Supporting Information

Polyphosphoestered Nanomedicines with Tunable Surface Hydrophilicity for Cancer Drug Delivery

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Materials and Methods

Materials

Docetaxel (DTX), 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and stannous octoate ($Sn(Oct)_2$) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) dye was purchased from Aladdin (Shanghai, China). 1, 1'-dioctadecyl-3,3,3',3'- tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) and 1,1'-dioctadecyltetramethyl indotricarbocyanine Iodide (DiR) dyes were purchased from Lumifore Biotech (Hefei, Anhui, China). Caprolactone (CL) was purchased from Acros Organics (Geel, Belgium), and was treated with calcium hydride and distilled prior to use. Polycaprolactone (PCL) was synthesized following previous work[1], and the structure was proved by ^{1}HNMR. Methanol, ethanol propanol, 2-methoxyethanol, Triethylamine, Tetrahydrofuran (THF), and Benzoic alcohol were purchased from Sinopharm Chemical Reagent (Shanghai, China). Methanol, ethanol propanol, and 2-methoxyethanol were distilled before use. Triethylamine was firstly refluxed with phthalic anhydride and then mixed with calcium hydride and distilled before use. THF was refluxed over potassium-sodium alloy under N_2 atmosphere and distilled before use. Benzoic alcohol was dried by stirring with calcium hydride overnight and distilled before use. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was purchased from Sangon Biotech (Shanghai, China). Other chemicals and reagents were of analytical grade and used as received, unless noted otherwise.

Characterization.

^{1}H NMR spectra were recorded on a Bruker AV300 NMR spectrometer (Bruker, Switzerland) using deuterated chloroform ($CDCl_3$) containing 0.03% tetramethylsilane (TMS) as the solvent. Phosphoric acid (85%) was used as an external reference for ^{31}P NMR analyses. Gel permeation chromatography (GPC) measurements on a waters GPC system (Waters, Milford, MA), which was equipped with a Waters 1515 HPLC solvent pump, a Waters 2414 refractive index detector, and four Waters styragel high-resolution columns (HR4, HR2, HR1, and HR0.5 with effective molecular weight ranges 5000−600000, 500−20000, 100−5000, and 0−1000, respectively). UV-Vis absorption spectra were recorded on a Agilent Cary 60 UV-Vis-NIR spectrophotometer (Agilent, Palo Alto, CA) with a quartzcuvette of 1 cm optical path length. Fluorescence spectra were recorded by F-4600 fluorescence spectrophotometer (Hitachi, Japan). The size and surface charge of micelles was detected by a Malvern Zeta sizer Nano ZS90 dynamic light scattering system (DLS, Worcestershire, UK) with a He-Ne (633 nm) and 90^{o} collecting optics. The data was analyzed by Malvern Dispersion Technology Software 7.0.2. The morphology of micelles was observed on a JEOL 2010 high-resolution cryogenic transmission electron microscope (Cryo-TEM) with an accelerating voltage of 200 kV.

Synthesis of MOEEP, MEP, EEP and PEP monomers

MOEEP and EEP were synthesized following the previous works[2]. MEP was synthesized by following procedures: Methanol (6.21 g, 201.5 mmol) and triethylamine

(20.40 g, 201.6 mmol) were dissolved in 100 mL THF and cooled to 0 °C. 2-chloro-2-oxo-1,3,2-dioxaphospholane (28.61 g, 201.3 mmol) in 100 mL THF was added dropwise with fast stirring. The mixture reacted for 12 h at -5 °C, and the precipitation was filtered off using a Schlenk technique in N_2. The filtrate was condensed and distilled twice under vacuum to acquire the transparent colorless product (bp 102 °C, 20 Pa), yield 72%. The yield of MEP, EEP and PEP were 42%, 77% and 82%, respectively.

Calculated logP (ClogP)

Clop values were obtained from ChemBioDraw software (version 19.0, Cambridgesoft, PerkinElmer, Waltham).

Synthesis of PMOEEP, PMEP, and PEEP homoplolymers

The reactions were performed at 25 °C in a glove box with water and oxygen percentage less than 0.1 ppm. Typically, initiator benzoic alcohol (0.014 g, 135 μmol in 100 μL THF) was added to the of EEP (1.99 g, 13.1 mmol) THF (10.07 g) solution. Then catalyst $Sn(Oct)_2$ (0.057 g, 135 μmol in 100 μL THF) was added. After 1 h, the reaction was deactivated with 20-fold benzoic acid. The resultant solution was concentrated and precipitated into excess methanol/diethyl ether (1:20, v/v). The precipitate was dried under vacuum overnight at room temperature (yield 63%). PMEP and PMOEEP homopolymers were synthesized following a similar protocol.

Synthesis of PCL-*b*-PMOEEP, PCL-*b*-PMEP, PCL-*b*-PEEP, and PCL-*b*-PPEP

block copolymers.

The reactions were performed at 25 °C in a glove box with water and oxygen percentage less than 0.1 ppm. Typically, the catalyst $Sn(Oct)_2$ (0.06 g, 0.14 mmol) was added into THF solution containing PCL (0.8 g, 0.14 mmol) and monomers of MOEEP (2.18 g, 11.9 mmol). After 2 h, the reaction was terminated. The products were condensed and then precipitated in diethyl ether/ methanol (20/1, v/v) for three times. The precipitate was dried under vacuum overnight at room temperature and analyzed by GPC. PCL-*b*-PMEP, PCL-*b*-PEEP homopolymers and PCL-*b*-PPEP were synthesized following a similar protocol. Yield of four copolymers were 70%, 75%, 65% and 78%, respectively. The monomer conversion of MOEEP, MEP, EEP and PEP in the synthesis of PCL-*b*-PPEs were 77%, 76%, 72% and 81%, respectively.

Cell lines and Animals

Human breast cancer cell line MDA-MB-231 and murine melanoma cell line B16 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured respectively in Dulbecco's modified Eagle's medium (DMEM, Gibco, Eggenstein, Germany) and Roswell Park Memorial Institute medium (RPMI 1640, Gibco) supplemented with 10% fetal bovine serum (FBS, ExCell Bio, Shanghai, China) and 1% penicillin-streptomycin (Gibco) at 37 ^{o}C under a 5% CO_2 atmosphere. The MDA-MB-231 cells with green fluorescent protein (GFP) expression were obtained by transfection with a retro-virus according to a standard protocol as described previously[3].

BALB/c nude mice, ICR mice and C57BL/6 mice were purchased from Beijing

HFK Bioscience (Beijing, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of the Chinese Animal Care and Use Committee.

Preparation and Characterization of Nanoparticles

Polymers of PCL-*b*-PPE (10 mg) in 1 mL dimethyl formamide (DMF) were added into a round bottom flask. Then three times volumes of Milli-Q ultrapure water (Millipore, 18.2 MΩ, Bedford, MA) was added under vigorous stir. After 10 min, the mixture was dialyzed against ultrapure water in a dialysis bag (molecular cutoff = 14,000 Da) overnight to remove DMF. Then the mixture was centrifuged at 3,000 g for 10 min to remove aggregates. For fluorescence imaging experiment, DiO, DiD or DiR fluorescent dye (0.4 wt %) was encapsulated into nanoparticles during preparation. For anti-tumor experiment, docetaxel (10 wt%) was encapsulated into nanoparticles during preparation.

MST Assay

FITC-conjugated BSA (BSA-FITC) and homopolymers were dissolved in 0.01 M PBS at concentrations of 100 nM and 100 μM, respectively. The homopolymers were respectively diluted into a series of concentrations by PBS and mixed with the same concentration of BSA solution. Afterwards, the mixture were filled into capillaries and detected by MST (Monolith, Kirkland, WA) with 40% MST power and 40% LED power. The data was analyzed by NT Analysis Software 1.5.41.

Protein Absorption Assay

Nanoparticles were incubated in ultrapure water containing 20% volume of FBS at 37 °C for 1 h. Then nanoparticles were collected by centrifugation at 12,000 g for 1.5 h and washed for three times with cold ultrapure water. Adsorbed proteins in nanoparticles were separated by adding 0.1% SDS solution and quantified by BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA).

Cellular Uptake of Nanoparticles *in vitro*

For flow cytometric analysis, MDA-MB-231 cells (1×10^5 cells per well) were seeded in 24-well plates and cultured overnight. After culture, the medium was replaced by fresh medium containing DiO-labeled nanoparticles at an equivalent dose. At predetermined time points, the cells were collected for flow cytometric analysis using BD FACSVerse™ flow cytometer (BD Biosciences, San Jose, CA).

For confocal laser scanning microscope (CLSM, LSM 710, Carl Zeiss, Jena, Germany) observation, MDA-MB-231 cells (8×10^4 cells per well) were seeded in 24-well plate containing round slides in each well and cultured overnight. After culture, the medium was replaced by fresh medium containing DiO-labeled nanoparticles at an equivalent dose. After 4 hours, the cells in round slides were washed three times with cold PBS and then fixed with 4% paraformaldehyde for 10 min at room temperature. Afterwards, the cytoskeleton and cell nucleus were stained by Alexa fluor 568 phalloidin (Invitrogen, Carlsbad, CA) and DAPI, respectively. The slides were observed by CLSM.

Pharmacokinetic Studies

For analyzing the pharmacokinetics of n-PMOEEP, n-PMEP and n-PEEP, female ICR mice (8 weeks) were placed on the microscope stage and anesthetized by 2% isoflurane. The ear of mice was attached onto a coverslip with a plastic tape. DiD-labeled nanoparticles were *i.v.* injected into the mice and then the blood vessel in the skin of ear was visualized by CLSM in real-time.

For analyzing the tumor accumulation of hybrid nanoparticles, DiR-labeled nanoparticles were *i.v.* injected into female ICR mice (8 weeks) at an equivalent dose of 5 μg DiR per mouse (n = 3). At predetermined time points, the blood was collected into anticoagulant tubes and centrifuged for 10 min at 3,000 g to collect plasma. The fluorescence intensity in the plasma was detected by IVIS Spectrum (Perkin Elmer Health Sciences Pvt. Ltd., Waltham, MA).

Tumor Accumulation

For analyzing the tumor accumulation of nanoparticles, BALB/c nude mice were subcutaneously inoculated with MDA-MB-231 cells (5×10^6 cells per mouse) at the fat pad of second nipple. When tumor volume reach about 200 mm^3, DiR-labeled nanoparticles were *i.v.* injected at an equivalent dose of 10 μg DiR per mouse. At predetermined time points, the mice were imaged with IVIS Spectrum.

In *vitro* DTX Release.

DTX-loaded nanoparticles were added into dialysis bags (molecular cutoff = 14,000 Da) in tubes with 20 mL PBS solution containing 0.1% Tween-80. The tubes were placed

at 37 °C and shaken at 120 rpm for 48 h. At predetermined time points, the solution in tubes was collected and analyzed by fluorescence spectrophotometer.

Cytotoxicity of DTX-loaded Determined by MTT Assay

MDA-MB-231 cells were seeded in a 96-well plate at 5×10^3 cells per well in 100 μL medium for 24 h and treated with different concentrations of free DTX, n-PMOEEP@DTX, n-PMEP@DTX and n-PEEP@DTX. After 72 h incubation, MTT was added into the wells at a final concentration of 1 mg/mL. After another 2 h incubation, medium was carefully removed and then 100 μL DMSO was added. The plates were measured at 490 nm using an ELx800™ absorbance microplate reader (BioTek Instruments, Winooski, VT, USA). Cell viabilities were normalized to that of cells cultured in medium.

MDA-MB-231 Tumor Growth Inhibition

MDA-MB-231 tumor-bearing BALB/c nude mice were used for anti-tumor experiment. When the volume of MDA-MB-231 tumor reached about 80 mm^3, the mice received *i.v.* injection with DTX-loaded nanoparticles or free DTX at an equivalent dose of DTX (1 or 2 mg/kg body weight) for every two days. Tumor volumes of mice were monitored during treatment and the tumor volume was calculated as 0.5×length×width×width.

Tumor Cellular Uptake *in vivo*

BALB/c nude mice were subcutaneously inoculated with GFP-expressing MDA-

MB-231 (5×10^6 cells per mouse) at the fat pad of the second nipple. When the tumor reached approximately 80 mm^3, the mice received *i.v.* injection with DiD-labeled nanoparticles (n = 3). Mice were sacrificed at 12 h post-injection and then tumors were collected and digested with 1% collagenase I (Sigma-Aldrich, Saint Louis, MO). Flow cytometric analysis the uptake of nanoparticles in GFP-expressing cells.

Anti-metastasis Efficacy

C57BL/6 (7 week) were *i.v.* injected with 2×10^5 B16 cells per mouse suspended in 100 μL PBS. Then the mice were randomly divided into four groups (n = 13, ten mice for survival analysis and three for lung metastasis evaluation): PBS, free DTX, n-PMOEEP@DTX and n-PMEP@DTX. The mice received *i.v.* injection of above formulations every two days at an equivalent dose of DTX (3 mg/kg body weight). The body weight and survival of mice were monitored daily. At day 20 of treatment, the three mice in each group were sacrificed, and their lungs were excised for evaluating the lung metastatic niches.

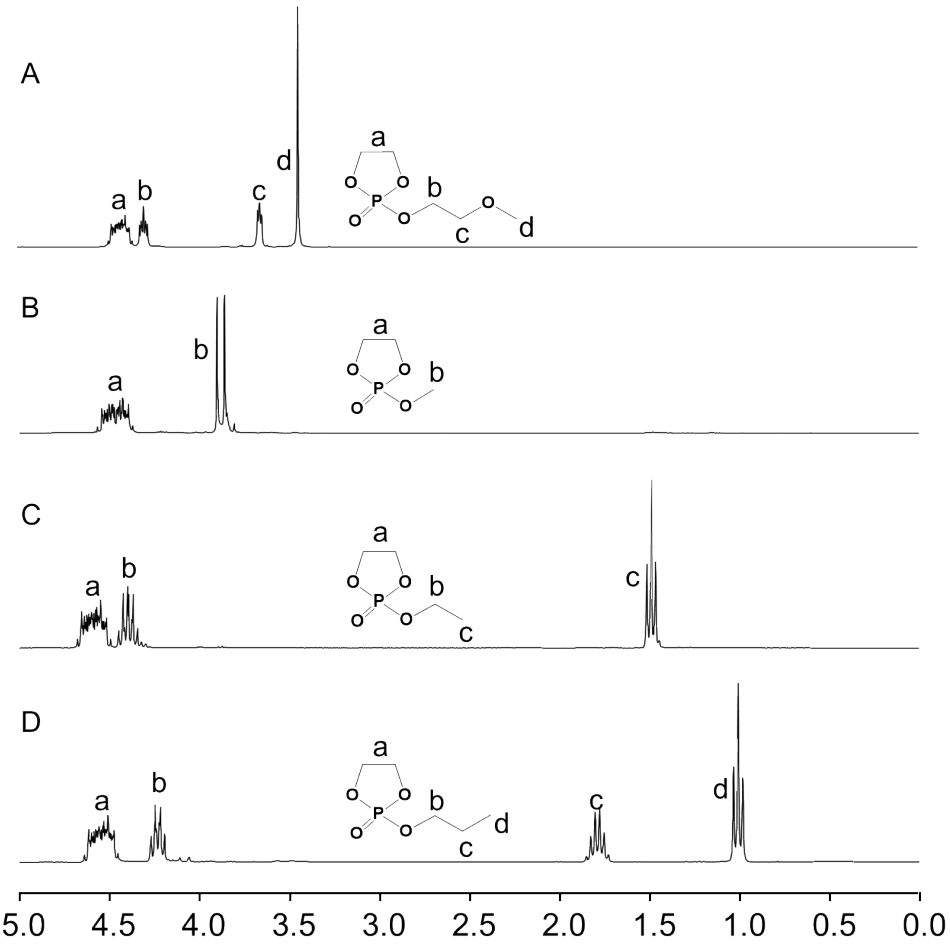


Figure S1. ^{1}H NMR spectra of (A) MOEEP, (B) MEP, (C) EEP, and (D) PEP monomers at room temperature 25 ℃ in $CDCl_3$ (300 MHz).

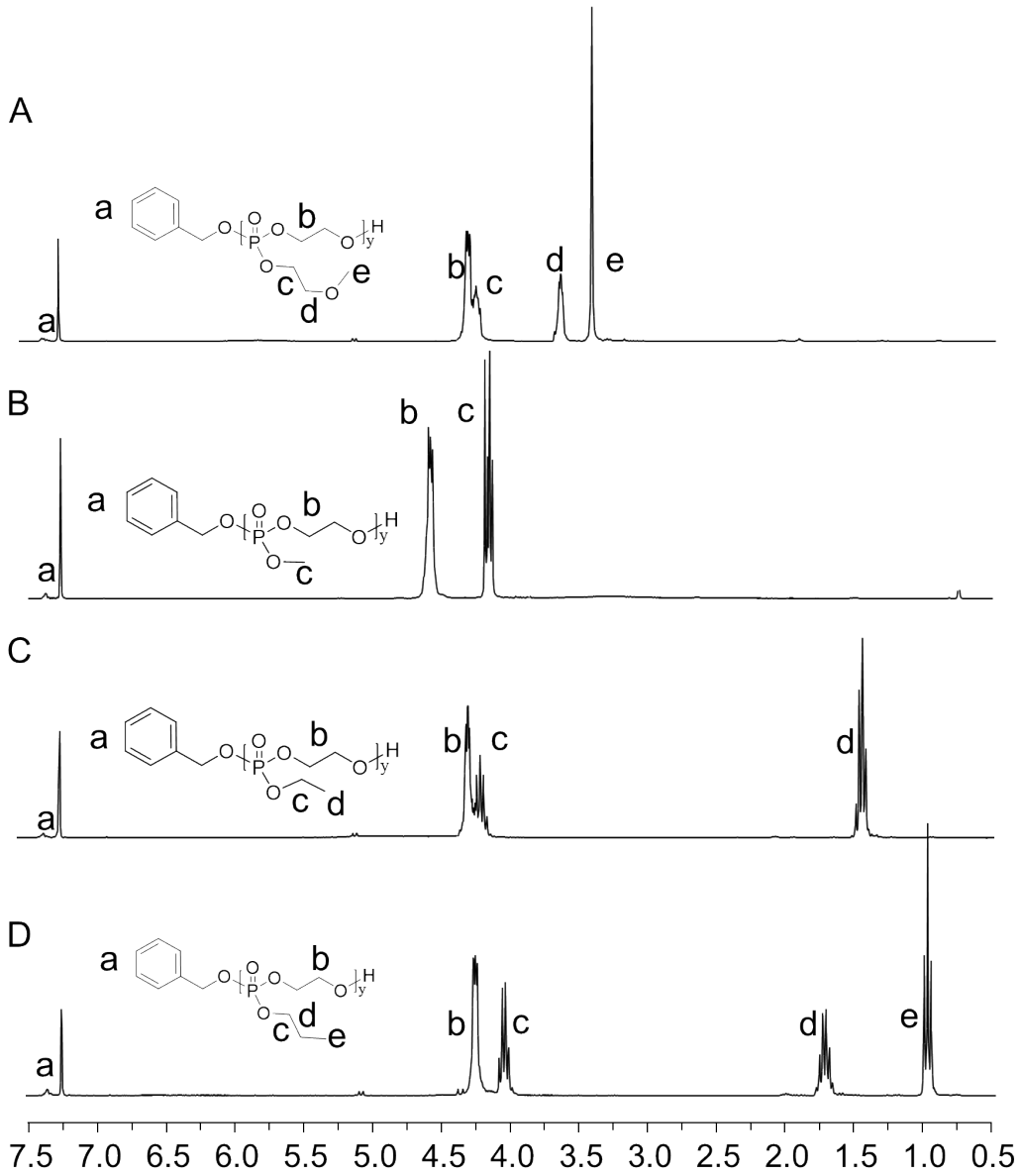


Figure S2. ^{1}H NMR spectra of (A) PMOEEP, (B) PMEP, (C) PEEP and (D) PPEP homopolymers used for MST assay at 25 ℃ in $CDCl_3$ (300 MHz).

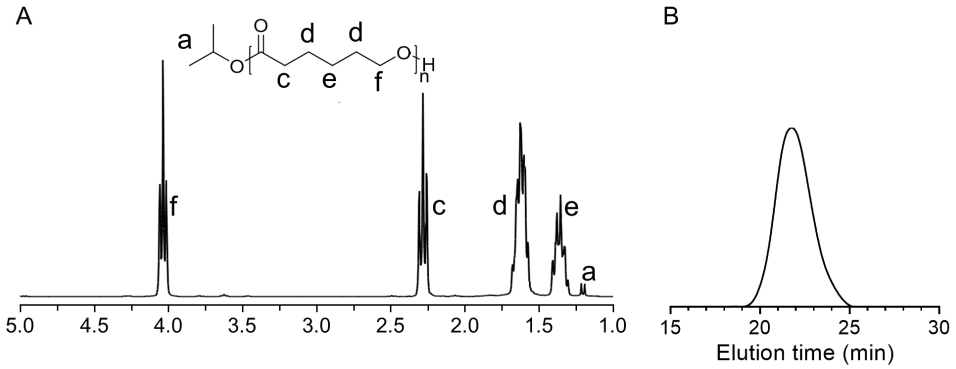


Figure S3. (A) ^{1}H NMR spectra of PCL_{40} at 25 ℃ in $CDCl_3$ (300 MHz). (B) GPC trace of PCL. GPC analysis was measured using THF as solvent.

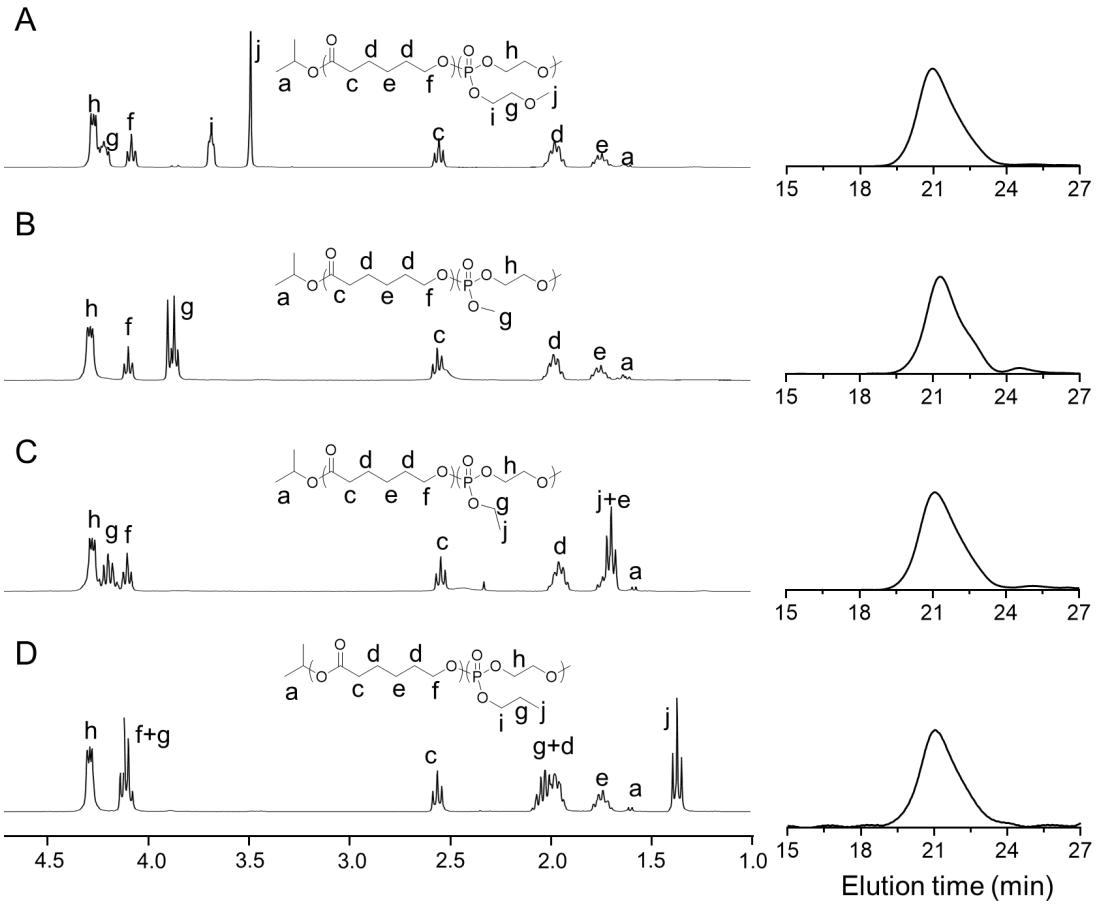


Figure S4. ^{1}H NMR spectra (left part) and GPC trace (right part) of PCL-*b*-PPs, (A) PCL_{40}-*b*-$PMOEEP_{65}$, (B) PCL_{40}-*b*-$PMEP_{65}$, (C) PCL_{40}-*b*-$PEEP_{58}$ and (D) PCL_{40}-*b*-$PPEP_{55}$) used for nanoparticles preparation at room temperature in $CDCl_3$ (300 MHz). GPC analysis was measured using THF as solvent.

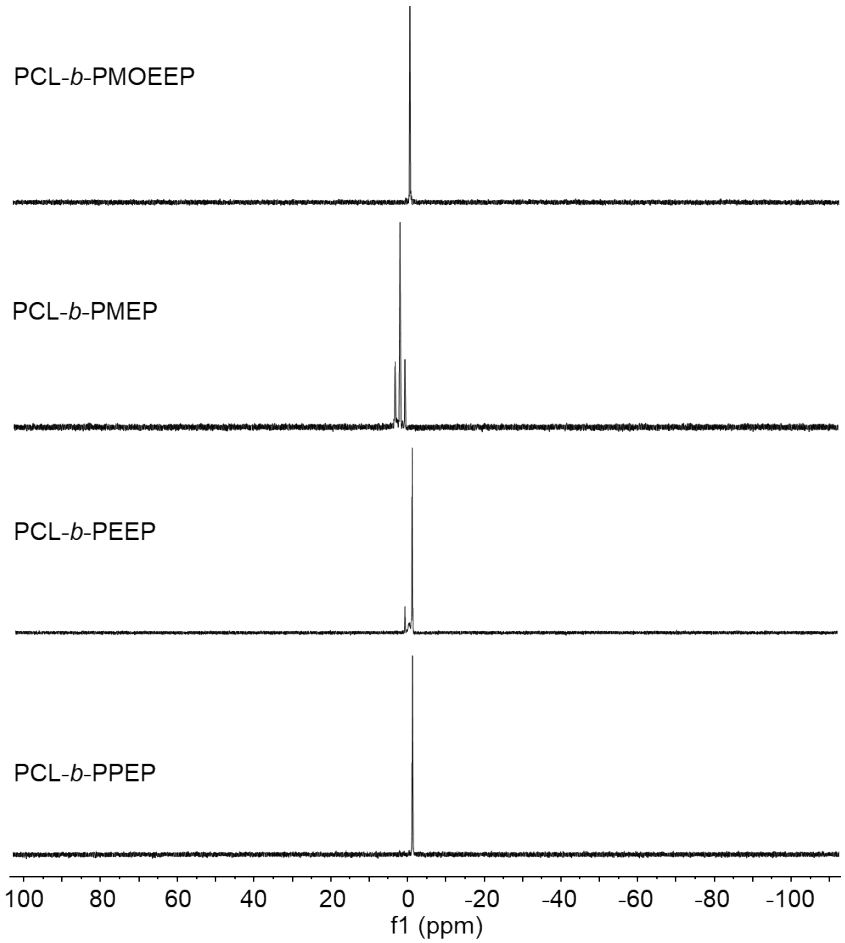


Figure S5. ^{31}P NMR spectra of PCL-*b*-PPs used for nanoparticles preparation at room temperature in $CDCl_3$ (121 MHz). Phosphoric acid (85%) was used as an external reference for ^{31}P NMR analyses.

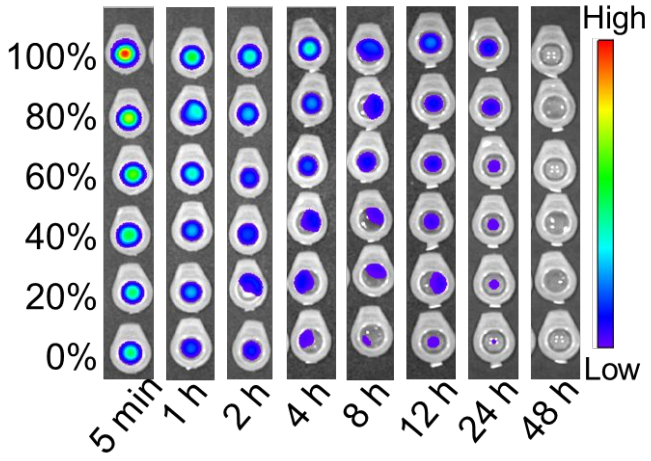


Figure S6. Fluorescent images of plasma samples collected from mice at different time points after *i.v.* injection of DiR-labeled hybrid nanoparticles.

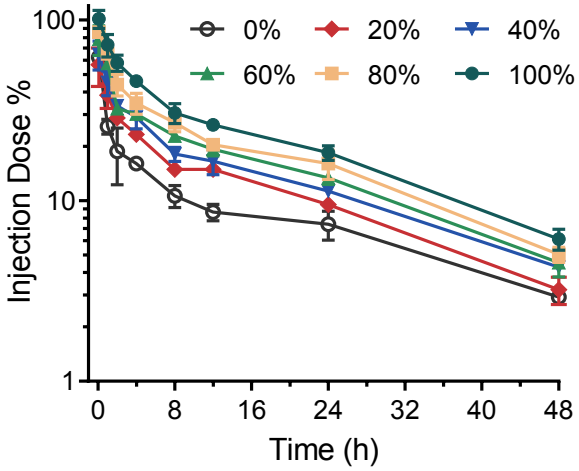


Figure S7. Corresponding quantification of average fluorescence intensity of DiR in the blood.

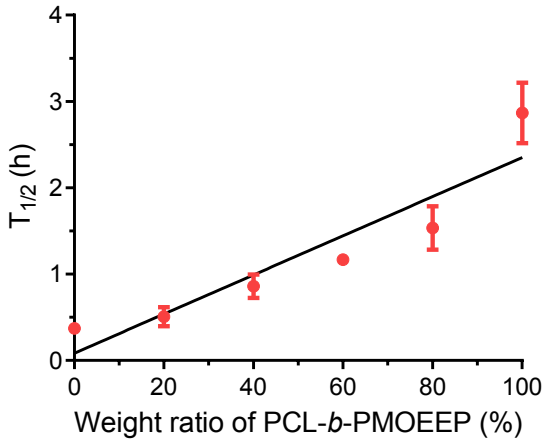


Figure S8. Scatter plot showing the correlation between half-life ($t_{1/2}$) of hybrid nanoparticles and weight ratio of PCL-*b*-PMOEEP in them. The $t_{1/2}$ of hybrid nanoparticles was analyzed in a non-compartment model.

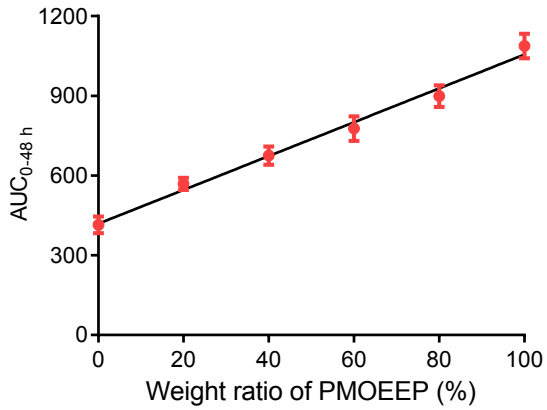


Figure S9. Area under curve (AUC) of hybrid nanoparticles. Data are shown as mean ± SD (n = 3).

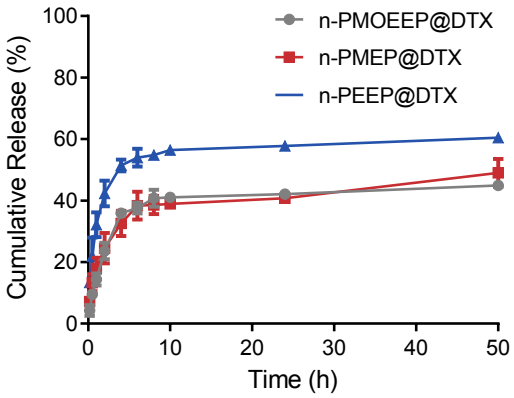
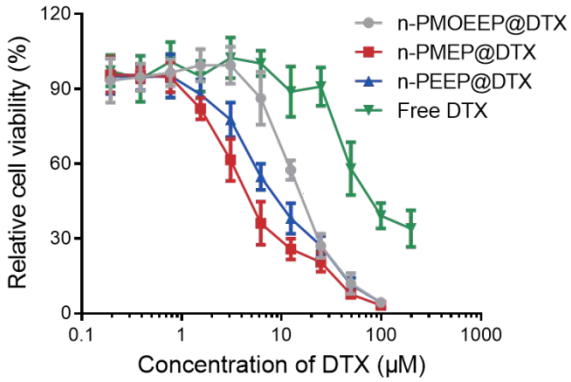


Figure S10. Drug release profiles of n-PMOEEP@DTX, n-PMEP@DTX, and n-PEEP@DTX.



	IC_{50} (μM)
n-PMOEEP@DTX	26.28±9.42
n-PMEP@DTX	4.484±1.032
n-PEEP@DTX	9.591±2.150
Free DTX@DTX	116.2±65.17

Figure S11. Analyze the variability of MDA-MB-231 tumor cells treated with n-PMOEEP@DTX, n-PMEP@DTX, n-PEEP@DTX and free DTX for 48 h by MTT method (n = 5).

References:

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(3) Wang, H. X.; Zuo, Z. Q.; Du, J. Z.; Wang, Y. C.; Ye, X. D.; Wang, J. L.; Leong, K. W.; Wang, J., Surface Charge Critically Affects Tumor Penetration and Therapeutic Efficacy of Cancer Nanomedicines. Nano Today 2016, 11, 133-144.