Supplementary Information

Polymer-Liposome Complexes with a Functional Hydrogen-Bond Crosslinker for Preventing Protein Adsorption and Improving Tumor Accumulation

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S1. Synthesis of mPEG-P(HPMA-co-His)-Chol

(1) Modification of Cholesterol

First, cholesterol (Chol, 1 mmol), succinyl anhydride (1.2 mmol), DPTS (0.3 mmol), and a small amount of triethyl amine were dissolved together in DCM. After reaction for 12 h, the solution was extracted with an aqueous solution three times to obtain Chol-COOH. The conversion was 100 % by ^{1}H-NMR ($CDCl_3$) determination. Then, Chol-COOH (1 mmol), N-hydroxy succinimide (1.2 mmol), DPTS (0.2 mmol), and DCC (3 mmol) were dissolved in DCM. The reaction was carried out at 4°C for 24 h. The mixture was then extracted with an aqueous solution three times and then dried by rotary evaporation and a vacuum oven to obtain cholesterol-NHS ester. The conversion of Chol-NHS ester was about 98% by ^{1}H-NMR ($CDCl_3$) determination.

Cholesterol-COOH: ^{1}H-NMR ($CDCl_3$; ppm): δ 0.6-2.4 (m, from cholesterol), 2.6 (t, -COO-C<u>H</u>$_2$-CH_2-COOH), 2.7 (t, -COO-CH_2-C<u>H</u>$_2$-COOH), 4.6-4.7 (br., -C<u>H</u>-O-), 5.4 (d, -C=C<u>H</u>-).

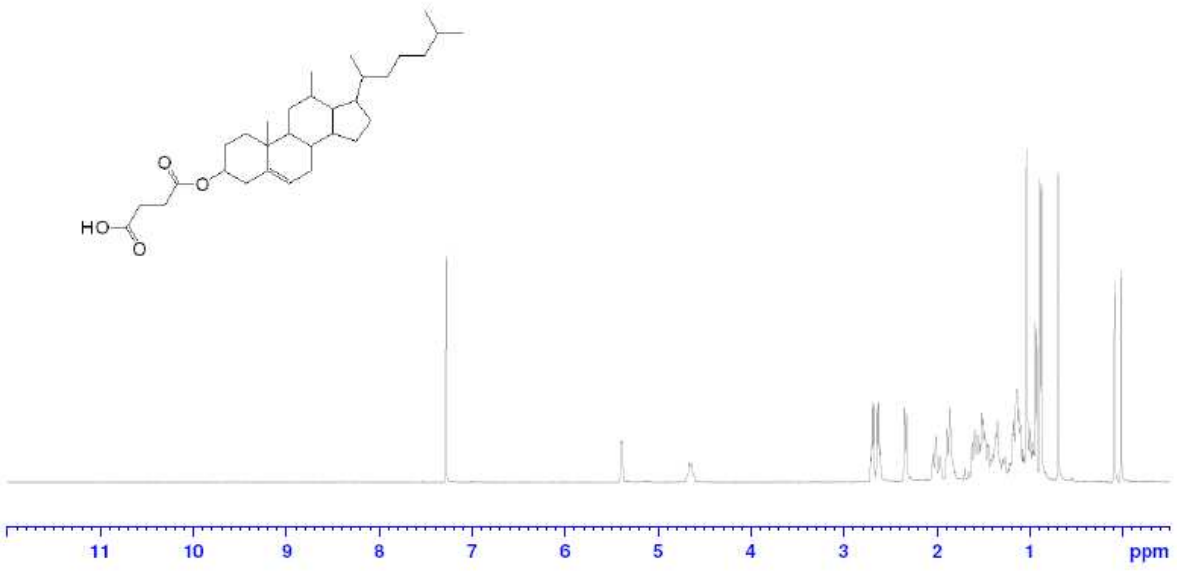


Figure S1. ^{1}H-NMR spectrum of cholesterol-COOH.

Cholesterol-NHS ester: ^{1}H-NMR ($CDCl_3$; ppm): δ 0.6-2.4 (m, from cholesterol), 2.7-2.8 and 2.9-3.0 (t, -COO-C<u>H</u>$_2$-C<u>H</u>$_2$-COO-), 2.8-2.9 (s,-C<u>H</u>$_2$-C<u>H</u>$_2$- from NHS), 4.6-4.7 (br.-C<u>H</u>-O-), 5.3-5.4 (d, -C=C<u>H</u>-).

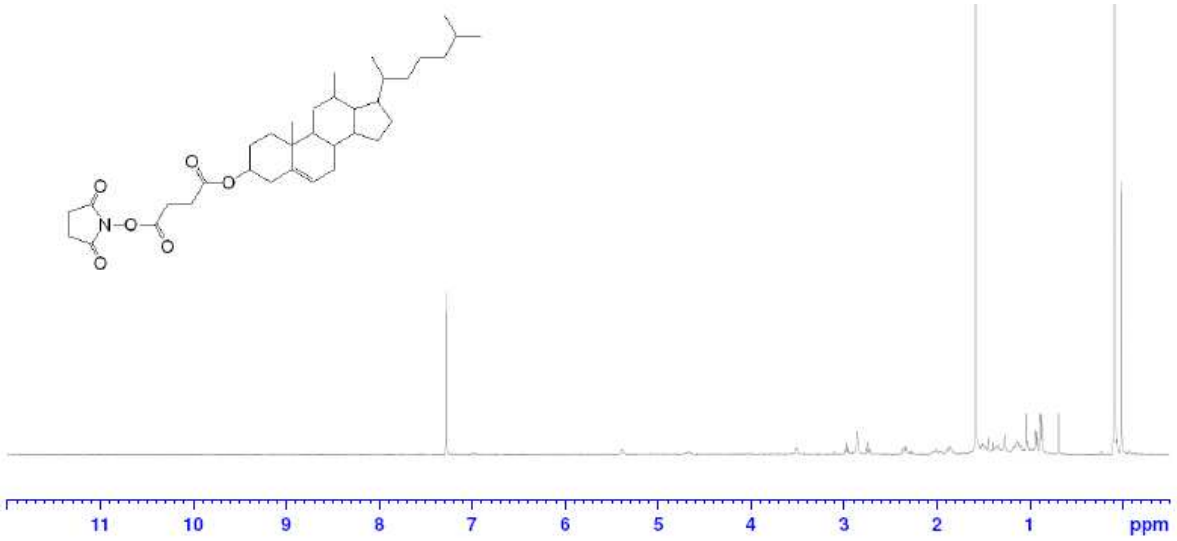


Figure S2. ^{1}H-NMR spectrum of cholesterol-NHS ester.

(2) Synthesis of mPEG2-ABCPA

The mPEG (M.W. 5000, 2 mmol), 4,4'-azobis-(4-cyanopentanoic acid) (ABCPA, 1 mmol), and 4-(dimethylamino)pyridinium-4-toluenesulfonate (DPTS, 0.3 mmol) were dissolved in DCM under N_2. The N,N '-Dicyclohexyl carbodiimide (DCC, 3 mmol) was also dissolved in DCM under N2, and then was slowly dropped into the mPEG solution at 4°C for reaction for 24 h. The crude mPEG2-ABCPA was obtained by precipitating diethyl ether and drying in a vacuum oven. The dried product was further purified by ultrafiltration (membrane: Millipore, MWCO 10K) and was then freeze-dried. The product was then characterized by ^{1}H-NMR (D_2O) and FT-IR (KBr). The polydispersity index of mPEG2-ABCPA was 1.02, as determined by gel permeation chromatography (GPC). mPEG2-ABCPA: ^{1}H-

NMR (D_2O; ppm): δ 1.6-1.7 (s, -CH_3), 1.7-1.8 (s, -C$\underline{H}_2$-C-CN), 2.3-2.6 (m, -C$\underline{H}_2$-CO-), 3.3 (s, -OCH_3), 3.4-3.9 (br., from mPEG), 4.2-4.3 (t, from mPEG segment which conjugated with ABCPA); FT-IR (KBr): 1738 cm^{-1}.

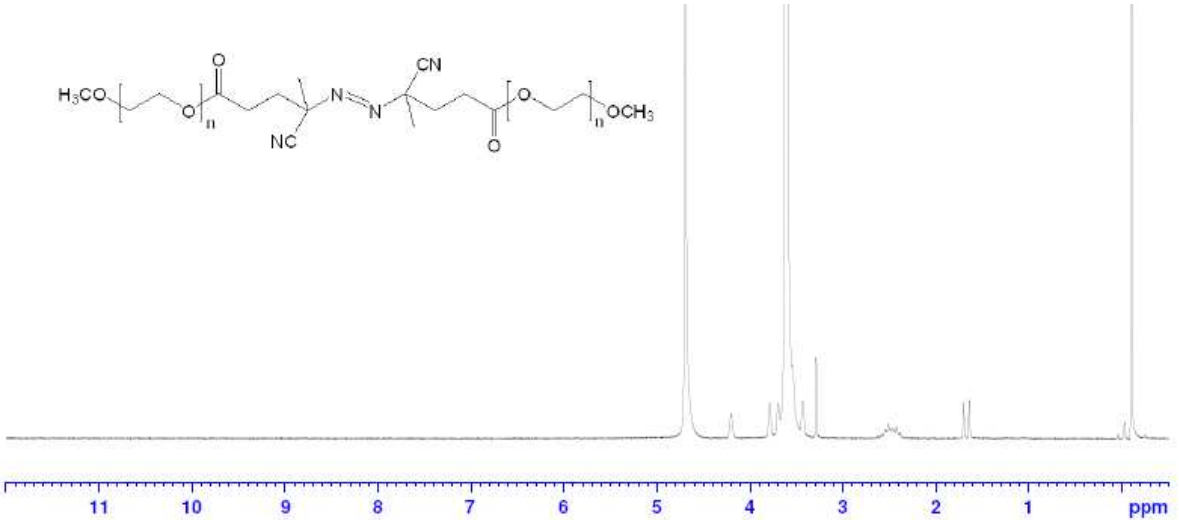


Figure S3. ^{1}H-NMR spectrum of mPEG2-ABCPA.

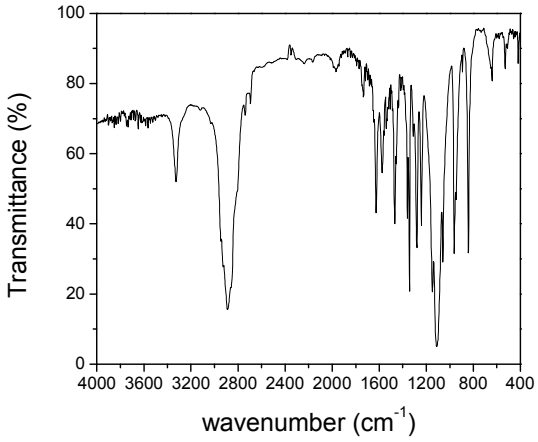


Figure S4. FT-IR spectrum of mPEG2-ABCPA.

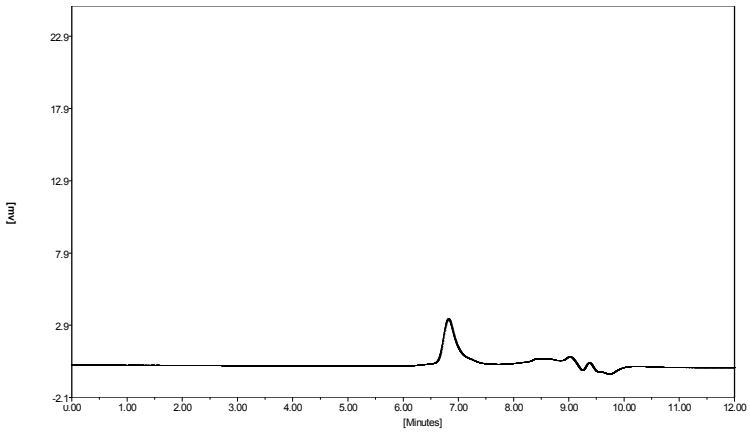


Figure S5. GPC spectrum of mPEG2-ABCPA.

(3) Synthesis of mPEG-P(HPMA-co-His)-Chol

First, mPEG2-ABCPA as a macroinitiator, 2-aminoethanethiol hydrochloride (AET-HCl) as a chain transfer reagent, and N-(2-hydroxypropyl) methacrylamide (HPMA) as a monomer were dissolved in ethanol under N_2. The reaction was conducted at 70°C for 24 h. The product, mPEG-PHPMA-NH_2, was purified by precipitation in diethyl ether, and then characterized by ^{1}H-NMR (DMSO-d_6). The mPEG-PHPMA-NH_2 (1 mmol) was then reacted with Chol-NHS ester (1 mmol) in methanol for 24 h to obtain mPEG-PHPMA-Chol. This product was also purified by precipitation and ^{1}H-NMR (THF-d_8+methanol-d_4) was also used to identify its chemical structure. Finally, mPEG-PHPMA-Chol, histidine (His), DPTS, and DCC were mixed together to prepare mPEG-P(HPMA-co-His)-Chol by the typical esterification protocol as described above. The chemical structure and compositions of mPEG-P(HPMA-co-His)-Chol were determined by ^{1}H-NMR (DMSO-d6), FT-IR (KBr), and GPC.

mPEG-PHPMA-NH_2: ^{1}H-NMR (DMSO-d_6; ppm): δ 0.7-1.3 (br., -C$\underline{H}_3$), 1.6-1.9 (br., -C$\underline{H}_2$-), 2.7-2.8 (s, -S-C$\underline{H}_2$-), 2.8-2.9 (s, -C$\underline{H}_2$-NH_2), 3.4-3.55 (br., from mPEG), 3.55-3.9 (br., -NH-C$\underline{H}_2$-C$\underline{H}$-), 4.1-

4.2 (t, from mPEG segment which conjugated with AET-HCl), 4.8-4.9 (br., -OH), 7.6-7.8 (m, -NH_2).

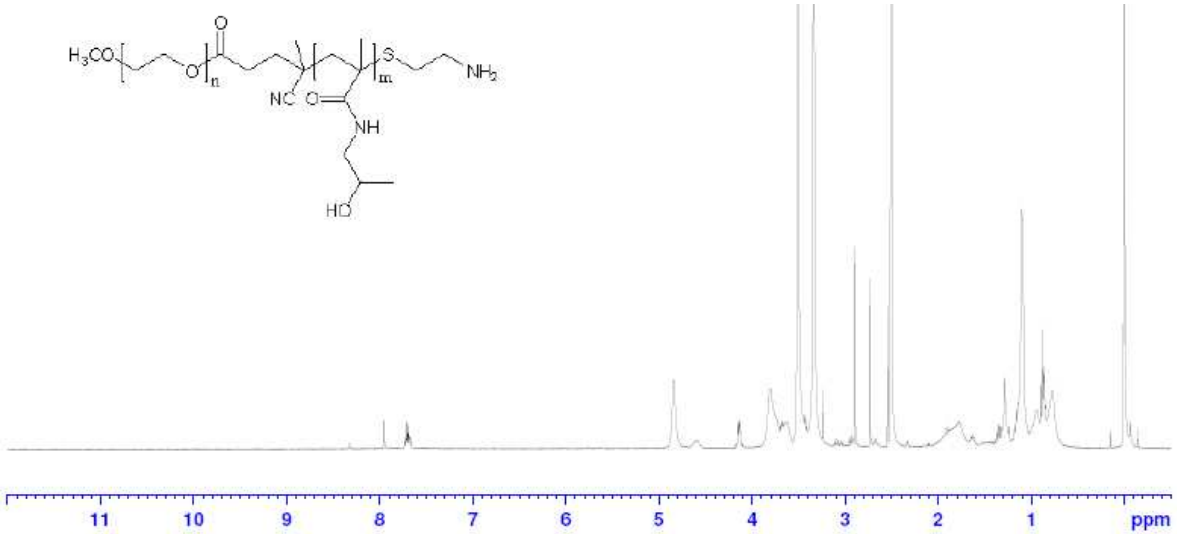


Figure S6. ^{1}H-NMR spectrum of mPEG-PHPMA-NH_2.

mPEG-PHPMA-Chol: ^{1}H-NMR (THF-d_8 + methanol-d_4; ppm): δ 0.7-2.4 (br., overlap from cholesterol and PHPMA), 2.7-2.9 (br., -S-C<u>H</u>$_2$-C<u>H</u>$_2$-NH-), 3.4-3.6 (br., from mPEG), 3.7-4.0 (br., -NH-C<u>H</u>$_2$-C<u>H</u>-), 4.1-4.2 (br., from mPEG segment which conjugated with cholesterol), 4.6-4.8 (br., -OH), 5.5 (s, -C=C<u>H</u>-).

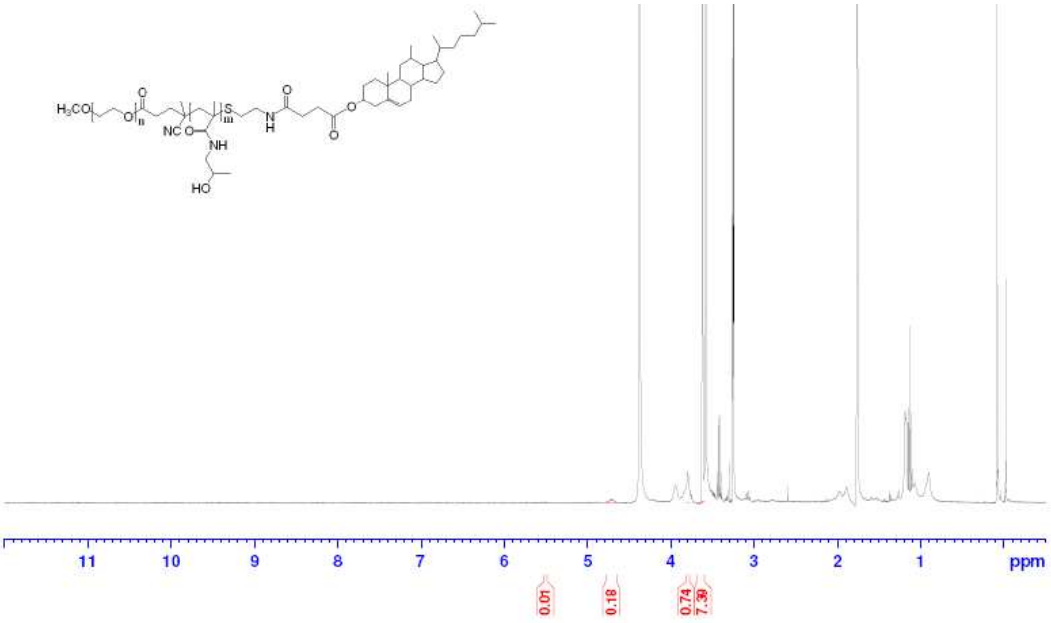


Figure S7. ^{1}H-NMR spectrum of mPEG-PHPMA-Chol.

mPEG-P(HPMA-co-His)-Chol: ^{1}H-NMR (DMSO-d_6; ppm): δ 0.7-2.3 (br., overlap from cholesterol and PHPMA), 2.6-3.0 (br., -S-C<u>H</u>$_2$-C<u>H</u>$_2$-NH-), 3.4-3.7 (br., from mPEG), 3.7-4.0 (br., -NH-C<u>H</u>$_2$-C<u>H</u>-), 4.0-4.3 (br., from mPEG segment which conjugated with cholesterol), 4.6-4.7 (br., -OH), 5.7 (s, -C=C<u>H</u>-), 7.0-7.6 (m, -C<u>H</u>-NH-C<u>H</u>- from histidine), 9.0 (-N<u>H</u>$_2$); FT-IR (KBr): 1722 cm^{-1}, 3421 cm^{-1}.

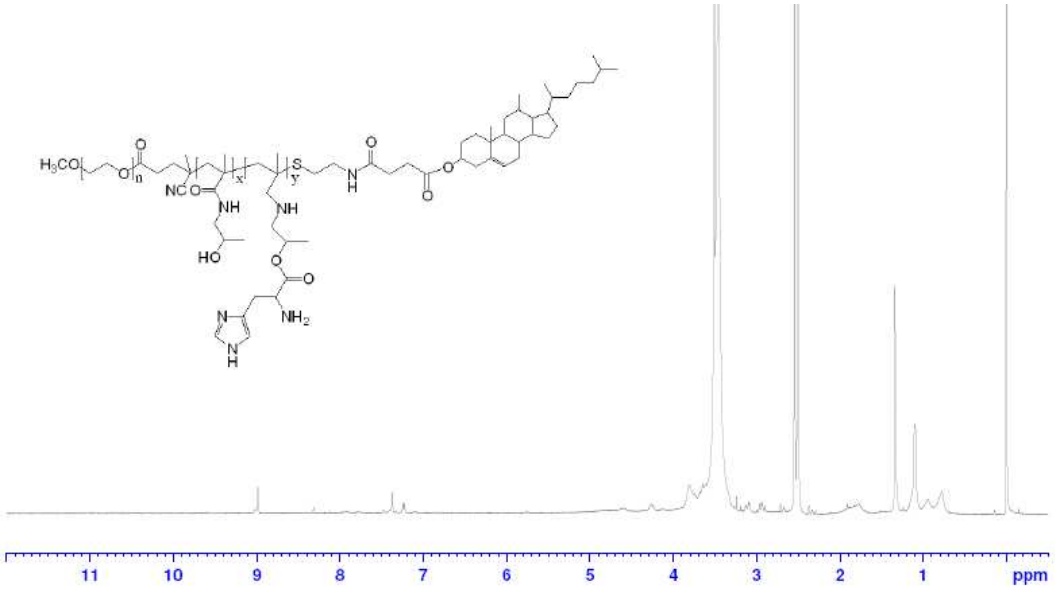


Figure S8. ^{1}H-NMR spectrum of mPEG-P(HPMA-co-His)-Chol.

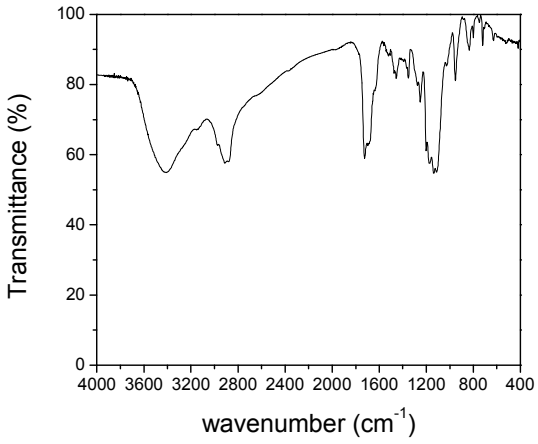


Figure S9. FT-IR spectrum of mPEG-P(HPMA-co-His)-Chol.

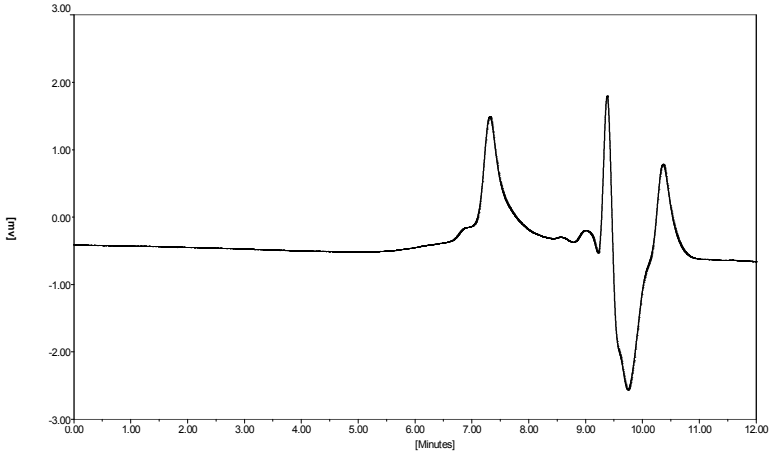


Figure S10. GPC spectrum of mPEG-P(HPMA-co-His)-Chol.

S2. Synthesis of biotin2-PEG

Biotin (2.4 mmol), polyethylene glycol (PEG with M.W. 200, 1 mmol), DPTS (0.2 mmol), and DCC (3 mmol) were dissolved in DMSO, and then reacted for 24 h. After reaction, biotin2-PEG, the product, was obtained after precipitation by diethyl ether. Its chemical structure was determined by ^{1}H-NMR (DMSO-d6) and FT-IR (KBr).

Biotin2-PEG: ^{1}H-NMR (DMSO-d_6; ppm): δ 1.1-1.9 (br., -CH2- from biotin), 2.2-2.4 (br., -OCO-CH_2-), 2.7-2.9 (d, -S-CH_2-CH- from biotin), 3.1 (s, -S-CH-CH- from biotin), 3.4-3.7 (br., -CH_2-CH_2-O- from PEG), 4.1 (br., -NH-CH-CH_2-), 4.3 (br., -NH-CH- from biotin), 6.3 and 6.5 (br., -NH-); FT-IR (KBr): 1704 cm^{-1}, 3329 cm^{-1}.

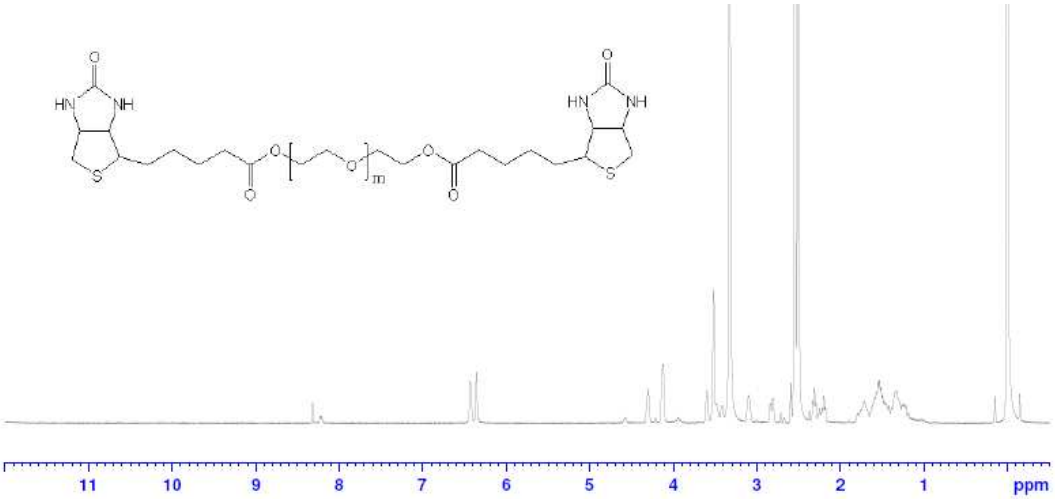


Figure S11. ^{1}H-NMR spectrum of biotin2-PEG.

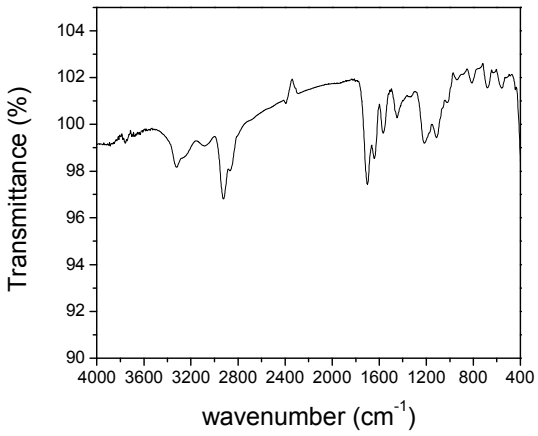


Figure S12. FT-IR spectrum of biotin2-PEG.

S3. Preparation of Liposomes.

The PEG-P(HPMA-g-His)-cholesterol (1 mmole) and DPPC (34 mmole) were dissolved in DCM/methanol (1/1 v/v). A polymer-incorporated lipid thin film was formed by rotary evaporation at room temperature. Then PBS at pH 7.4 was added to rehydrate the thin film, and the solution was subjected to sonication for 6 min. To prepare polymer-incorporated liposomes, the solution was extruded by a 0.22-μm PVDF filter twice and by a 0.1-μm PVDF filter five times. To prepare ECM-

targeting liposomes, cross-linking agents (12.5 mmol) were added to the polymer-incorporated liposomes solution, shaken for 5 min, and the solution was extruded with a 0.1-μm PVDF filter twice. The particle size of each liposome was determined by dynamic laser scattering (DLS) (Malvern zetasizer 3000), and morphology was observed by TEM (JEM-2000 EXII) with 2% uranyl acetate staining. To determine the crosslinking level, the ECM-targeting liposomes solution was centrifuged by Amicon Ultra centrifugal filter (MWCO 10000) at 4000 rpm for 10 min. The solution in the filtrate collection tube was treated with 4-hydroxyazobenzene-2-carboxylic acid (HABA)/avidin reagent and analyzed by UV-Vis spectrometer at 500 nm, and the crosslinking level was then calculated.

S4. Small-angle X-ray scattering (SAXS) determination.

Measurements of small-angle X-ray scattering (SAXS) were performed with a Nano-Viewer (Rigaku) using Cu Kα X-rays (30 kV and 40 mA) with wavelength λ = 1.54 Å and exposure durations 1 h for SAXS. For analysis of SAXS data, the scattered intensity was obtained. According to the scattered intensity, the form factor, which was related to the shapes of liposomes could be given by the Fourier transform of the distributions of the electron density. The SAXS profiles were fitted with IGOR Pro software, and the unilamellar vesicle model and four shell model were utilized for analysis of the SAXS data.[S1]

The model of the unilamellar vesicle for DPPC liposomes analysis was showed as follows:[S2]

$$I(q) = \frac{scale}{Vshell}\left[\frac{3V1(\rho 1-\rho 2)j1(qR1)}{qR1} + \frac{3V2(\rho 2-\rho solvent)j1(qR2)}{qR2}\right]\text{^}2 + bkg$$

The equation is utilized to calculate the form factor for a monodisperse and unilamellar vesicle.

The form factor was normalized by the volume of shell.[S2] $\rho 1$ and $\rho 2$ are represented the density of the core and the shell, respectively. The term of R1 was defined as the radius of the core; the term of R2 was defined as the thickness of the shell plus the radius of the core. Therefore, the volume of the core and the shell can be spectualted, and $j1(i) = (\sin x - \cos x)/x^2$.

The four shell model was described the monodisperse spherical particle system.[S2] Polymer-incorporated liposomes and ECM-targeting liposomes were assumed that a phospholipid bilayer sandwiched between the polymer inner and outer the bilayer, while the outer polymer could be separated into the P(HPMA-co-His) (or P(HPMA-co-His with Biotin2-PEG crosslinker) and the PEG layer.[S3] Therefore, the four shell model was chosen to apply in analysis of Polymer-incorporated liposomes and ECM-targeting liposomes.

In addition, the power law was fitted to the slope of SAXS data to estimate the surface rougness of liposomes[4]. The power law equation was as follows:[S2]

$$I(q) = Aq^{-m} + BGD$$

The exponent, m represented the slopes of of the SAXS data which was plotted into logarithm scales.

S5. Preparation of Dox-Loaded Liposomes.

Dox-loaded polymer-incorporated liposomes were prepared by the typical protocol as described above, in addition to using ammonium sulfate solution (250 mM) for rehydration. Dox in PBS (10 mg/mL) was mixed with the liposomes solution at 60°C for 2 h. The solution was then extruded by 0.22-μm and 0.1-μm PVDF filters. Dox-loaded polymer-incorporated liposomes were obtained after

adjusting the pH of solution to 7.4. Cross-linking agents were then added into the solution to prepare Dox-loaded ECM-targeting liposomes. Excess Dox was removed by a Sephadex G-50 with the PBS mobile phase.

S6. Hydrogen-Bond Determination.

(1) By UV/Vis determination: biotin2-PEG, polymer-incorporated liposomes, and polymer-incorporated liposomes plus biotin2-PEG under a fixed concentration as well as for liposomes preparation were dissolved/ suspended in pH 7.4 PBS. The electronic absorption spectra ranging from 190 to 500 nm were monitored with a UV/Vis spectrophotometer. A base line correction was made for the spectra recorded in pH 7.4 PBS.

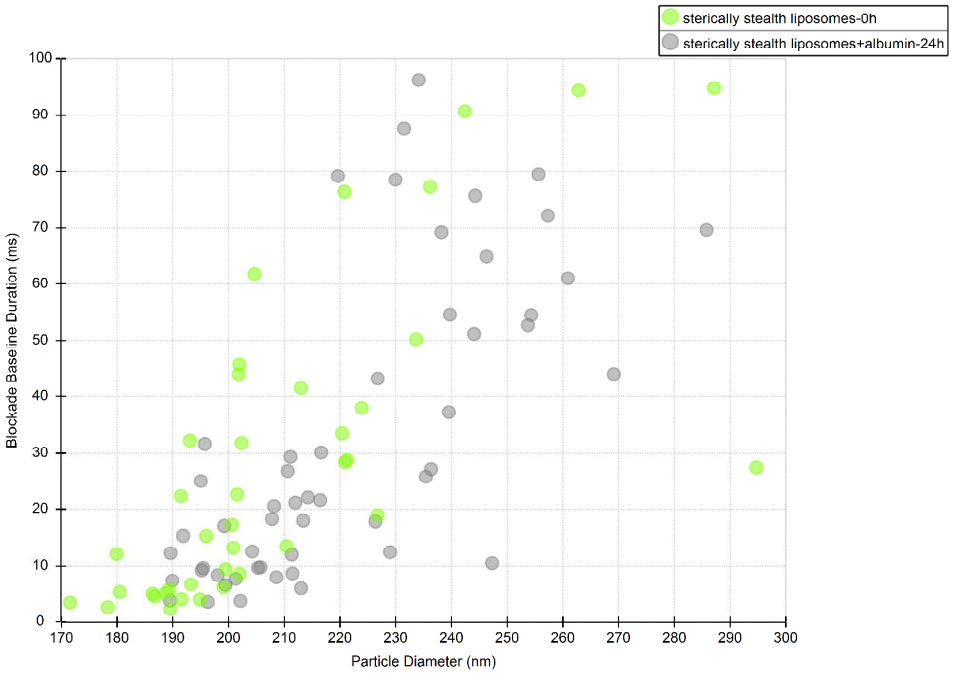
(2) By FT-IR measurement: PEG-P(HPMA-g-His)-cholesterol and biotin2-PEG were dissolved in methanol and coated on a NaCl salt plate by a typical thin film method. FT-IR spectra were recorded with a resolution of 1 cm^{-1} in the range from 4000 to 400 cm^{-1}. A base line correction was made for the spectra that were recorded in air.

S7. Relative Surface charge of liposomes.

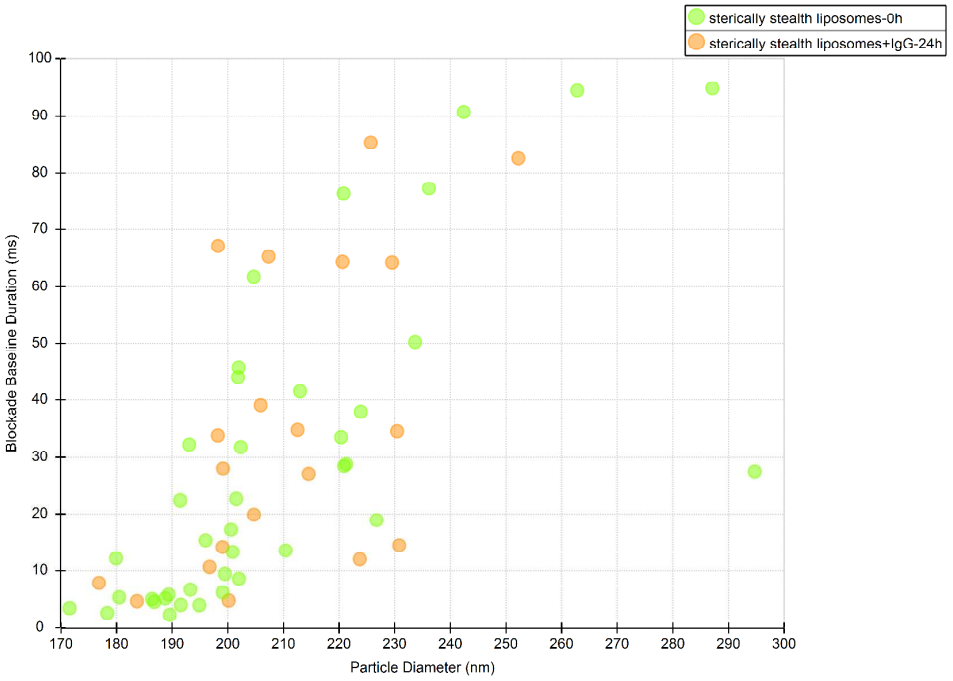
For qNano experiments, 40 μL of particle PBS solution with 4 wt% of HSA or with 0.1 wt% of fibrinogen was added to the fluid cell of qNano instrument and a minimum of 100 particles were recorded for each experiment. The analog digital converter operates at 1 MHz, which is reduced to a sampling rate of 50 KHz through electronic filtering. Measurements were undertaken at 50 mm of applied stretch. Fluid flow rate measurements were conducted at 0.18V. Polystyrene nanoparticles with

50 nm were used as a standard.

(a)



(b)



(c)

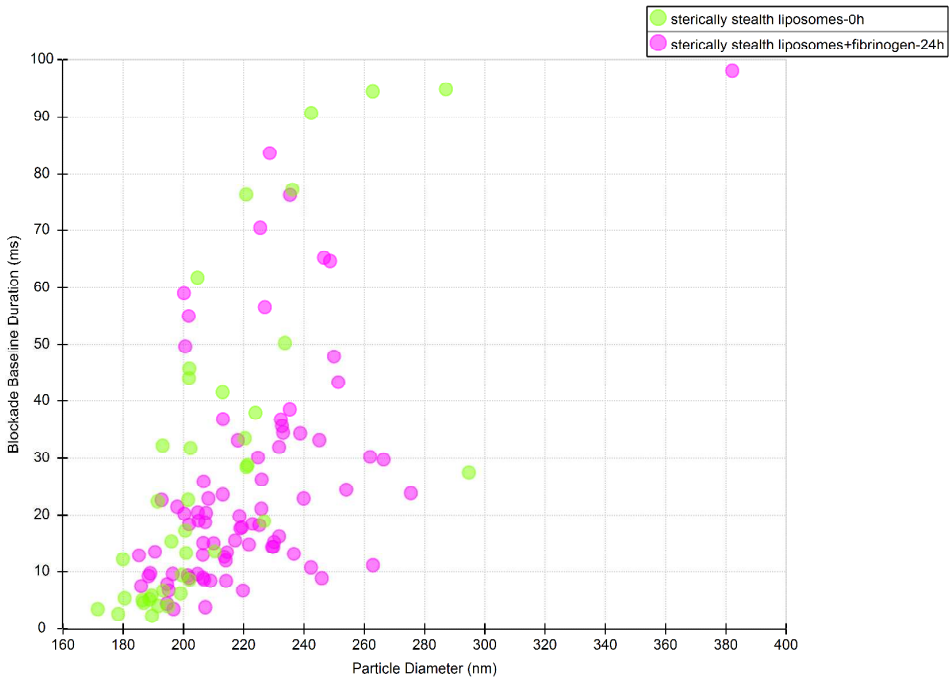
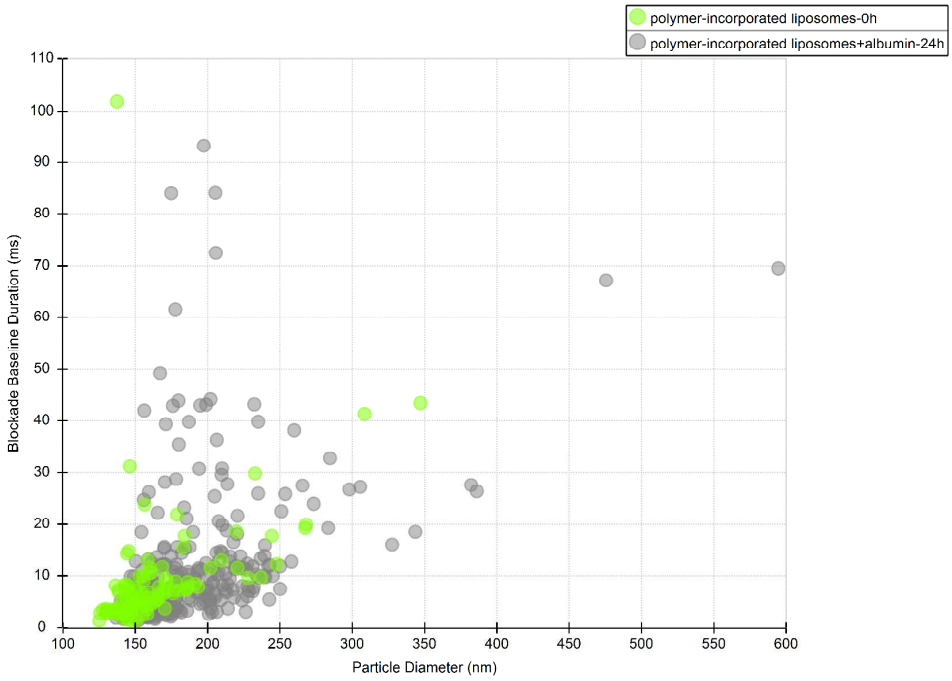
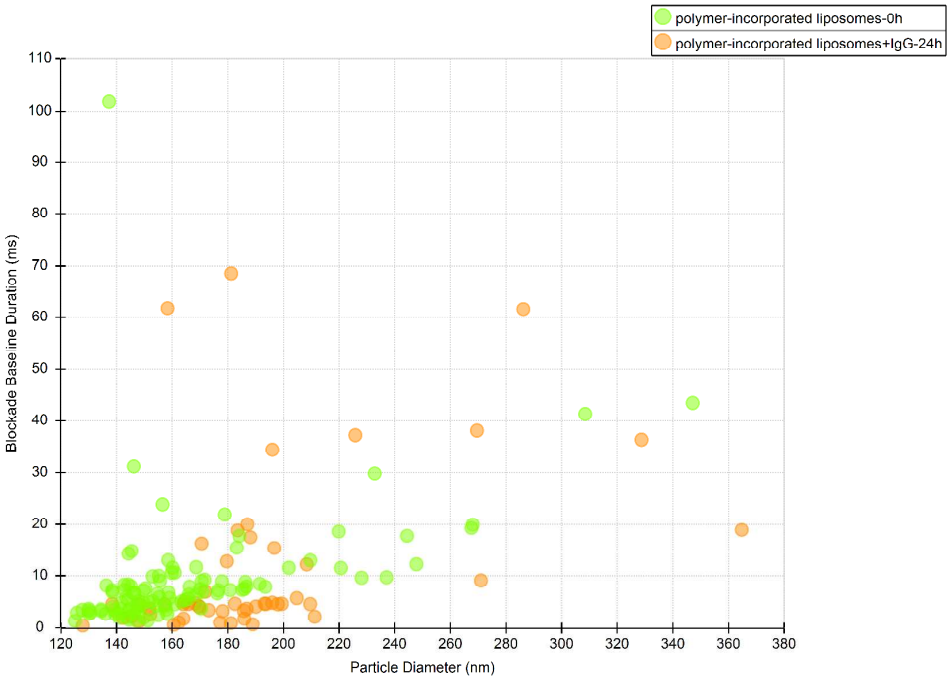


Figure S13. Blockade baseline duration (relative surface charge) of stealth liposomes treated with (a) HSA, (b) IgG, and (c) fibrinogen.

(a)



(b)



(c)

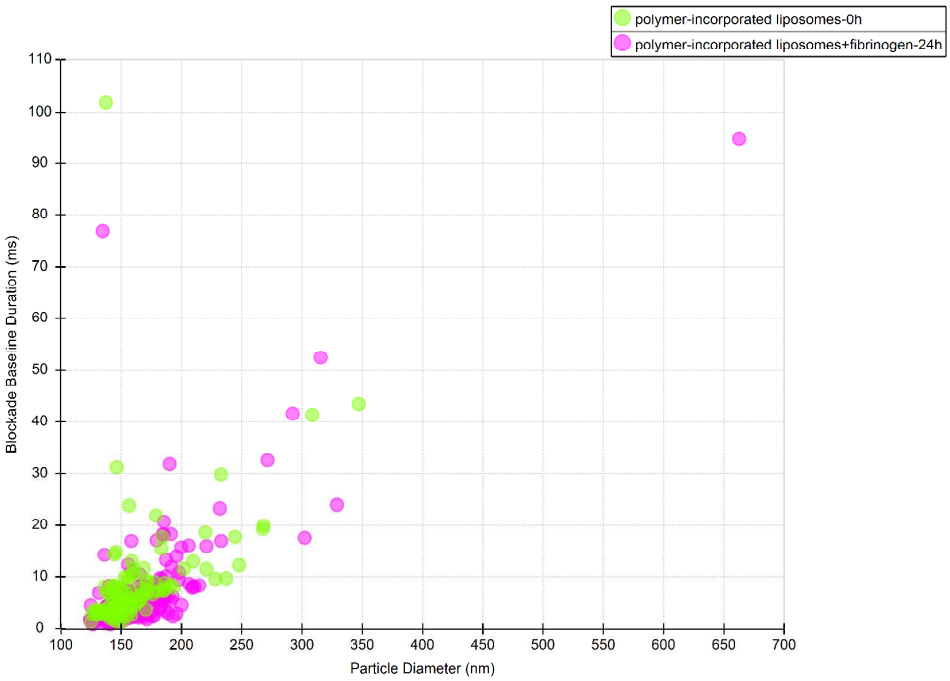
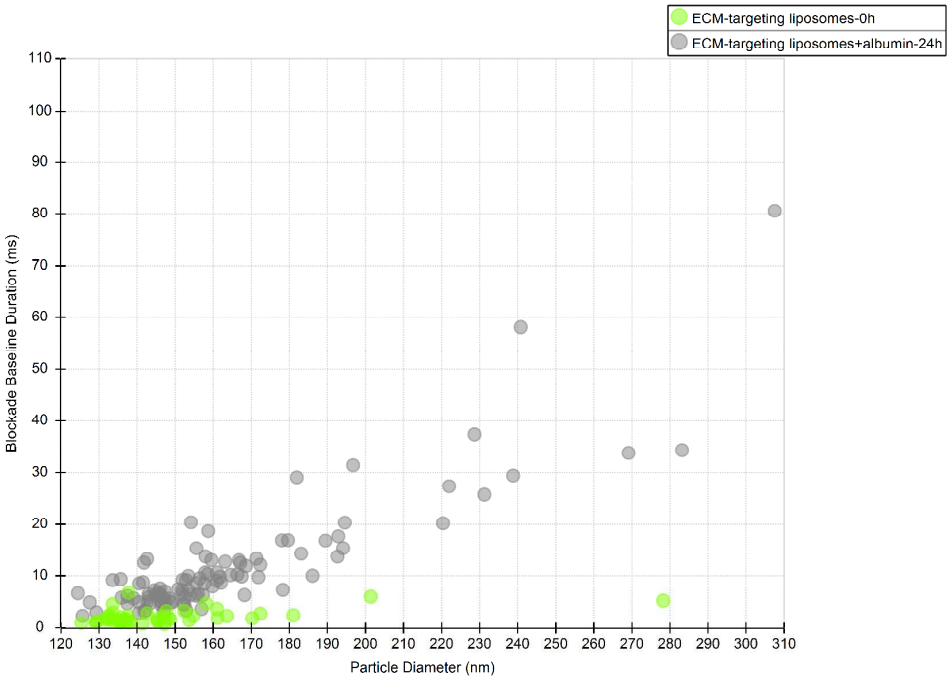
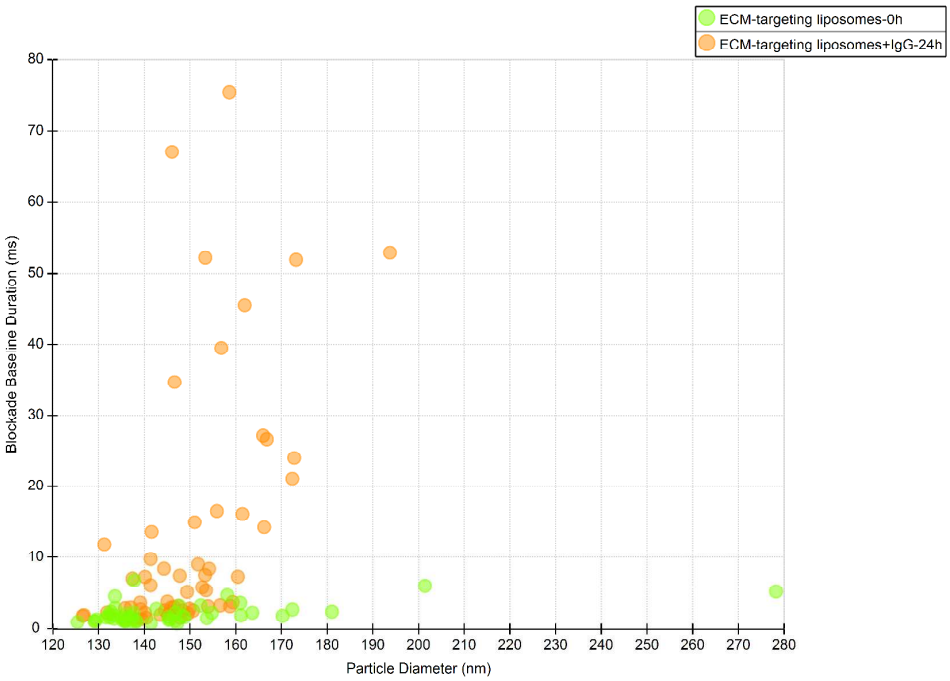


Figure S14. Blockade baseline duration (relative surface charge) of polymer-incorporated liposomes treated with (a) HSA, (b) IgG, and (c) fibrinogen.

(a)


ECM-targeting liposomes-0h
ECM-targeting liposomes+albumin-24h
Blockade Baseline Duration (ms)
Particle Diameter (nm)

(b)


ECM-targeting liposomes-0h
ECM-targeting liposomes+IgG-24h
Blockade Baseline Duration (ms)
Particle Diameter (nm)

(c)

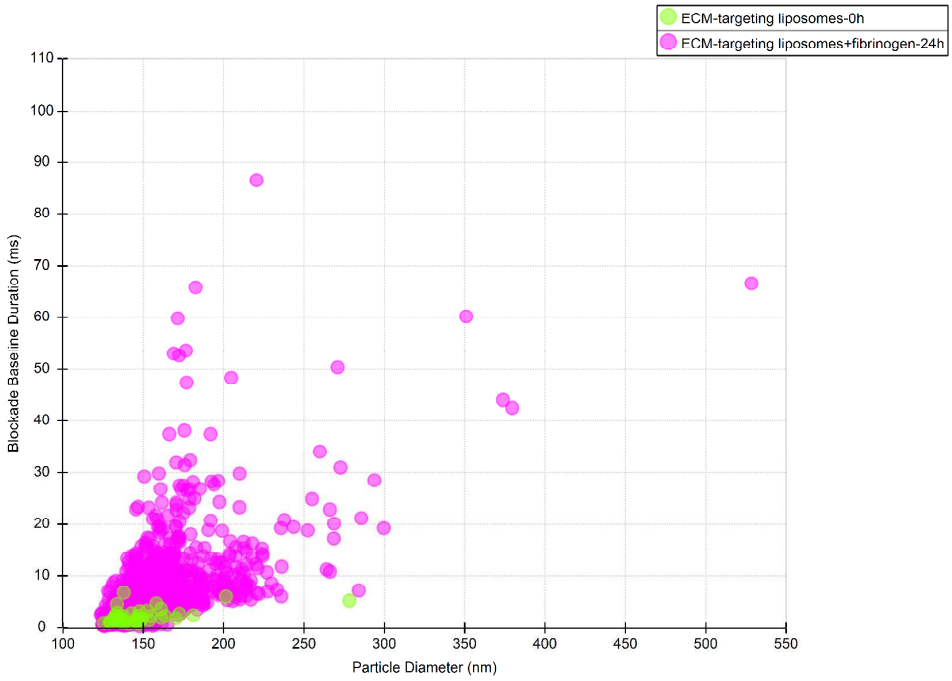


Figure S15. Blockade baseline duration (relative surface charge) of ECM-targeting liposomes treated with (a) HSA, (b) IgG, and (c) fibrinogen.

S8. ECM-targeting Liposomes at acidic surroundings.

(1) By UV/Vis determination: biotin2-PEG, polymer-incorporated liposomes, and ECM-targeting liposomes under a fixed concentration as well as for liposomes preparation were dissolved/ suspended in pH 7.4 or 6.8 (adjusted by by 1 M HCl) PBS. After 1 and 4 h, the electronic absorption spectra ranging from 190 to 500 nm were monitored with a UV/Vis spectrophotometer. A base line correction was made for the spectra recorded in pH 7.4 PBS.

(2) Crosslinker departure from ECM-targeting liposomes: ECM-targeting liposomes were suspended in pH 6.5 PBS for 4 h, and the solution was then centrifuged by Amicon at 4000 rpm for 10 min to collect the departed crosslinkers. The crosslinking level could be obtained by avidin-biotin complex

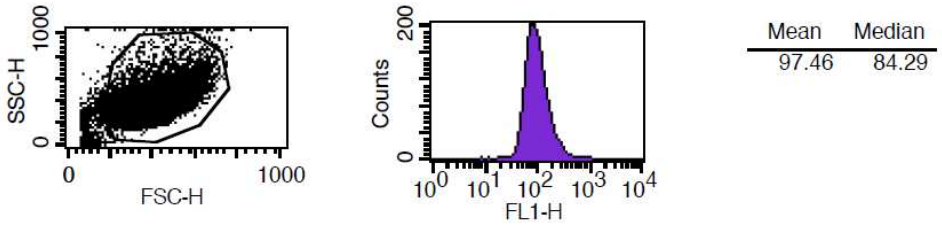
method (HABA/avidin reagent test).

S9. HCT116 Cancer Cell Uptake by Flow Cytometer Measurement.

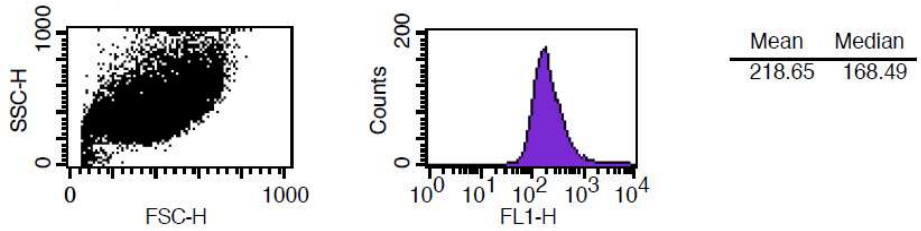
The cellular uptake experiments were performed using flow cytometer. First, HCT116 cells ($1x10^6$) were seeded in 6-well culture plates and grown overnight. The cells were then treated with liposomes for 0.5 h at pH 6.5 and 37°C. The concentration of each sample was fixed at 2nM. Cells were then washed twice with PBS to remove untrapped liposomes and were cultured under pH 7.4 medium. After 1 and 3 h, the cells were washed with PBS three times and were lifted using trypsin and washed with PBS twice. Each sample was analyzed by BD FACSCalibur flow cytometer using the 488 nm argon/krypton laser line and a 520 nm band pass FL1-H emission filter.

1 h

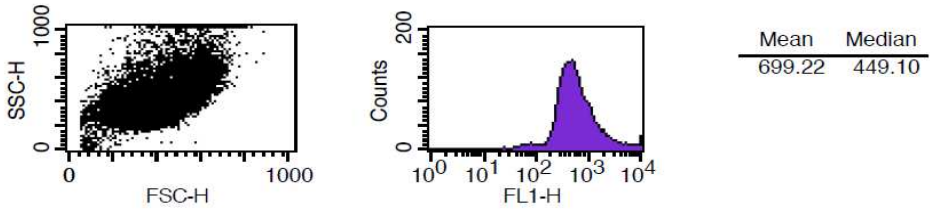
Control



Polymer-incorporated liposomes

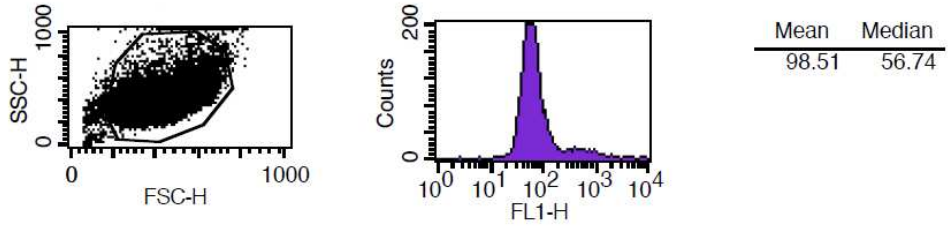


ECM-targeting liposomes



3 h

Control



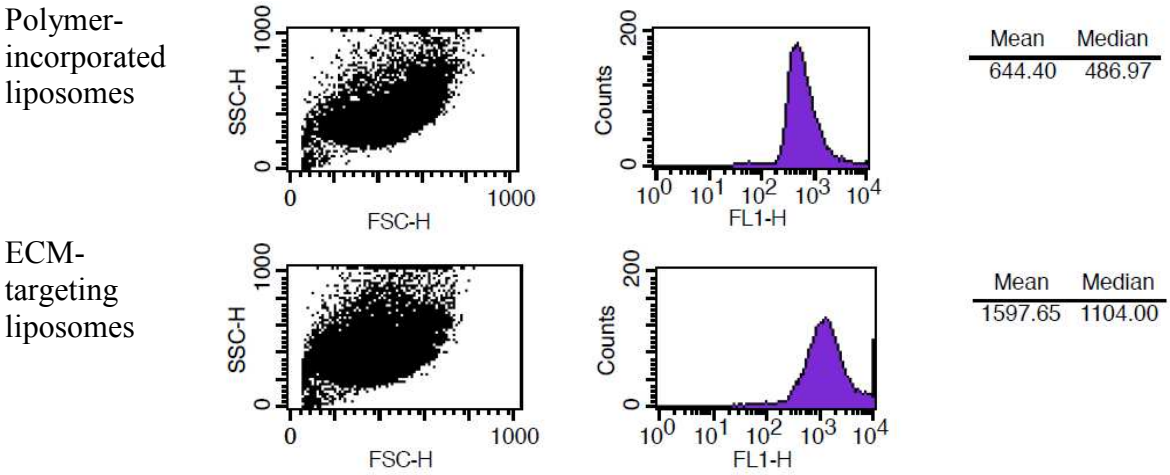


Figure S16. Flow cytometry analysis of liposomes incubated with HCT116 cells. Over 20,000 cells were counted per sample.

S10. CLSM observation.

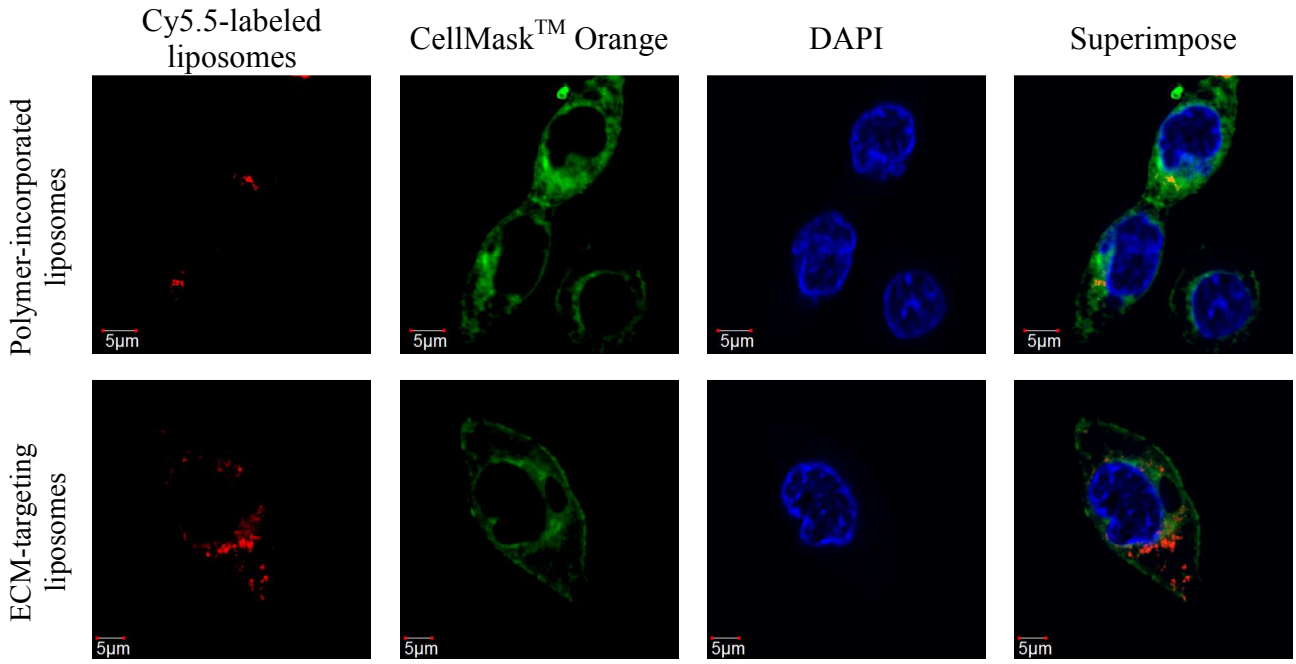


Figure S17. Confocal images of HCT116 cells incubated with polymer-incorporated liposomes and ECM-targeting liposomes for 0.5 h at pH 6.5 and 4°C. Red fluorescence indicates Cy5.5-labeled particles. Green and blue fluorescence indicate CellMask™ Orange and DAPI, respectively.

S11. Biodistribution (at 24 h post-injection).

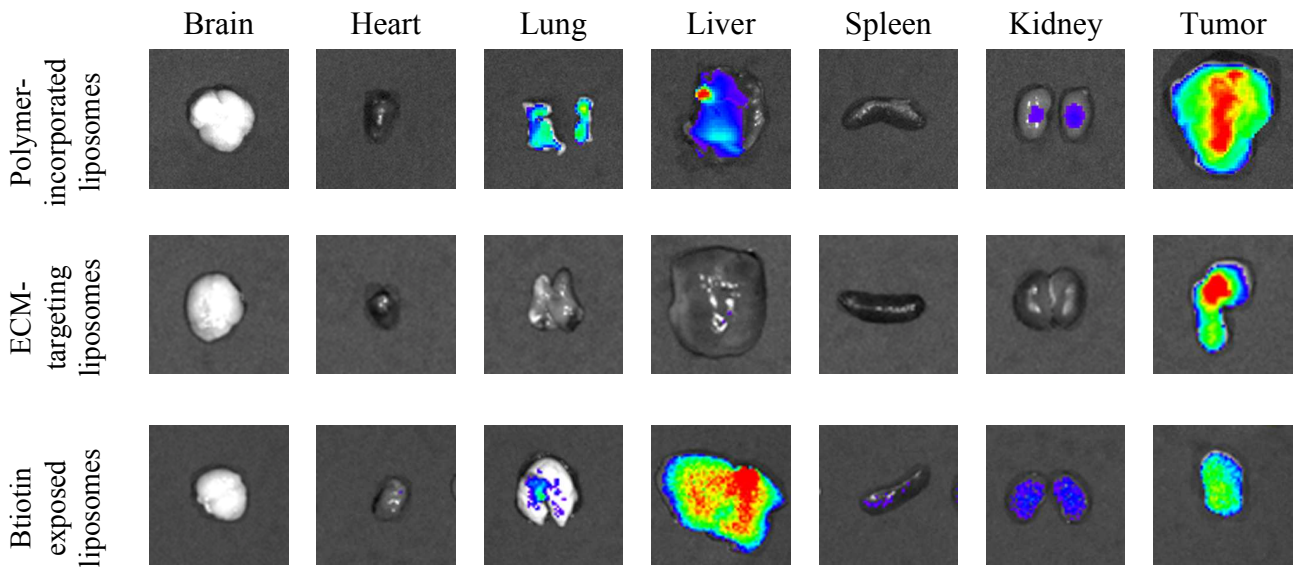


Figure S18. Organs distribution of HCT116 tumor xenografted Balb-C/nude mice administrated with polymer-incorporated liposomes, ECM-targeting liposomes, and biotin exposed liposomes.

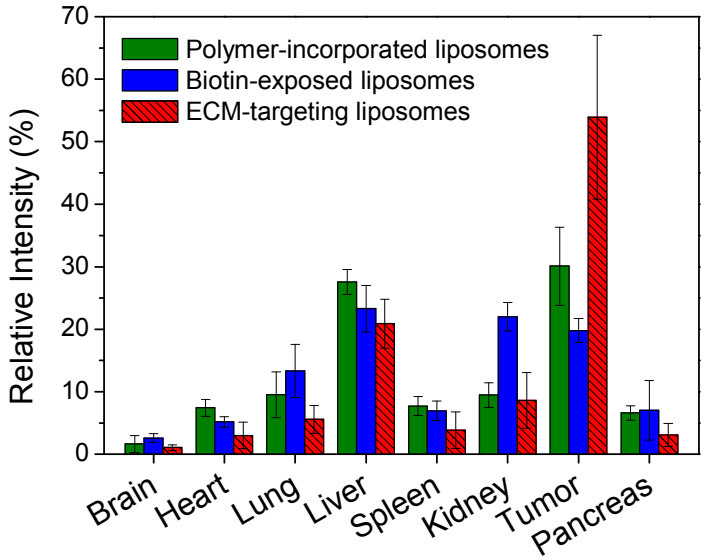


Figure S19. Quantitative analysis of organs distribution after 24 h post-injection.

Reference:

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