Supporting Information

Macrophage Membrane-Coated Nanoparticles for Tumor-Targeted Chemotherapy.

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Materials and methods

Materials. Octylamine, 2-aminoethyldiisopropylamine, 1,4-butanediol diacrylate and phenylmethanesulfonyl fluoride (PMSF) were purchased from Energy Chemical (Shanghai, China). 1,4-Butanediol diacrylate was used after extra purification by column chromatography. α-Methoxy-ω-amino-poly(ethylene glycol) (mPEG-NH_2, MW 5000) and α-azido-ω-amino-poly(ethylene glycol) (N_3-PEG-NH_2, MW 5000) were purchased from Jenkem Biotech (Beijing, China). Paclitaxel, docetaxel standard substance and agarose were purchased from Meilun

Biotech (Dalian, China). The *D*-form IGF1R targeting peptide (sequence: 6-alkynylcaproic acid-cskc) were synthesized by ChinaPeptides Co. Ltd (Nanjing, China). High concentration Matrigel Matrix (Phenol Red-free) was purchased from BD Biosciences (San Jose, USA). Polyoxyethylated castor oil, thioglycolate broth, coumarin, phenylarine oxide, colchicine, filipin complex and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, USA). Hoechst 33342 trihydrochloride, LysoTracker Deep Red and BODIPY FL were purchased from Molecular Probes (Waltham, USA). One step TdT-mediated dUTP Nick-End Labeling (TUNEL) apoptosis assay kit and annexin V-FITC/PI apoptosis detection kit were purchased from KeyGEN BioTECH (Nanjing, China). Bradford protein quantification kit was purchased from Beyotime (Shanghai, China). Other reagents without specified explanation were purchased from Sinopharm Chemical Reagent (Shanghai, China). MDA-MB-231 human breast cancer cell line and 293 cell were purchased from cell bank of Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells were carefully maintained in high glucose DMEM medium supplemented with 10% heat-inactivated FBS and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% CO_2. For construction of tumor spheroid, 4000 cells in 400 μL complete DMEM medium were seeded in 100 μL 4% agarose pretreated 48 well plate and maintained for 7 days before aspirating out for further experiment. Female Balb/c nude mice of about 20 g weight were purchased from Department of Experimental Animals, Fudan University and raised under standard SPF laboratory conditions (Shanghai, China). All animal experiments were strictly conducted according to the guidelines stipulated and approved by the animal ethics committee of Fudan University. For construction of breast cancer bearing mice model, 2×10^6 MDA-MB-231 cells in a suspension solution of 25% Matrigel in 100 μL serum-

free DMEM medium were subcutaneously inoculated into the right flank of the mice. Avanti mini extruder was bought from Avanti Polar Lipids (Alabaster, USA).

Synthesis and characterization of polymers.

PBAE polymer was synthesized *via* a high throughput method as reported.[1] Briefly, the octylamine or 2-aminoethyldiisopropylamine was neatly reacted with 1.05 equivalent of 1,4-butanediol diacrylate under strict argon protection at 80 □ for 48 h and purified by repeated wash with cold anhydrous hexane. The as-prepared polymer was then dissolved in dimethyl sulfoxide with 2 equivalent of mPEG-NH_2 for polyethylene glycol (PEG) conjugation. Targeting ligand-modified polymer was synthesized by conjugating the targeting peptide to N_3-PEG-NH_2 before the above-mentioned PEG conjugation procedure. Conjugation of peptide ligand to the end of N_3- PEG-NH_2 was performed via click reaction in the presence of Cu (I) as catalyst and excessive ascorbic acid as reductant. The molecular weight was calculated as Mn by GPC and further confirmed by ^{1}H-NMR. The enveloped drug loading nanoparticle was formulated by the as-prepared Bola-amphiphilic PEG conjugation PBAE.

Titration of polymers.

HCl (4 mM) was added incrementally to titrate PPiP and PPC8 solution (1mg/mL). The pH change was measured by a pH meter (Mettler Toledo). [2]

Nanoparticle preparation.

PTX was first dissolved in DMF with PPC8, PPiP or cskc-PPiP at designated weight ratio to the final concentration of 20 mg/mL. The solution was then added dropwise into 2 mL PBS with quick and foamless stirring for 30 min before dialyzed against the same hydrating medium to remove the extra organic solvent. Drug loading was calculated as DL = *w*(drug)/[*w*(drug)+

w(polymer)] and drug loading efficiency was calculated as LE = *w*(drug in NP)/[*w*(initial drug added)].

Critical micelle concentration (CMC) determination.

To series of tubes was added 200 ng Nile Red (20 μg/mL ethanol stock solution, 10μL) and dried under vacuum. PPiP and PPC8 were dissolved in PBS (7.4, 6.5 and 5.0) to designated concentration and then added to the Nile Red applied tubes before shaken for 1 h. The fluorescence of the solution was then measured by fluorescence spectrometer (λex=557 nm, λem= 601 nm). The CMC value was determined by calculating the intersection value of linear fittings of high and low concentration region.[2]

Preparation of membrane coated nanoparticle and characterization.

Fresh mouse peritoneal macrophage was harvested according to previously reported method.[3] The mouse was intraperitoneal injected with 1 mL 4% thioglycolate broth for 3 days before sacrificed and disinfected by immersing in 75% ethanol. The body was then injected with 2 mL DMEM medium in enterocoelia followed by full massage. The withdrawn DMEM medium containing fresh peritoneal macrophage could be maintained in CO_2 incubator. The harvested macrophage ghost was prepared by 3 cycle of freezing and thawing in liquid nitrogen of macrophage suspension in 50 nM PMSF added 0.25× PBS. The lysate was washed with the buffer and centrifuged at 5000 rpm to collect macrophage ghost. The as-prepared fresh macrophage ghost was then sonicated for 5 min with newly made nanoparticle solution before subsequently extruded through 400 nm and 100 nm polycarbonate porous membranes by an Avanti mini extruder.[8] Size/PDI and zeta-potential of prepared membrane coated nanoparticle and uncoated formulations were measured by dynamic light scattering detector (DLS) (Zetasizer

Nano-ZS, Malvern, UK). In consideration of avoiding possible shrinkage and damage during the TEM sample preparation as well as presenting the vesicles' natural form in bio-mimic hydrating milieu, we used high power lens of confocal microscope to observe the morphological change of different formulations in adjusted pH conditions which mimic the physiology and tumor microenvironment. The morphological examination of membrane coated nanoparticle was performed using Biology Transmission Electron Microscope (B-TEM, Tecnai G2 spirit Biotwin, FEI, USA) and confocal fluorescence microscope (Carl Zeiss LSM710, Zeiss, Germany) for morphological analysis of membrane coated nanoparticle under hydrating circumstance.

***In vitro* PTX release study.**

The *in vitro* PTX release profile of cskc-PPiP/PTX, PPC8/PTX, cskc-PPiP/PTX@Ma and PPC8/PTX@Ma at different pH were measured through dialysis method (n=3). Briefly, 500 μL nanoparticle solution was sealed in a dialysis bag (MWCO 3500) before suspended in a centrifuge tube containing 10 mL buffer (pH 7.4, 6.5 or 5.0). An aliquot of solution (150 μL) was withdrawn from the outwear tube with same volume renewal of release medium at various time points while shaken at 100 rpm at 37 °C water bath. The concentration of PTX was measured by HPLC by the above mentioned method. The release medium in outwear tube was supplemented with 0.1% tween 80 to meet the sink condition.

Cellular uptake and internalization mechanism studies.

Cellular uptake was determined by the intracellular PTX amount on the strength of total crude protein. MDA-MB-231 cells were seeded in 6-well plates followed by incubating for 24 h until reaching a confluence of 80% - 90%. The cells were then incubated with free PTX, cskc-PPiP/PTX, PPC8/PTX, cskc-PPiP/PTX@Ma, PPC8/PTX@Ma for 3 h before being trypsinized

and extracted with 1 mL methyl tertiary butyl ether per well. All the formulations were calibrated for PTX loading efficiency and dilute to ensure a normalized PTX concentration of 10 μg/mL. The cskc-PPiP/PTX@Ma, PPC8/PTX@Ma particle was pre-incubated in PBS (pH 6.5) before applied on cells in order to mimic the minor acidic microenvironment in extracellular tumor tissue. For the internalization mechanism study, 1 μg/mL colchicine, 0.2 μg/mL phenylarine oxide (PhAsO) and 0.4 μg/mL filipin complex treated cells as endocytic inhibitors for macropinocytosis, clathrin and caveolin pathway respectively. The HPLC method for PTX level determination was as follow (Agilent C18 column. 250 mm, 1.0 mL/min, 65%-45% acetonitrile + 35%-55% 0.1% trifluoroacetic acid, injection volume 10 μL). Cells without any treatment were used as control to evaluate the matrix effect. Cellular uptake was also presented by fluorescent photos using coumarin-loaded formulations instead of PTX to track the nanoparticles' endocytosis process with same treatment criteria as above. The cells was then observed and photographed under inverted fluorescence microscope.

***In vitro* and *in vivo* penetration study.**

For *in vitro* penetration study, fluorescent dye loaded formulations were given to the tumor spheroids of about 200 μm in diameter which were constructed as mentioned above. The tumor spheroids were incubated with BODIPY-loaded PPiP@Ma, cskc-PPiP@Ma and PPC8@Ma in different medium (pH 7.4 and pH 6.5) and then washed, fixed and transferred to a flat glass-bottom petri dish for observation under confocal fluorescence microscope. For *in vivo* penetration study, fluorescent dye loaded formulations were given to the breast cancer bearing mice to evaluate penetration efficiency in solid tumor. Grouped mice were intravenous injected with PPiP/dye@Ma, cskc-PPiP/dye@Ma and PPC8/dye@Ma respectively and sacrificed after 12 h. Tumor tissue was excised after perfusion and fixed in 4% neutral paraformaldehyde. Cryo-

sections of 10 μm were made followed by staining with CD4 antibody and DAPI to demonstrate vessel and nucleus. The sections were then observed and photographed under fluorescence microscope to follow the nanoparticles' trace in tumor region.

***In vivo* imaging study.**

In vivo imaging study of the nanoparticle distribution was performed on breast cancer bearing mice models intravenously injected with BODIPY loaded cskc-PPiP@Ma at a dose of 1 mg per mouse. The mice for imaging study were of 25 ± 1 g in body weight and 200 ± 15 mm^3 in tumor volume. Anesthetized by isoflurane, the mice were photographed under IVIS (*In Vivo* Imaging Instruments, Perkin Elmer, Waltham, USA) spectrum imaging system at Ex/Em 650 nm / 665 nm at 2 h, 4 h, 8 h, 12 h and 24 h post injection. The tissues and main organs (heart, liver, spleen, lung, kidney and tumor) were collected and photographed after rinsed in saline.

Biodistribution studies.

Biodistribution study was performed on breast cancer bearing mice models intravenously injected with free PTX, PPiP/PTX, cskc-PPiP/PTX, PPC8/PTX, PPiP/PTX@Ma, cskc-PPiP/PTX@Ma, PPC8/PTX@Ma at a dose of 10 mg PTX/kg (n=4). The mice were sacrificed after 24 h. The tumor tissue and main organs (heart, liver, spleen, lung and kidney) were weighted and homogenized after fully rinsed in saline. PTX concentration in tissues was determined by LC-MS according to the previously reported method.[4] The biological samples (blood or tissue homogenate) were centrifuged twice (5000 rpm, 10 min) and the supernatant were collected. For each sample, 2 mL methyl tertiary butyl ether was added to 100 μL of the supernatant to precipitate proteins before centrifuged (12,000 rpm, 5 min) to collect the supernatant. The supernatant was dried under nitrogen before re-dissolving in 100 μL methanol

and injected into LC-MS(Agilent C18 150 mm, 0.2 mL/min, column temperature 35 □, 80% methanol + 20% 0.1% formic acid, injection volume 2 μL, ion pair for peak integration: PTX: 854.400/569.400 DTX: 830.400/549.300) for PTX quantification.

***In vitro* antitumor efficacy study.**

In vitro antitumor efficacy study was characterized by MTT assay and cell apoptosis assay to evaluate the released drug loading nanoparticles' antitumor efficacy. As for MTT assay, MDA-MB-231 cells were seeded in 96-well plates at a density of 1×10^3 cells/well and routinely maintained until reaching a confluence of 80%. The cells were treated with Taxol, PPC8/PTX, PPiP/PTX and cskc-PPiP/PTX at various concentrations for 48 h. Afterwards, the medium was removed and cells were washed before 100 μL MTT solution (0.5 mg/mL) was added and incubated for 4 h. After removal of the MTT solution, 100 μL DMSO was added to dissolve the formazan crystal on a 37 □ shaker for 10 min. The absorbance of formazan was read at 570 nm using microplate spectrophotometer. Cells without any treatment were served as control. For cell apoptosis assay, MDA-MB-231 cells were seeded in 24-well plates at 1×10^4 cells/well and routinely maintained until reaching a confluence of 90%. The cells were treated with Taxols, PPC8/PTX@Ma, PPiP/PTX@Ma and cskc-PPiP/PTX@Ma for 24 h at 37 °C. The cskc-PPiP/PTX@Ma, PPC8/PTX@Ma particle was pre-incubated in PBS (pH 6.5) before applied on cells in order to mimic the rupture and particle escape behavior in minor acidic microenvironment of extracellular tumor tissue. The cells were then washed and stained by annexin V-FITC/PI apoptosis detection kit before observed and photographed by the fluorescence microscope (Leica, Wetzlar, Germany, 488 nm channel for Annexin V-FITC, 540 nm channel for PI).

***In vivo* antitumor efficacy study**.

Breast cancer bearing mice models were randomized into different groups (n=6) which treated with free PTX, PPiP/PTX, cskc-PPiP/PTX, PPC8/PTX, PPiP/PTX@Ma, cskc-PPiP/PTX@Ma, PPC8/PTX@Ma respectively every four days at a dose of 10 mg PTX/kg. The body weight and tumor volume of mice was recorded every other day. Tumor volume was calculated by Tumors excised from the tumor model on day 22 were fixed in 4% neutral paraformaldehyde before dehydrated in 15%, 30% sucrose solution for 24 h respectively. Cryo-section of 10 μm thick tumor slices were collected on cationic polylysine coated glass slides and then stained with one step TUNEL apoptosis assay kit and hoechst 33342 before observed and photographed by fluorescence microscope according to the manufacturer's instructions.

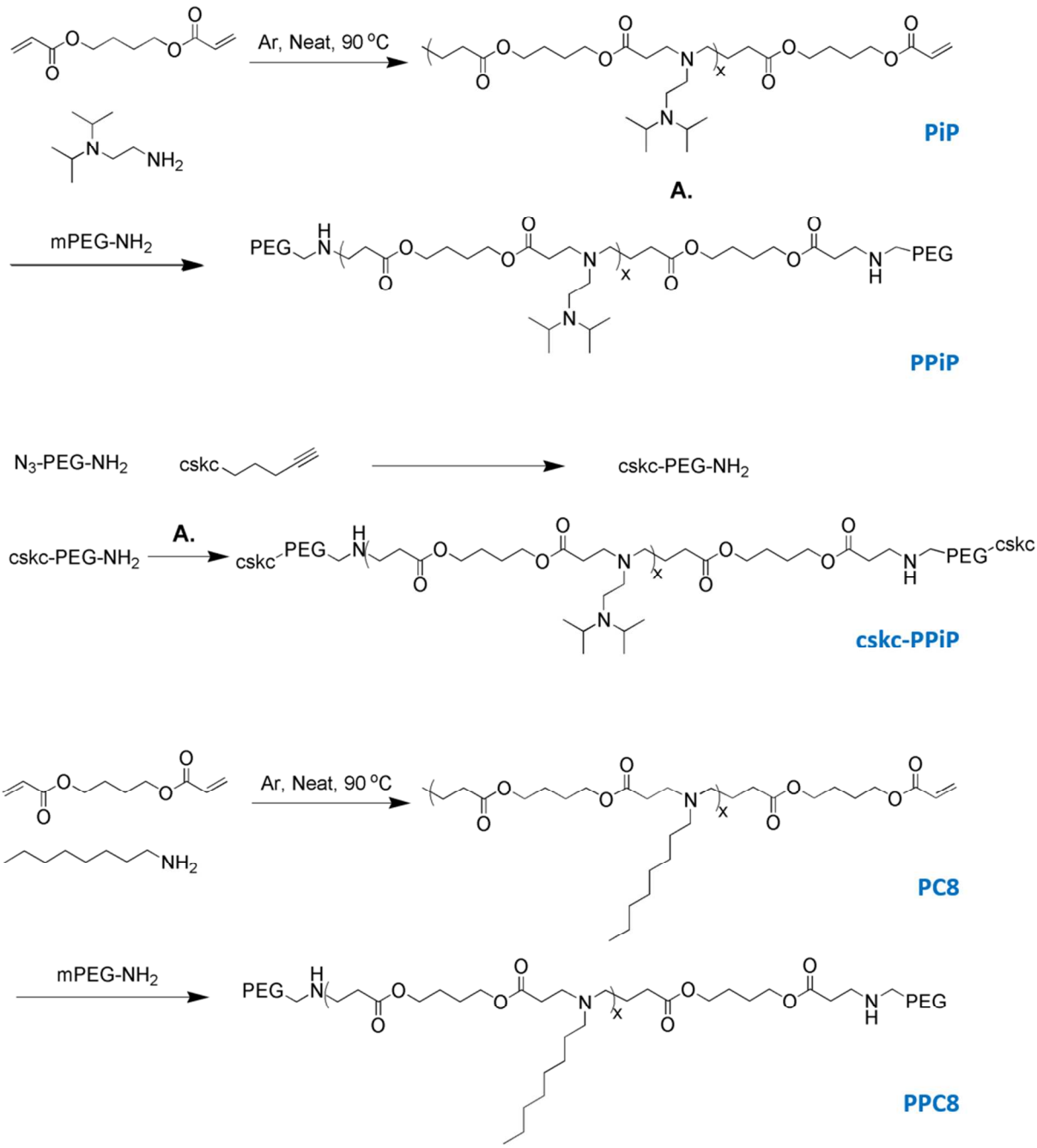
Toxicity study.

The *in vitro* toxicity of polymer and membrane materials was evaluated by MTT test. Off target uptake of released cskc-PPiP/PTX particles was tested on human renal epithelial cell line (293 cell) with same treating protocol as mentioned above for MDA-MB-231 uptake experiment. To further investigate the potential multi-dose toxic effects of membrane coated nanoparticle, the breast cancer bearing mice models from different groups were euthanatized on day 22 with main organs (heart, liver, spleen, lung and kidney) excised and fixed in 4% neutral paraformaldehyde solution. Tissue sections of 5 μm were stained with hematoxylin and eosin (H&E) before observation under microscope.

Statistical analysis.

GraphPad Prism software was used to analyze and present the data which showed as mean ± standard error (SD). Statistical comparisons were assessed by one-way ANOVA. The accepted level of significance was $P < 0.05$.

Supplemented figures



Scheme S1. General synthesis routes of polymers.

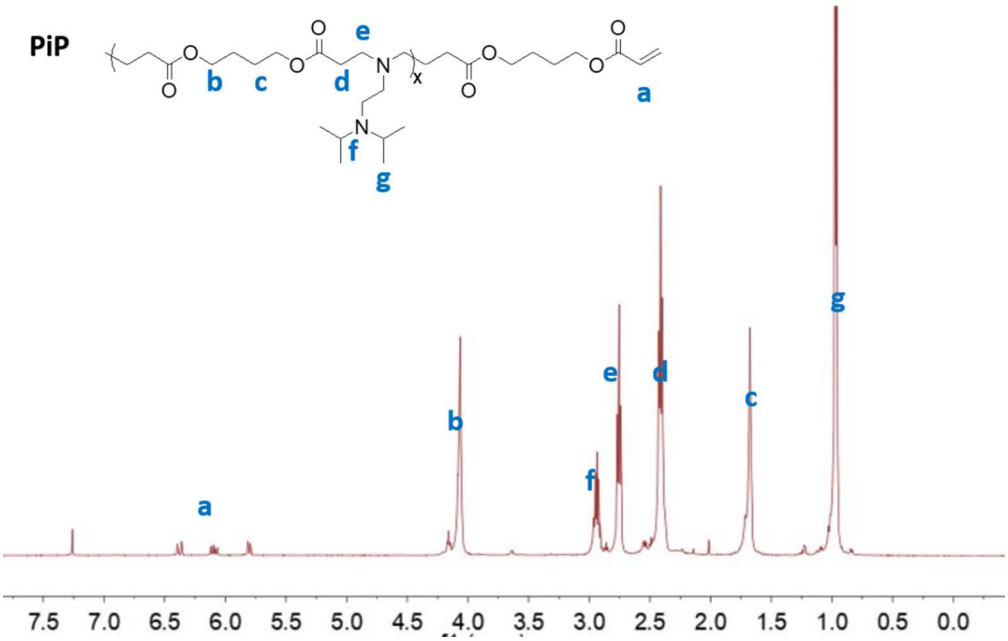


Figure S1. ^{1}H-NMR characterization of PBAE with diisoproyl side chains (PiP).

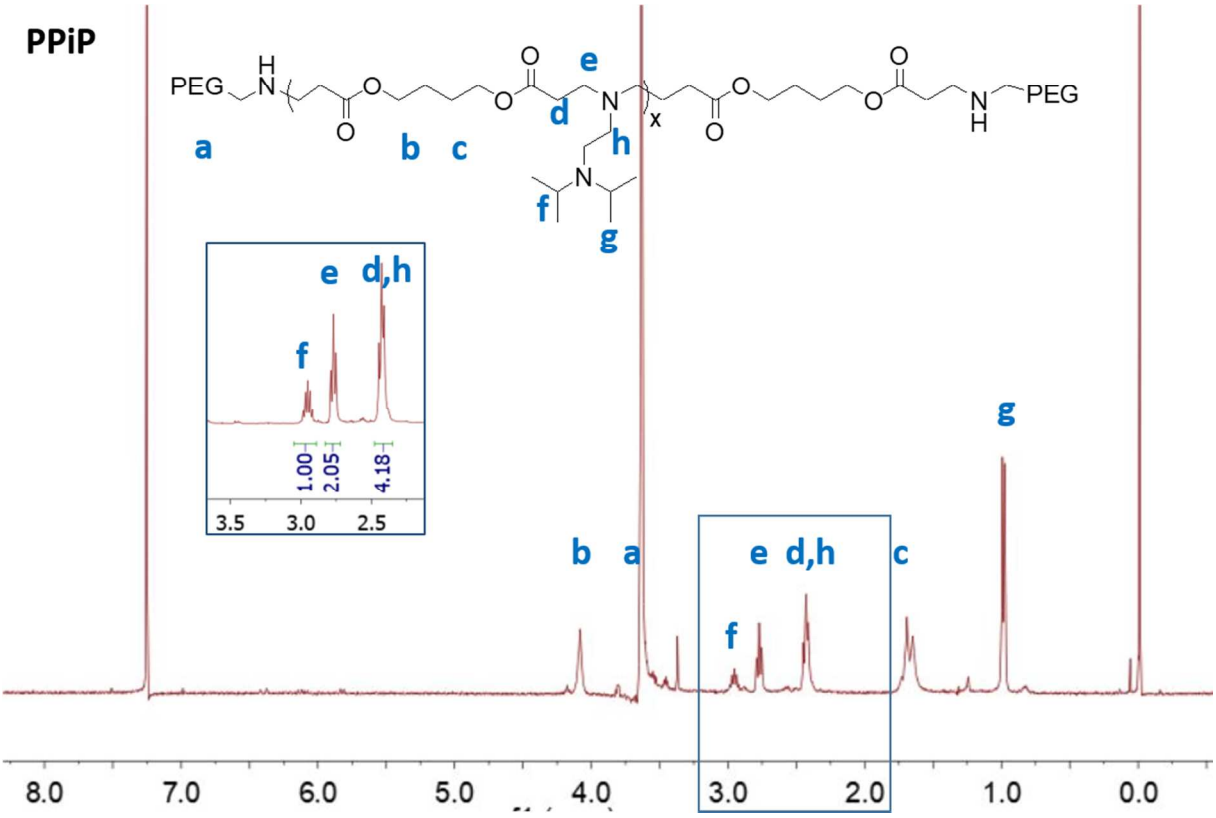


Figure S2. ^{1}H-NMR characterization of PEGylated PBAE with diisoproyl side chains (PPiP).

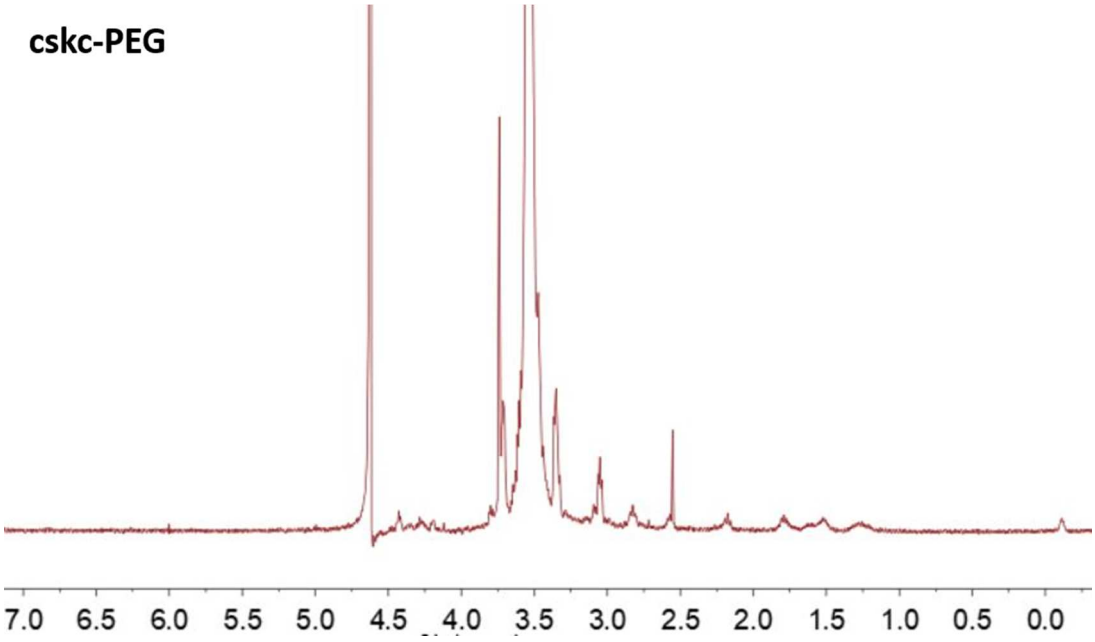


Figure S3. ^{1}H-NMR characterization of cskc peptide conjugated PEG-NH_2 (cskc-PEG).

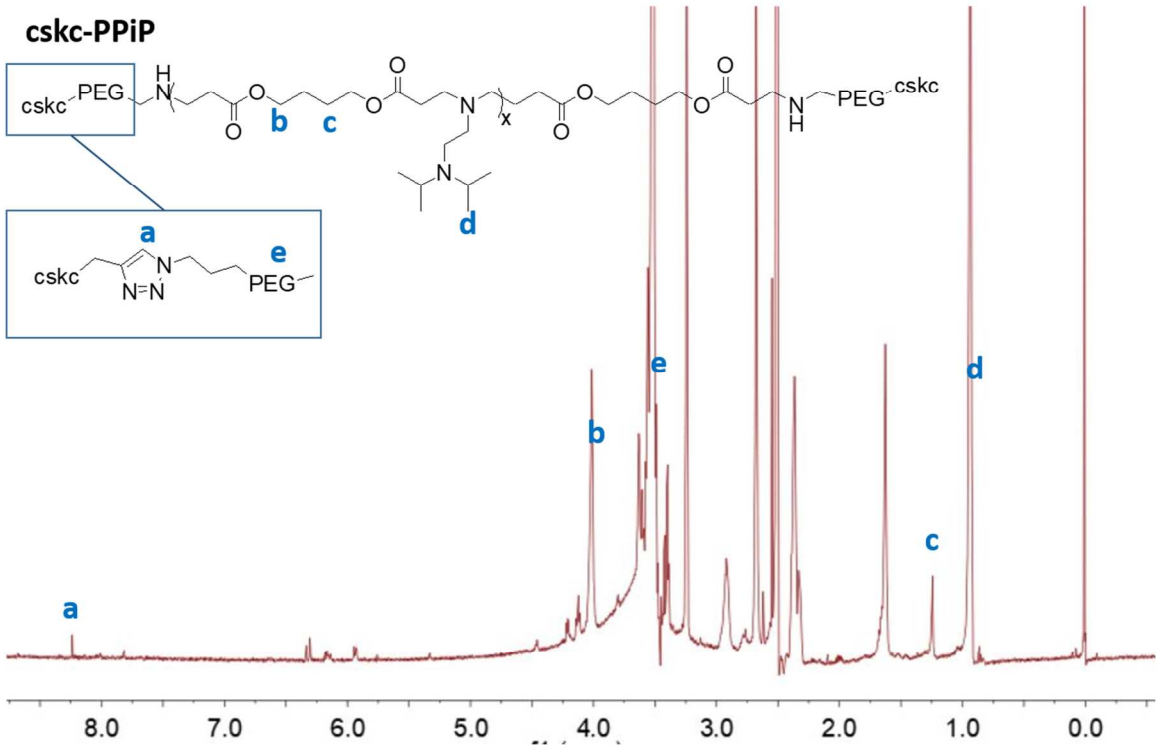


Figure S4. [1]H-NMR characterization of cskc-PEGylated PBAE with diisoproyl side chains (cskc-PPiP).

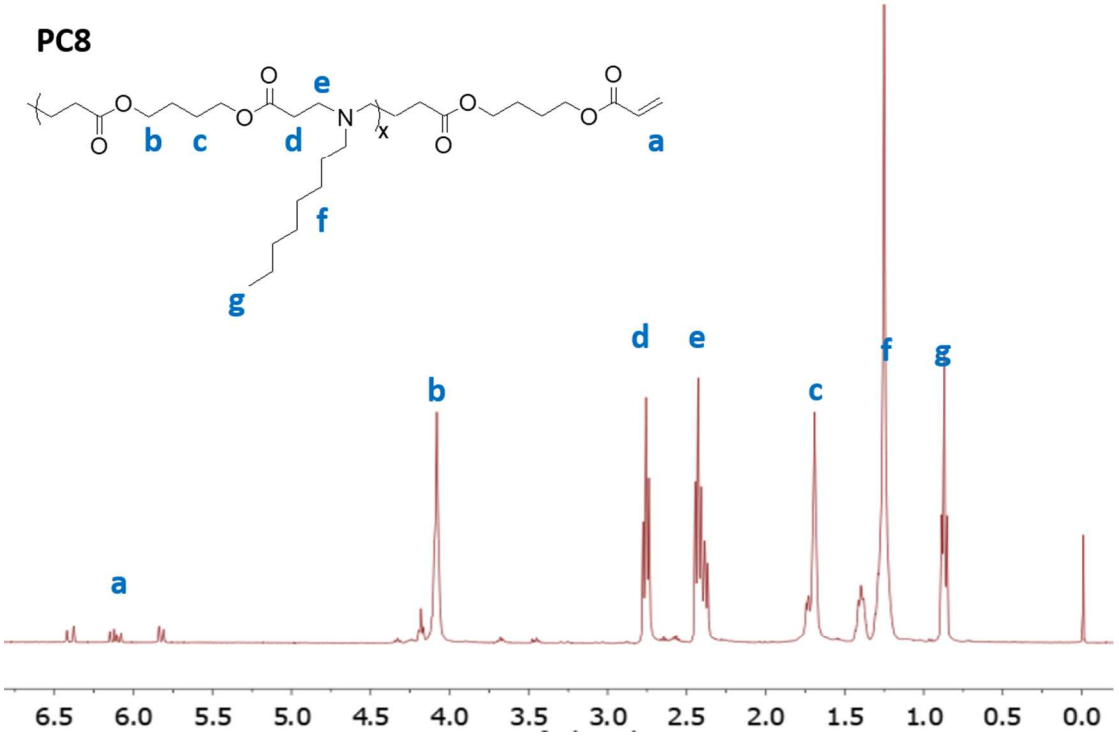


Figure S5. [1]H-NMR characterization of PBAE with octyl side chains (PC8).

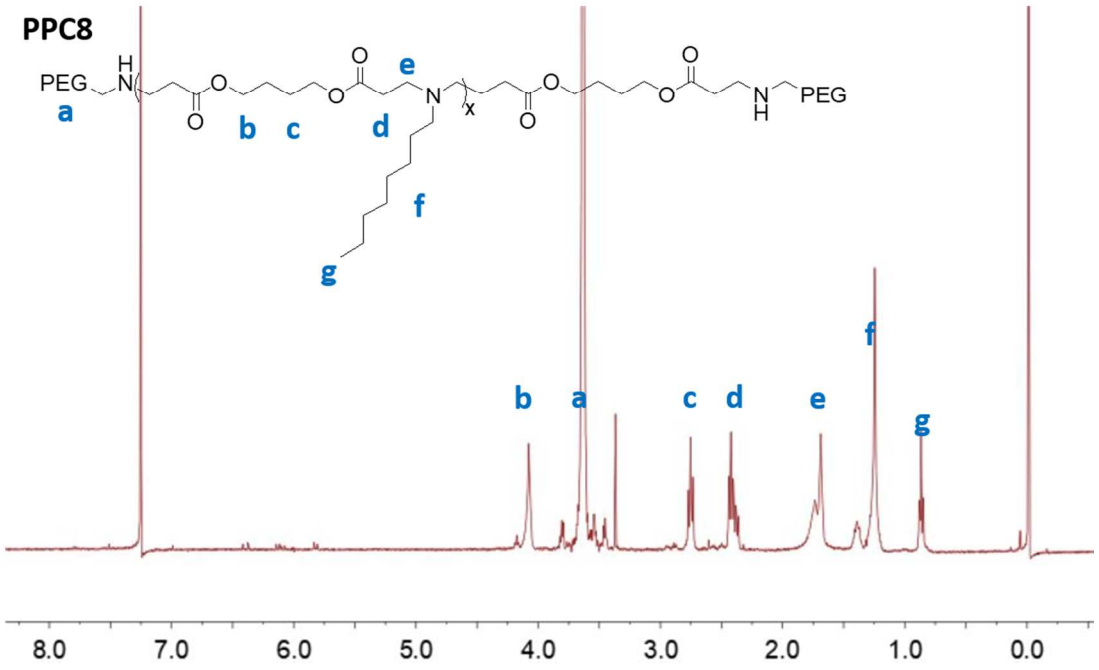


Figure S6. ^{1}H-NMR characterization of PEGylated PBAE with octyl side chains (PPC8).

Polymers	M_n (kDa)	PDI
PiP	8.1	2.03
PC8	7.7	1.78

Table S1. Molecular weight (presented as Mn) and PDI of different PBAE polymers.

Formulations	Diameter (nm)		Zeta potential (mV)	Drug loading	Loading efficiency
	pH 7.4	pH 6.5			
cskc-PPiP/PTX	82.3 ± 7.5	149.5 ± 31.2	13.1	9.88%	82%
PPiP/PTX	80.9 ± 9.7	151.1 ± 27.5	12.4	10.4%	85%
PPC8/PTX	78.4 ± 7.9	84.4 ± 9.5	3.70	10.2%	87%
cskc-PPiP/PTX@Ma	200.1 ± 19.4	-	-31.3	-	-
PPiP/PTX@Ma	207.2 ± 24.7	-	-33.9	-	-
PPC8/PTX@Ma	198.5 ± 20.8	200.6 ± 38.6	-30.1	-	-
Empty Ma	189.7 ± 28.1	192.5 ± 30.2	-32.4		

Table S2. Miscellaneous properties of PTX loading nanoparticles with or without membrane coating.

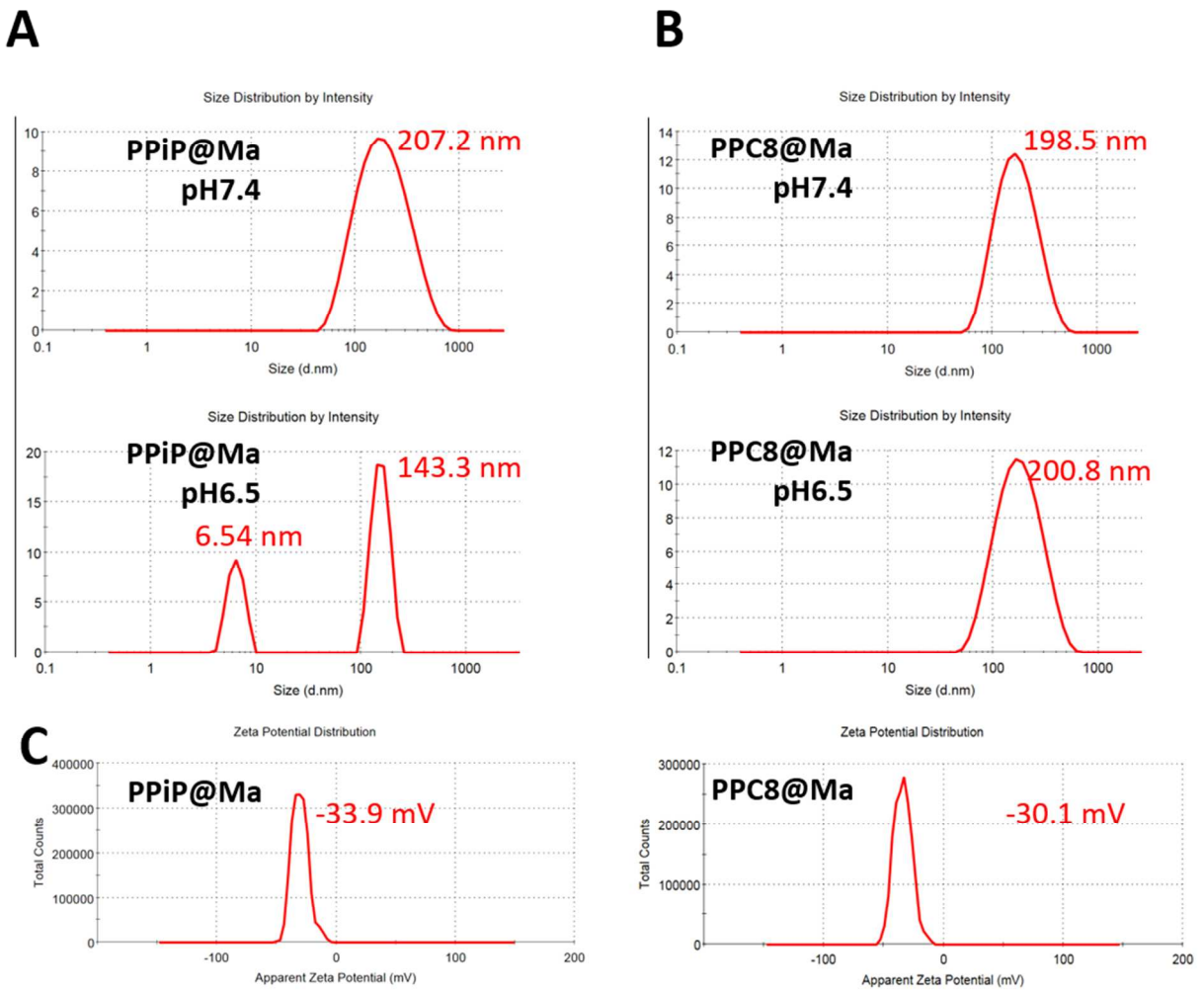


Figure S7. Size and zeta potential characterizations of membrane coating nanoparticles PPiP/PTX@Ma and PPC8/PTX@Ma. A. Size change of PPiP/PTX@Ma from pH 7.4 (a) to pH 6.5 (b). Zeta potential of PPiP/PTX@Ma (c) after membrane coating. B. Size change of PPC8/PTX @Ma from pH 7.4 (a) to pH 6.5 (b). Zeta potential of PPC8/PTX@Ma (c) after membrane coating.

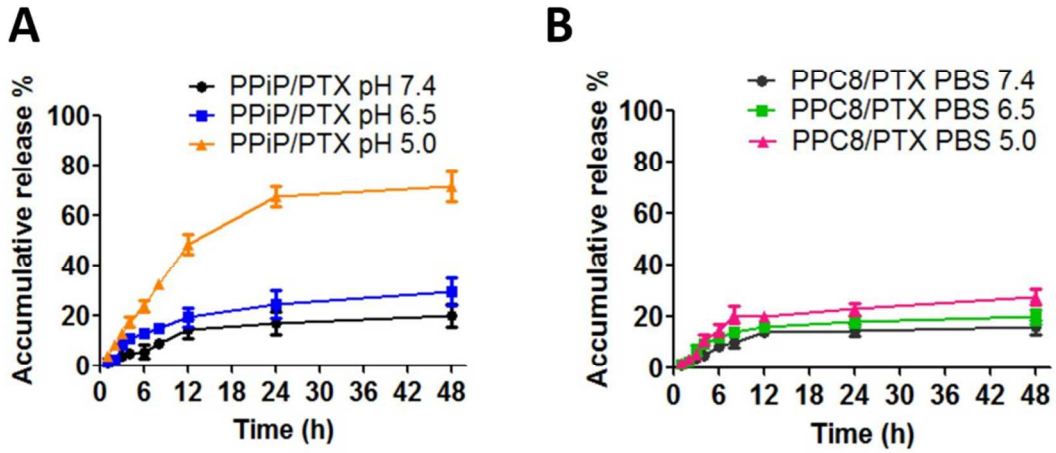


Figure S8. Drug release profile of A. PPiP/PTX and B. PPC8/PTX nude nanoparticles.

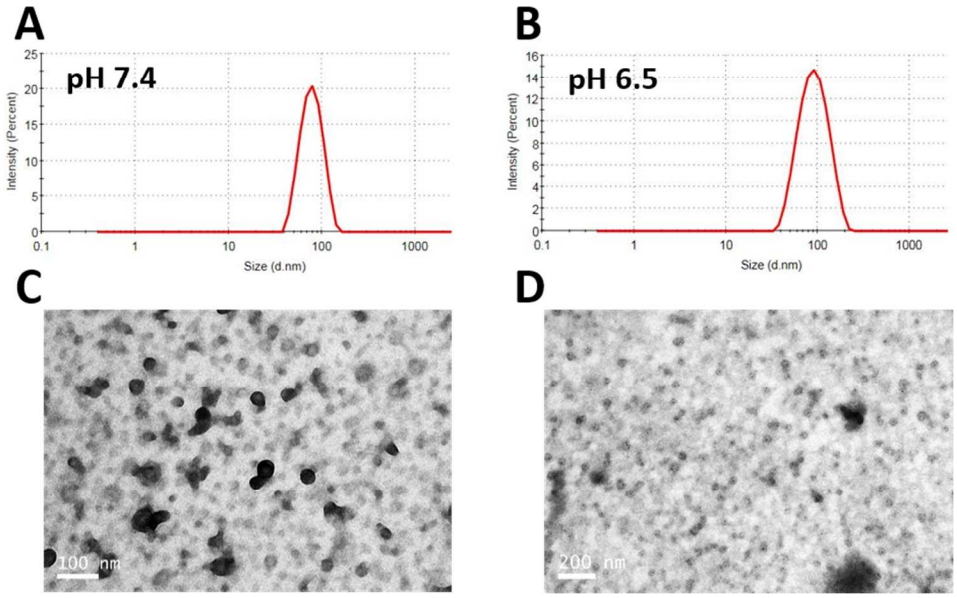


Figure S9. Size characterization of nude PPC8/PTX nanoparticle by DLS (A/B) and TEM (C/D) with sample staining by phosphomolybdic acid. A/C. PPC8/PTX nanoparticle at pH 7.4. B/D. PPC8/PTX nanoparticle at pH 6.5.

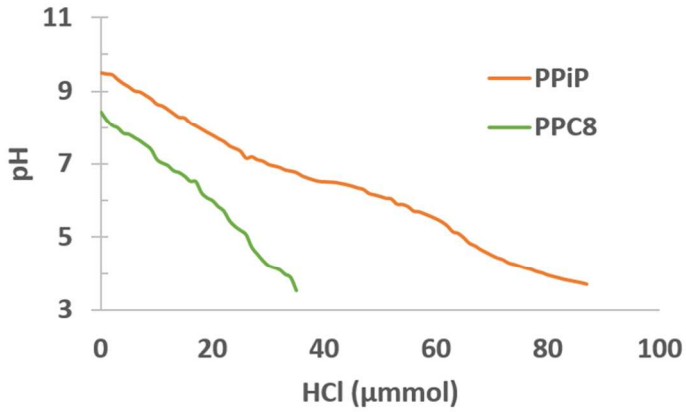


Figure S10. Titration curve of polymer PPiP and PPC8.

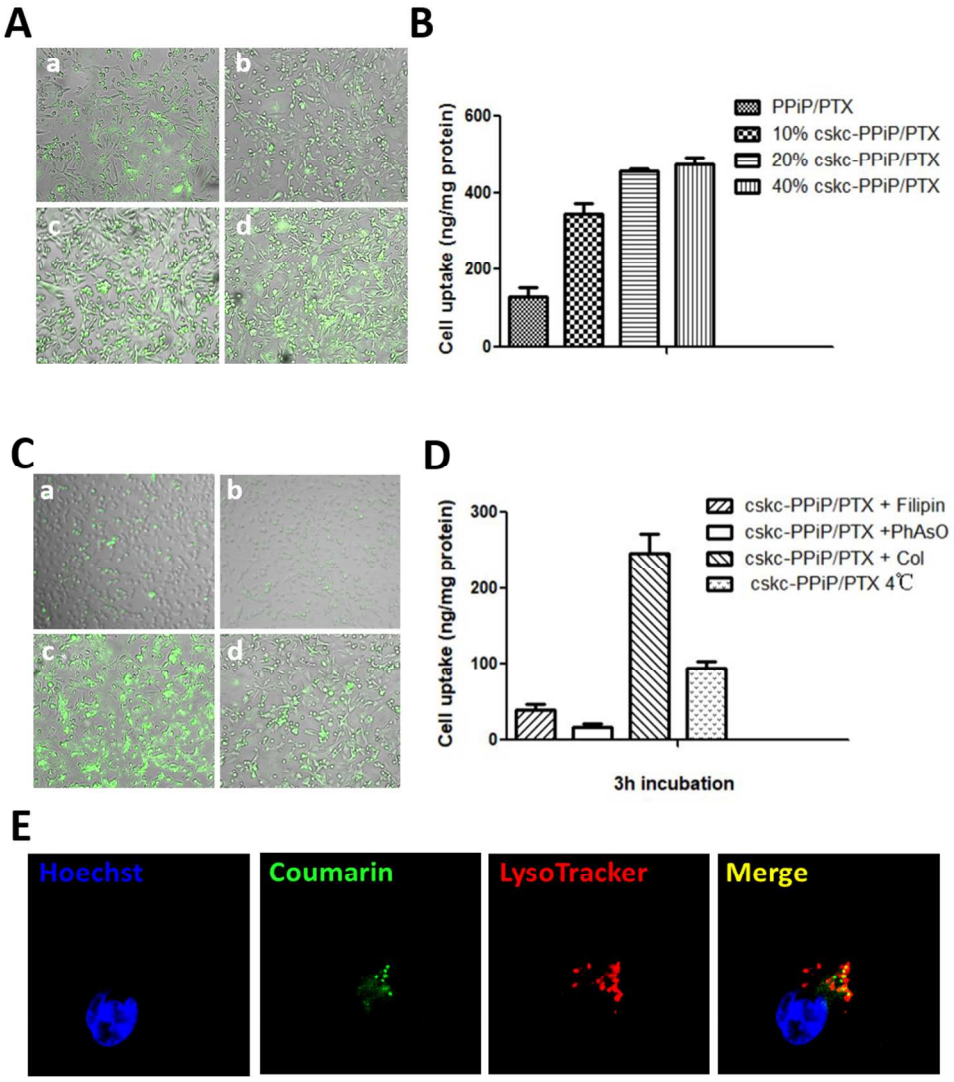


Figure S11. Screen of optimized targeting ligand modification rate and cell uptake mechanism of internal nanoparticle. A. Cells treated with coumarin loaded cskc-PPiP nanoparticles with 0% (a), 10% (b), 20% (c), 40% (d) of cskc-PPiP polymer involved in the particle formulating process. B. Quantitative PTX uptake of cells treated with PTX loaded cskc-PPiP nanoparticles with 0%, 10%, 20% and 40% modification rate. C. Fluorescence photographs of csck-PPiP/coumarin uptake by MDA-MB-231 cells treated with filipin complex (a), phenylarsine oxide (PhAsO, b), colchicine (col, c) and ice bath (4 □, d) respectively. D. PTX quantification of csck-PPiP/PTX uptake by MDA-MB-231 cells treated with filipin complex, phenylarsine oxide, colchicine and ice bath. E. Confocal images of MDA-MB-231 cells uptake csck-PPiP/coumarin with Hoechst 33342 stained nucleus and LysoTracker stained late endosome/lysosome.

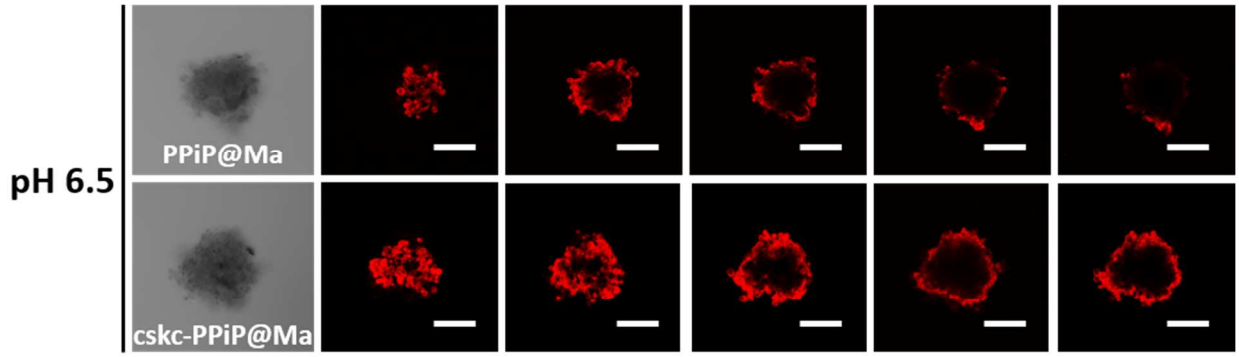


Figure S12. A. Tumor penetration on tumor spheroids at pH 6.5 treated with PPiP@Ma (top) and cskc-PPiP@Ma (bottom) for 45min. B. 3D reconstruction of general signals of above mentioned two groups, scale bar 100 μm, z-axis depth 20μm.

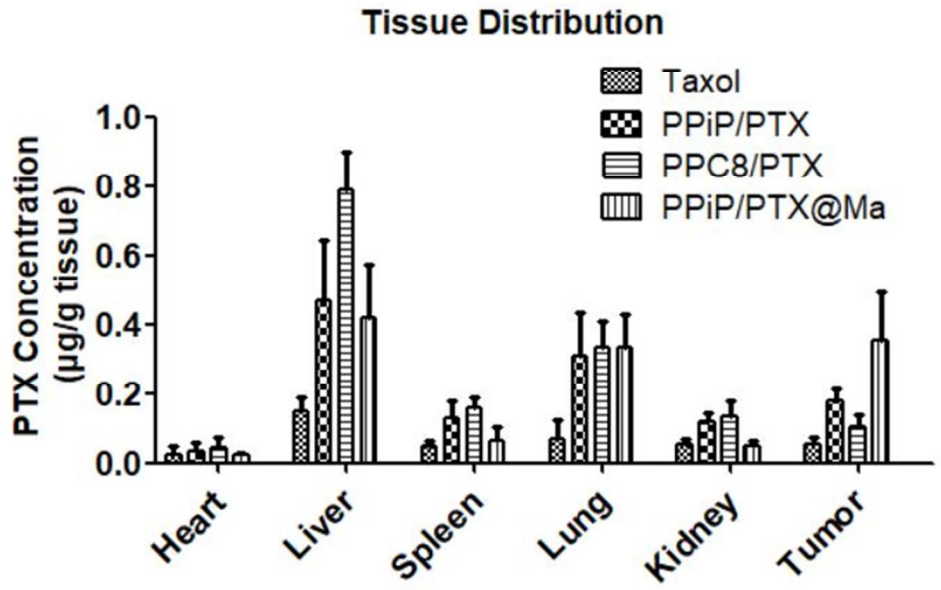


Figure S13. Quantitative biodistribution data of PPC8/PTX, PPiP/PTX and PPiP/PTX@Ma.

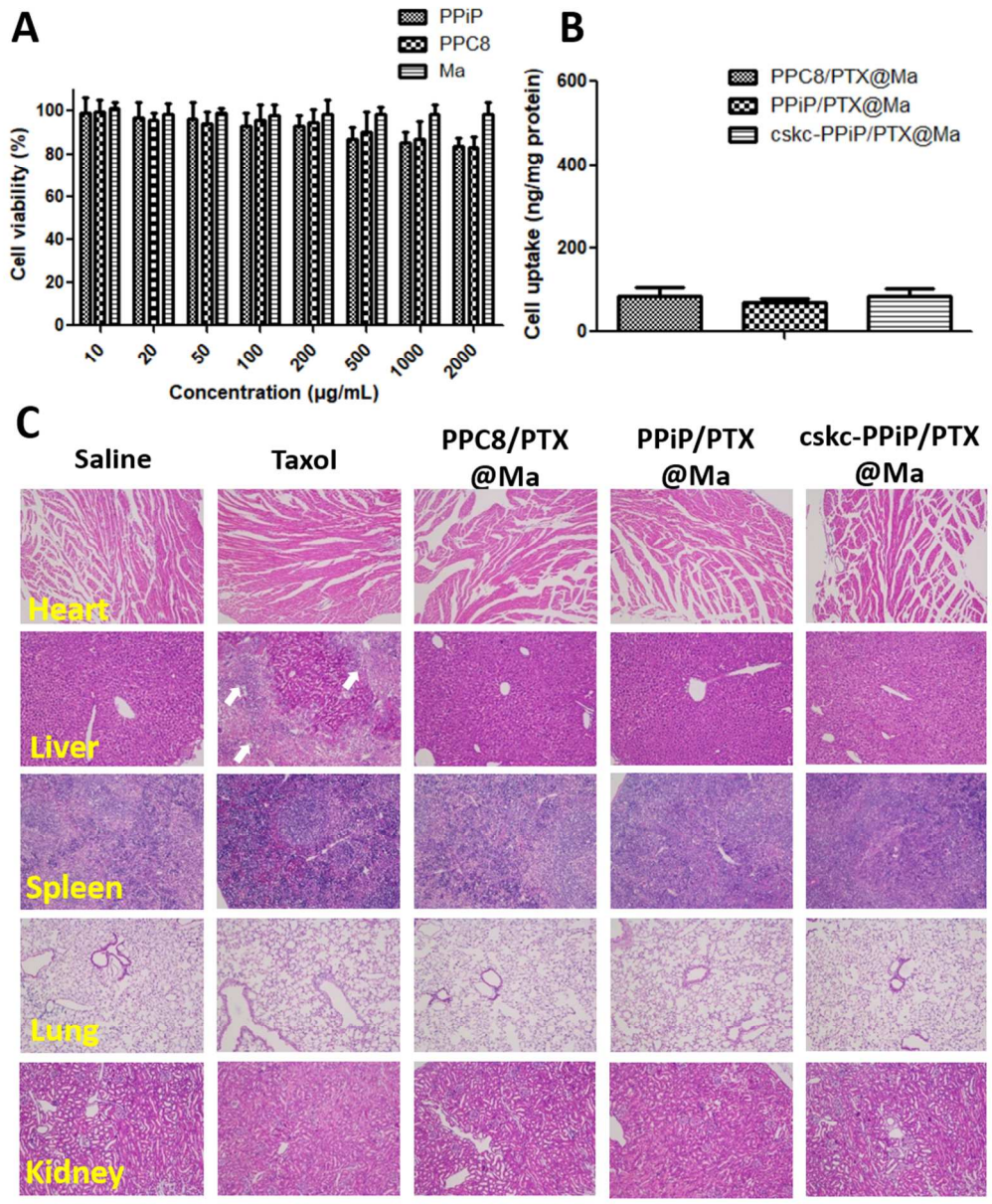


Figure S14. Materials safety study. A. Cell viability of 293 cells after incubation with PPiP, PPC8 and macrophage membrane at designated concentrations. B. Quantitative uptake of PTX in 293 cells incubated with PPC8/PTX@Ma, PPiP/PTX@Ma and cskc-PPiP/PTX@Ma. C. H&E staining images of organ sections excised from mice treated with multi dose saline, Taxol, PPC8/PTX@Ma, PPiP/PTX@Ma, and cskc-PPiP/PTX@Ma, magnification scale 100 ×.

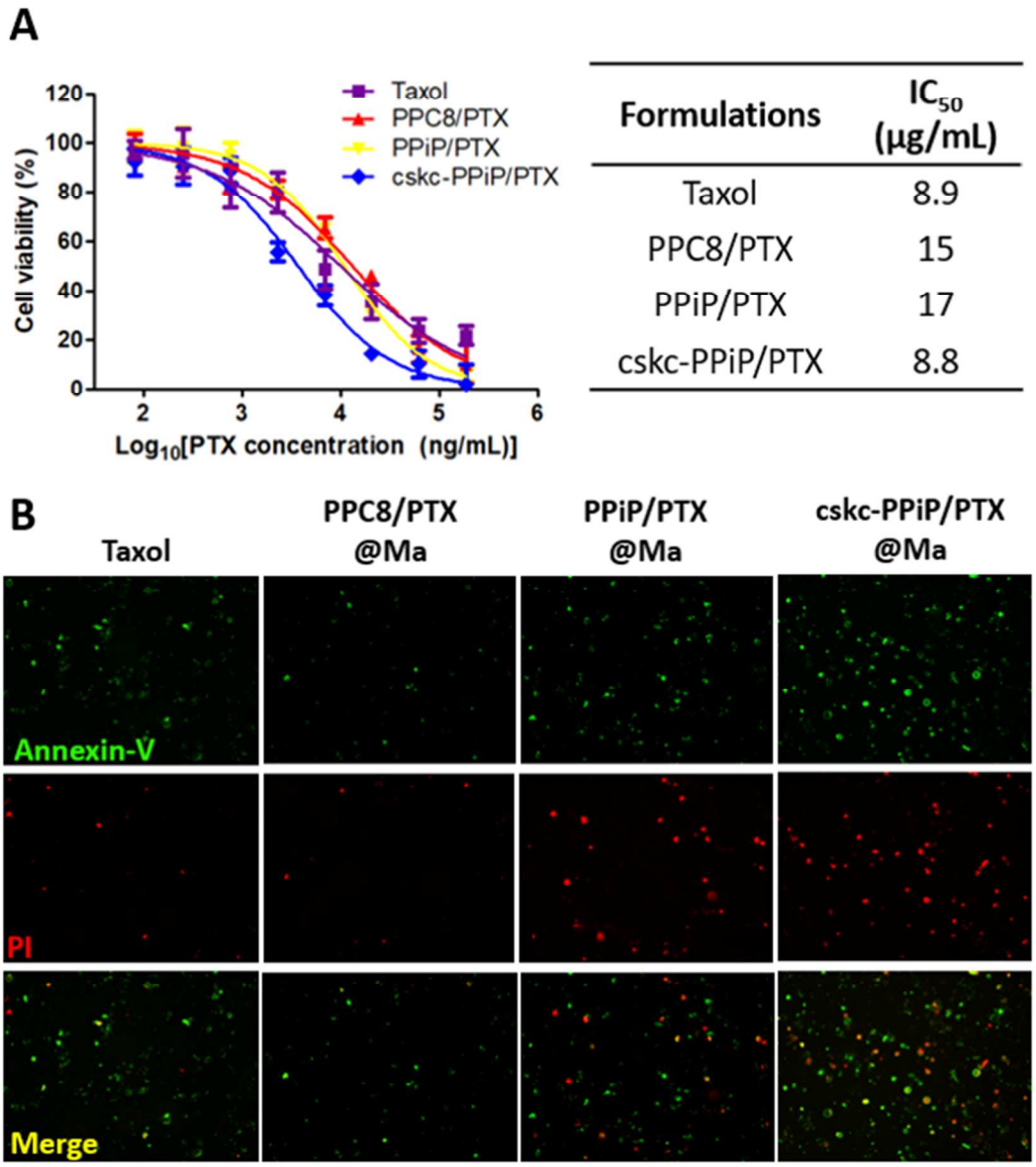


Figure S15. *In vitro* antitumor efficacy study. A. Cell viability of MDA-MB-231 cells incubated with Taxol, PPC8/PTX, PPiP/PTX, and cskc-PPiP/PTX. IC_{50} values were calculated and presented by side. B. Cell apoptosis test of MDA-MB-231 cells treated with Taxol, PPC8/PTX@Ma, PPiP/PTX@Ma, and cskc-PPiP/PTX@Ma. Single Annexin-V (green signals) marked early apoptosis. Dual-stained cells with PI (red signals) marked late apoptosis. magnification scale 200 ×.

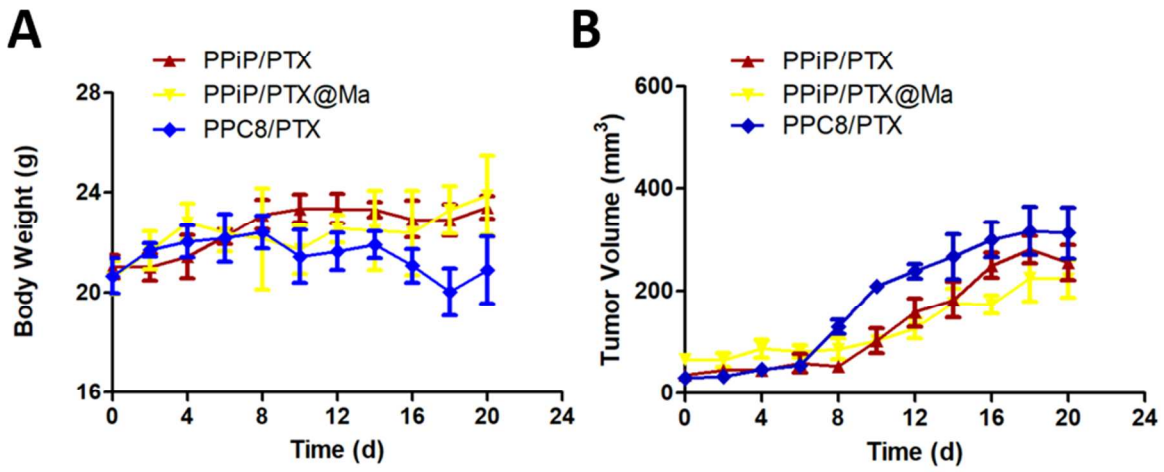


Figure S16. *In vivo* antitumor efficacy study. Body weight (A.) and tumor volume (B.) data of PPiP/PTX, cskc-PPiP/PTX and PPC8/PTX treated mice recorded every other day during 3 weeks treatment course.

Reference

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