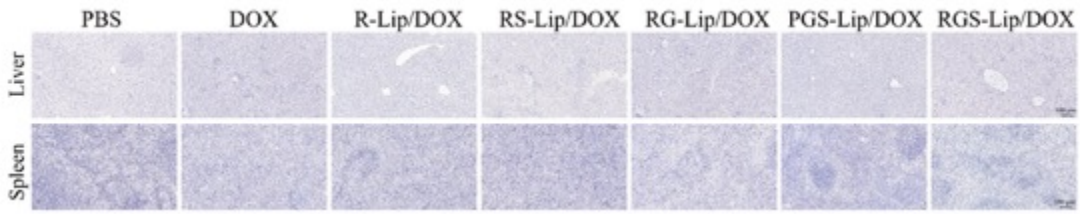

Figure S17. TUNEL staining of liver and spleen sections.

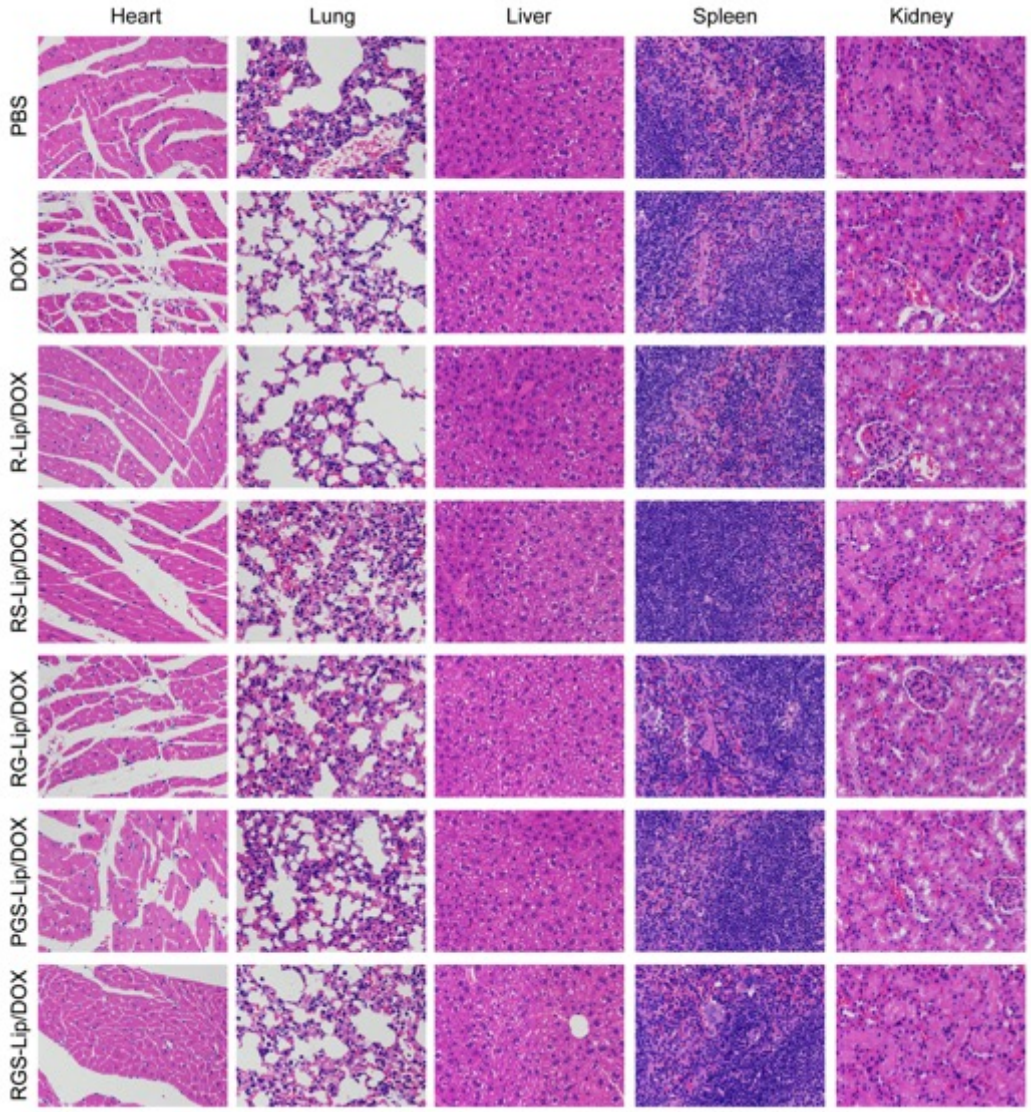

Figure S18. H&E staining of the main organs of the mice in different treatment groups.

Table S1. Formulations of the liposomes, the other ratio used in manuscript was descriped alone.

Liposomes	Formulations	Molar ratio
Lip	EPC:Cho	70:30
S-Lip	EPC:Cho:Pal-self	69.3:29.7:1
G-Lip	EPC:Cho: N-oleoyl galactose	69.3:29.7:1
GS-Lip	EPC:Cho:Pal-self: N-oleoyl galactose	68.6:29.4:1:1
R-Lip	EPC:Cho: DSPE-S-S-PEG2000	67.9:29.1:3
RS-Lip	EPC:Cho: DSPE-S-S-PEG2000: Pal-self	67.2:28.8:3:1
RG-Lip	EPC:Cho: DSPE-S-S-PEG2000: N-oleoyl galactose	67.2:28.8:3:1
RGS-Lip	EPC:Cho: DSPE-S-S-PEG2000: Pal-self: N-oleoyl galactose	66.5:28.5:3:1:1
PGS-Lip	EPC:Cho: DSPE-mPEG2000: Pal-self: N-oleoyl galactose	66.5:28.5:3:1:1

Table S2. Characterization of the liposomes (n=3).

Liposomes	Mean size(nm)	PDI	ζ-potential (mV)
Lip	92.5± 1.9	0.12± 0.02	-4.4± 0.1
(0.1%)S-Lip	87.2± 2.1	0.11±0.01	-4.3± 0.2
(0.5%)S-Lip	85.8± 1.9	0.13±0.02	-4.5± 0.2
(1%)S-Lip	87.3± 1.5	0.08±0.02	-4.3± 0.5
(3%)S-Lip	84.2± 1.7	0.09±0.02	-4.4± 0.3
(0.5%)G(1%)S-Lip	87.2± 2.3	0.10±0.01	-3.9± 0.2
(1%)G(1%)S-Lip	86.7± 1.8	0.09±0.02	-3.7± 0.5
(2%)G(1%)S-Lip	86.1± 1.5	0.11±0.02	-3.6± 0.4

Table S3. Characterization of the liposomes loaded with DOX (n=3).

Liposomes	Mean size (nm)	ζ-potential (mV)	EE (%)	DL (%)
Lip	95.3± 2.2	-4.7± 0.1	91.7± 2.3	8.3± 0.2
S-Lip	88.5± 1.6	-4.8± 0.1	92.6± 1.0	8.4± 0.1
G-Lip	89.3± 2.2	-4.2± 0.2	92.9± 0.7	8.4± 0.1
GS-Lip	88.3± 1.7	-4.5± 0.1	91.0± 0.9	8.3± 0.1
R-Lip	85.3± 2.3	-4.3± 0.2	91.1± 1.0	8.3± 0.1
RS-Lip	83.4± 1.8	-4.2± 0.1	91.9± 1.6	8.4± 0.2
RG-Lip	84.7± 1.3	-4.3± 0.2	91.3± 0.5	8.3± 0.1
PGS-Lip	84.6± 1.1	-4.5± 0.2	91.7± 1.0	8.3± 0.1
RGS-Lip	85.2± 1.9	-4.1± 0.1	90.9± 1.3	8.3± 0.1

References:

1. Tang, Y.; Wang, X.; Li, J.; Nie, Y.; Liao, G.; Yu, Y.; Li, C. Overcoming the Reticuloendothelial System Barrier to Drug Delivery with a "Don't-Eat-Us" Strategy. *ACS Nano* **2019**, *13*, 13015-13026.

2. Siu, F. Y.; Ye, S.; Lin, H.; Li, S. Galactosylated PLGA Nanoparticles for the Oral Delivery of Resveratrol: Enhanced Bioavailability and *in Vitro* Anti-Inflammatory Activity. *Int. J. Nanomedicine* **2018**, *13*, 4133-4144.

3. Kirpotin, D.; Hong, K.; Mullah, N.; Papahadjopoulos, D.; Zalipsky, S. Liposomes with Detachable Polymer Coating: Destabilization and Fusion of Dioleoylphosphatidylethanolamine Vesicles Triggered by Cleavage of Surface-Grafted Poly(ethylene Glycol). *FEBS Lett.* **1996**, *388*, 115-118.

4. Miller, C. R.; Bondurant, B.; McLean, S. D.; McGovern, K. A.; O'Brien, D. F. Liposome-Cell Interactions *in Vitro*: Effect of Liposome Surface Charge on the Binding and Endocytosis of Conventional and Sterically Stabilized Liposomes. *Biochemistry* **1998**, *37*, 12875-12883.

5. Miyauchi, J. T.; Chen, D.; Choi, M.; Nissen, J. C.; Shroyer, K. R.; Djordevic, S.; Zachary, I. C.; Selwood, D.; Tsirka, S. E. Ablation of Neuropilin 1 From Glioma-Associated Microglia and Macrophages Slows Tumor Progression. *Oncotarget* **2016**, *7*, 9801-9814.