Supporting Information

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SI Materials and Methods

Materials. Doxo·HCl was purchased from Bosche Scientific. D,L-lactide (LA) was purchased from TCI America. (*S*)-2,2′,2′′,2′′′-(2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid (*p*-SCN-Bn-DOTA) was purchased from Macrocyclics, Inc. All of the other chemicals were purchased from Sigma-Aldrich. Anhydrous THF, DMF, and dichloromethane (DCM) were purified by alumina columns and kept anhydrous by using molecular sieves. D-luciferin potassium was purchased from Regis Technologies.

Instrumentation. The molecular weights of PLA were determined by gel permeation chromatography (GPC, also known as size-exclusion chromatography) equipped with an isocratic pump (model 1100; Agilent Technologies), a DAWN HELEOS 18-angle laser light scattering detector, and an Optilab rEX refractive index detector (Wyatt Technology). The wavelength of the HELEOS detector was set at 658 nm. The size-exclusion columns (Phenogel columns 100, 500, 10^3 and 10^4 Å, 5 μm, 300 × 7.8 mm; Phenomenex) used for the analysis of polymer–drug conjugates was serially connected on the GPC. The GPC columns were eluted with DMF (HPLC grade) containing 0.1 M LiBr at 65 °C at 1 mL/min. Data processing was performed with ASTRA V software (version 5.1.7.3; Wyatt Technology). HPLC analyses were performed on a System Gold system equipped with a 126P solvent module and a System Gold 128 UV detector (Beckman Coulter) equipped with a 126P solvent module, a System Gold 128 UV detector, and an analytical C18 column (Luna C18, 250 × 4.6 mm, 5 μ; Phenomenex). The NMR studies were conducted on a Varian UI500NB system (500 MHz). Infrared spectra were recorded on a PerkinElmer 100 serial FTIR spectrophotometer (PerkinElmer). The sizes and the size distributions of the NPs were determined on a ZetaPALS DLS detector (15-mW laser, incident beam 676 nm; Brookhaven Instruments). The ζ-potential of freshly prepared MPs was evaluated by Malvern Zetasizer. Lyophilization of the NPs was carried out on a benchtop lyophilizer (FreeZone 2.5; Fisher Scientific).

Cell Culture. Murine OS K7M2 cells transfected with GFP were provided by Chand Khanna. Murine OS K7M3 cells engineered with firefly luciferase were provided by Su Young Kim, National Cancer Institute, Bethesda. Both types of cells were cultured in DMEM containing 10% FBS and 100 units/mL aqueous penicillin G at 37 °C in 5% CO_2 humidified air.

Animals. Female BABL/c mice (8 wk old) were purchased from the National Cancer Institute , and 6- to 8-wk-old male CD1 Crl:CD1(ICR) mice were purchased from Charles River Laboratory. Feed and water were available for ad libitum consumption. Artificial light was provided in a 12-h/12- h cycle. The median age and weight of pet dogs was 8 y (range 6–11 y) and 40.1 kg (range 21.8–63.3 kg), respectively.

Preparation and Characterization of Pam-Doxo-NPs

Preparation of Doxo–PLA Polymer Conjugate. Doxo–PLA conjugates were synthesized by following procedures similar to those already published (34). In a glovebox, Doxo (5.4 mg, 0.01 mmol) was dissolved in anhydrous THF (500 μL) and mixed with a THF solution (500 μL) containing (BDI-EI)ZnN(TMS)$_2$ [(BDI) is 2-((2,6-diethylphenyl) amido)-4-((2,6-diisopropylphenyl)-imino)-2-pentene] (6.5 mg, 0.01 mmol). The mixture was stirred for 15 min. LA (14.4 mg, 10 eq) was dissolved in THF (500 μL) and added to the stirred (BDI-EI)ZnN(TMS)$_2$ and Doxo mixture. The reaction proceeded in the glovebox overnight. After LA was completely consumed, the reaction was stopped by quenching the polymerization solution with cold methanol solution (30 μL). The polymer was precipitated with ether (10 mL), collected by centrifugation, and dried by vacuum.

Synthesis of PLA-PEG-COOH Conjugate. A diblock polymer of PLA-PEG-COOH was synthesized by following similar procedures as described above by using HO-PEG-COOH (M_w of PEG: 5k Da) as the initiator. In a glovebox, HO-PEG-COOH (50 mg, 0.01 mmol) solution in anhydrous DCM (800 μL) was mixed with a DCM solution (500 μL) containing (BDI-EI)ZnN(TMS)$_2$ (6.5 mg, 0.01 mmol). The mixture was stirred for 15 min. LA (144.1 mg, 100 eq) was dissolved in DCM at 50 mg/mL concentration and added to the stirred solution of HO-PEG-COOH and (BDI-EI)ZnN(TMS)$_2$. The reaction proceeded in the glovebox overnight. After LA was completely consumed, the reaction was stopped by quenching the polymerization reaction with cold methanol solution (300 μL). The polymer was precipitated with ether (50 mL), collected by centrifugation, and dried by vacuum.

Synthesis of PLA-PEG-Pam Conjugate. To a DMF solution (2 mL) of PLA_{100}-PEG_{5k}-COOH (40.0 mg, 2.1 μmol) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (1.0 mg, 6.4 μmol) and NHS (0.8 mg, 6.9 μmol). After the solution was stirred at room temperature for 30 min, Pam (1.0 mg, 4.3 μmol) was added and the mixture was stirred overnight. The polymer conjugate was precipitated with ether (12 mL), collected by centrifugation, and dried by vacuum. The resulting polymer conjugate is characterized by ^{1}H NMR (Fig. S1).

Synthesis of PLA-PEG Diblock Polymer. A diblock polymer of PLA-PEG was synthesized by following similar procedures as described above by using mPEG-OH (M_w of PEG: 5k Da) as the initiator. In a glovebox, mPEG-OH (50 mg, 0.01 mmol) solution in anhydrous DCM (800 μL) was mixed with a DCM solution (500 μL) containing (BDI-EI)ZnN(TMS)$_2$ (6.5 mg, 0.01 mmol). The mixture was stirred for 15 min. LA (144.1 mg, 100 equiv) was dissolved in DCM at 50 mg/mL concentration and added to the stirred solution of mPEG-OH and (BDI-EI)ZnN(TMS)$_2$. The reaction proceeded in the glovebox overnight. After LA was completely consumed, the reaction was stopped by quenching the polymerization reaction with cold methanol solution (300 μL). The polymer was precipitated with ether (50 mL), collected by centrifugation, and dried by vacuum.

DLS Measurements. The hydrodynamic size was measured with 90Plus Particle Size Analyzer by dispersing the NPs in DI water at concentration of 0.5 mg/mL. The freshly prepared NPs were dispersed in DI water to a concentration of 0.5 mg/mL.

Release Kinetic Study of Pam-Doxo-NPs. Pam-Doxo-NPs were prepared by nanoprecipitation as previously described. The collected NP solutions (50 μg/mL, 320 μL) were mixed with PBS buffer (pH 7.4) or PBS-citrate buffer (pH 5.0) (480 μL) in a dialysis tube then incubated with 19.2 mL buffer solution in a scintillation vial in the dark at 37 °C. At scheduled time points, 2 mL of the solution was taken out for fluorescence measurements (λ_{ex} = 478 nm, λ_{em} = 595 nm) and then returned back to the vial. The Doxo concentration was quantified by standard curve measured under the same condition.

Stability Study of Pam-Doxo-NPs in FBS (50%). Pam-Doxo-NPs were prepared by nanoprecipitation as previously described. The obtained NPs were dispersed in FBS buffer (FBS:PBS 1:1, vol/vol). The particle sizes were measured by DLS.

Lyophilization of Pam-Doxo-NPs with Human Serum Albumin. Pam-Doxo-NPs (50%) were prepared by nanoprecipitation as previously described. Human serum albumin (HSA) was then added into the NP solution. The mixture was lyophilized for 16 h at −50 °C to obtain a white powder. The white powder was reconstituted in nanopure water (2 mL) and the solution was stirred for 5 min at room temperature. The resulting NP solution was analyzed by DLS to assess NP size and size distribution.

Pharmacokinetics Evaluation. Nontargeted Ctrl-NPs and Pam-NPs were radiolabeled by ^{64}Cu (denoted as ^{64}Cu-NPs and Pam-^{64}Cu-NPs) and purified for pharmacokinetics study. Nine male CD1 Crl:CD1(ICR) mice (6–8 wk old) were divided into three groups ($n = 3$), minimizing the weight differences. The three groups of mice were treated with ^{64}Cu-labeled Pam-NPs (10% and 50%) and nontargeted Ctrl-NPs. Blood was collected from mice facial veins or tail veins at scheduled time points (5, 15, 30, and 60 min and 2, 4, 7, 10, and 24 h postinjection) for pharmacokinetic profiling. The collected blood samples (one or two drops) were weighed and measured for radioactivity (^{64}Cu) with a gamma counter (Wizard2; PerkinElmer) using an appropriate energy window centered at a photo peak of 511 keV. Raw counts were corrected for background, decay, and weight. Corrected counts were converted to microcurie per gram of tissue with a previously determined calibration curve by counting the ^{64}Cu standards. The activity in each collected blood sample was calculated as percentage of injected dose per gram of plasma. The total concentration of NPs in plasma was calculated based on the assumption that the total plasma weight of a mouse was equal to 6% of its body weight. For this calculation, the blood radioactivity was corrected for the ^{64}Cu decay to the time of gamma-well counting:

$$\text{Plasma half-life} = 0.693 \times \text{time interval} / \left[\ln\left(\text{Concentration}_{\text{peak}}\right) - \ln\left(\text{Concentration}_{\text{trough}}\right)\right],$$

where time interval is the time between the peak and the end point, $\text{Concentration}_{\text{peak}}$ is the maximum concentration in plasma, and $\text{Concentration}_{\text{trough}}$ is the concentration at the end point.

In Vitro Studies of Pam Functionalized NPs

Synthesis of Cy5–PLA Conjugates. Cy5–PLA conjugates were synthesized by following procedures similar to those published (50). In a glovebox, Cy5 (5.0 mg, 0.01 mmol) was dissolved in anhydrous THF (900 μL) and mixed with a THF solution (500 μL) containing (BDI-EI)ZnN(TMS)$_2$ (6.5 mg, 0.01 mmol). The mixture was stirred for 15 min. LA (72.0 mg, 50 eq) was dissolved in THF (1 mL) and added to the stirred (BDI-EI)ZnN(TMS)$_2$ and Cy5 mixture. The reaction proceeded in the glovebox overnight. After LA was completely consumed, the reaction was stopped by quenching the polymerization solution with cold methanol solution (300 μL). The polymer was precipitated with ether (50 mL), collected by centrifugation, and dried by vacuum.

Preparation and Characterization of Cy5-Labeled Pam-NPs. A 100-μL DMF solution of Cy5–PLA (100 μL, 10 mg/mL) was mixed with a 100-μL DMF mixture of PLA-PEG (10 mg/mL) and PLA-PEG-Pam conjugate (10 mg/mL) at ratio of 1/0.8/0.2 and then added dropwise to rapidly stirred nanopure water (4 mL). The resulting Cy5-labeled Pam-NPs (Pam-Cy5-NPs) with different Pam contents were collected by ultrafiltration (5 min, 3,000 × *g*, Ultracel membrane with 10,000 NMWL; Millipore), washed with water, and then characterized by DLS for particle sizes and size distributions.

In Vivo Studies of Pam-Doxo-NPs in Mice

Synthesis of 1,4,7,10-Tetraazacyclododecane-1,4,7,10-Tetraacetic Acid–PLA Conjugate. The NH_2–PLA conjugate was synthesized by following a similar procedure as described above by using ethanolamine as the initiator. In a reaction vial containing NH_2–PLA conjugates (6.0 mg, 0.004 mmol) was added an anhydrous DMF solution (0.5 mL) of (*S*)-2,2′,2″,2‴-(2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid (*p*-SCN-Bn-DOTA) (9.9 mg, 0.018 mmol). The reaction mixture was stirred for 4 h under nitrogen at room temperature. The solvent and triethylamine were removed under vacuum to give DOTA–PLA conjugate, which was used directly without further purification.

Preparation of ^{64}Cu-Labeled Pam-NPs. The ^{64}Cu chloride (300 μCi, obtained from Washington University in St. Louis) was mixed with Pam-NPs (10%, 20 mg) in NH_4OAc buffer (pH 5.5, 0.1 M, 0.5 mL). The mixture was incubated for 1 h at 60 °C. To determine the labeling efficiency, the NPs were washed by ultrafiltration (10 min, 3,000 × *g*, Ultracel membrane with 10,000 NMWL; Millipore), and the radioactivity in the supernatant and the NP solution was measured at different time points (Fig. S5*A*). The purified ^{64}Cu-labeled Pam-NPs (Pam-^{64}Cu-NPs) were used for in vivo biodistribution study.

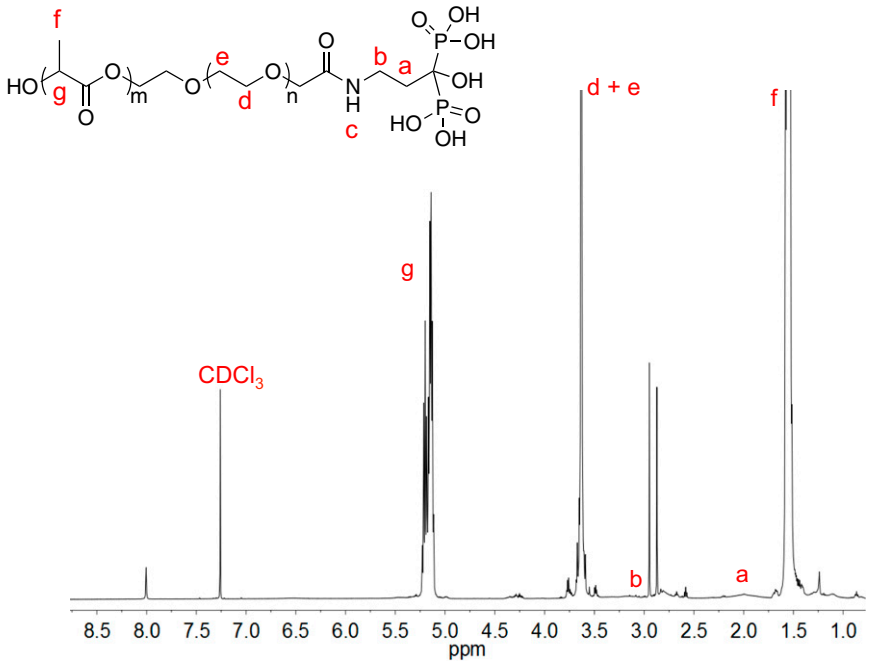


Fig. S1. ^{1}H NMR characterization of PLA-PEG-Pam conjugate.

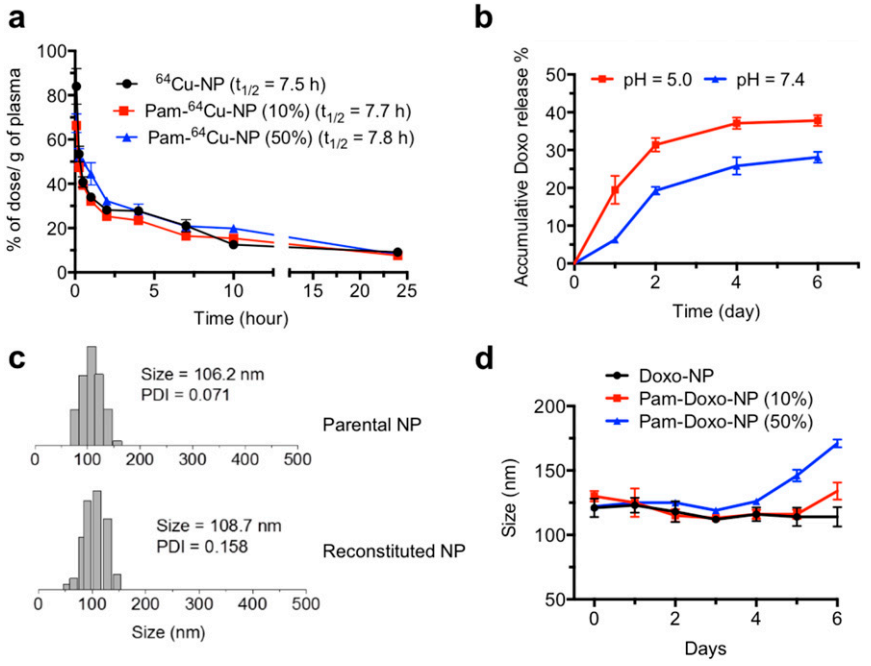


Fig. S2. Characterizations of Pam-Doxo-NPs. (*A*) In vivo circulation time of ^{64}Cu-labeled nontargeted NPs (black, denoted as ^{64}Cu-NPs) and ^{64}Cu-labeled Pam-NPs [10%, red; 50%, blue; denoted as Pam-^{64}Cu-NPs (10%) and Pam-^{64}Cu-NPs (50%), respectively]. All data are presented as average ± SEM; $n = 3$ in each group. (*B*) Release kinetic profile of Doxo from Pam-Doxo-NPs (10%) in PBS buffer at pH 5.0 and pH 7.4. (*C*) DLS analysis of NP reconstitution. Pam-Doxo-NPs (50%) in water (*Top*) and reconstituted Pam-Doxo-NPs (50%) after lyophilization in the presence of HSA (HSA:NP 10:1, mass/mass) (*Bottom*). (*D*) The stability of Pam-Doxo-NPs in FBS buffer (FBS:PBS 1:1, vol/vol) over the course of 6 d.

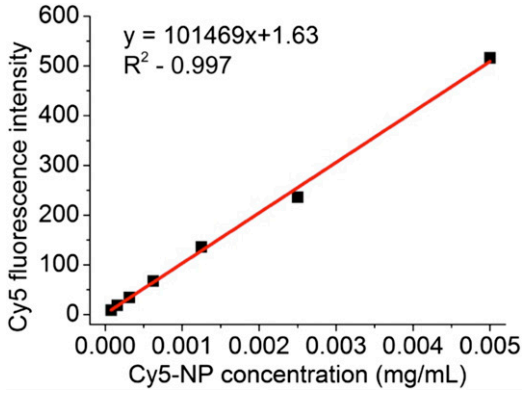


Fig. S3. Validation studies of Cy5 fluorescence measurement. Calibration curve of Cy5 fluorescence intensity with concentration of Cy5-NPs, measured by fluorescence spectrometer.

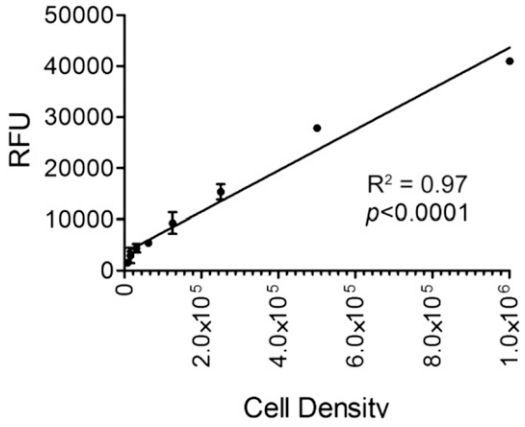


Fig. S4. Validation studies of GFP fluorescence measurement. Linear relationship between K7M2 cell density and relative fluorescent units (GFP) upon cell lysis.

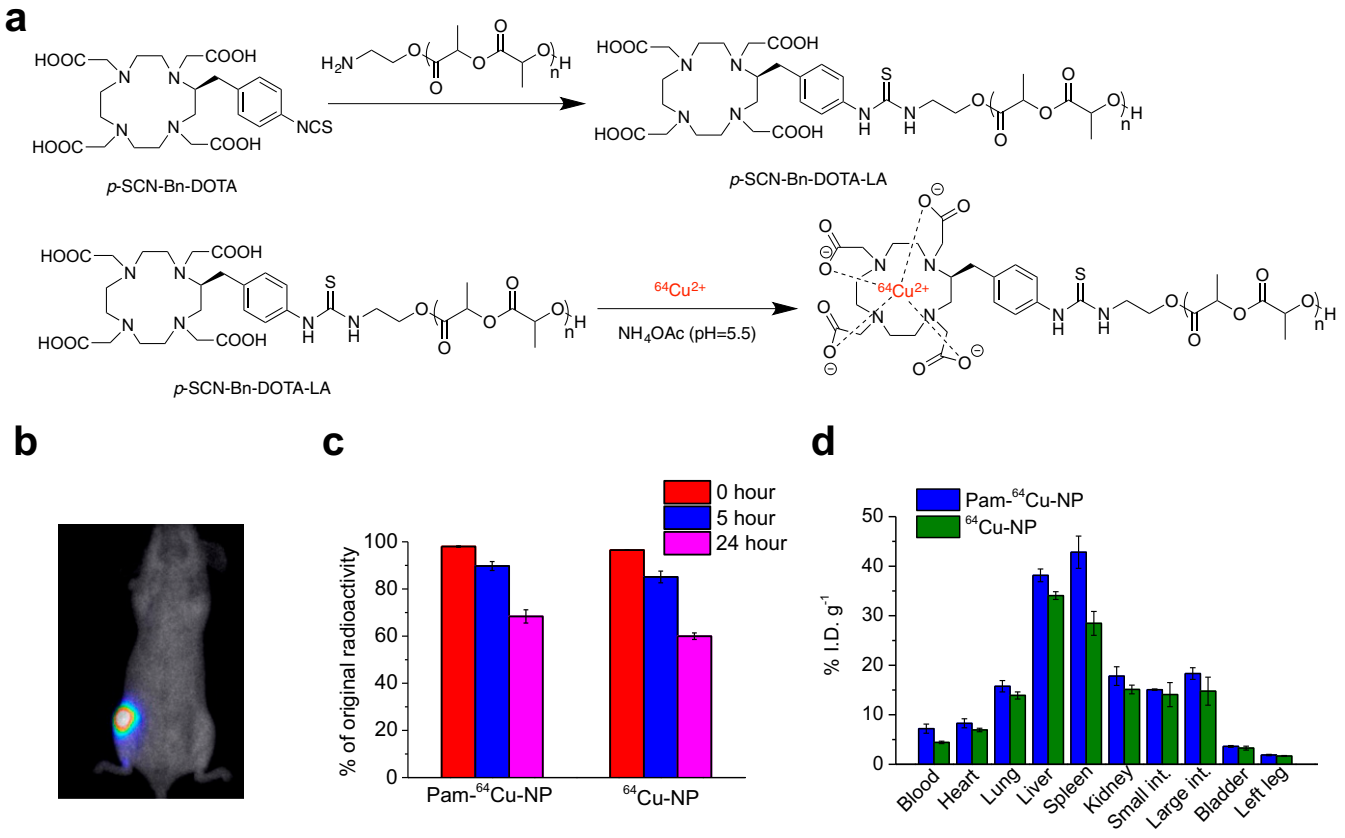


Fig. S5. In vivo biodistribution of ^{64}Cu labeled Pam-NPs in orthotopic murine OS tumor model. (*A*) Synthesis of Pam-^{64}Cu-NPs. (*B*) In vivo demonstration of K7M3 BLI in BABL/c mice. (*C*) Stability of ^{64}Cu labeling with Pam-^{64}Cu-NPs and ^{64}Cu-NPs in serum. (*D*) In vivo biodistribution of Pam-^{64}Cu-NPs (blue bar) and ^{64}Cu-NPs (green bar) in K7M3 OS-bearing mice, assessed by gamma counter. All of the data were presented as percentage of injected dose per gram of tissues.

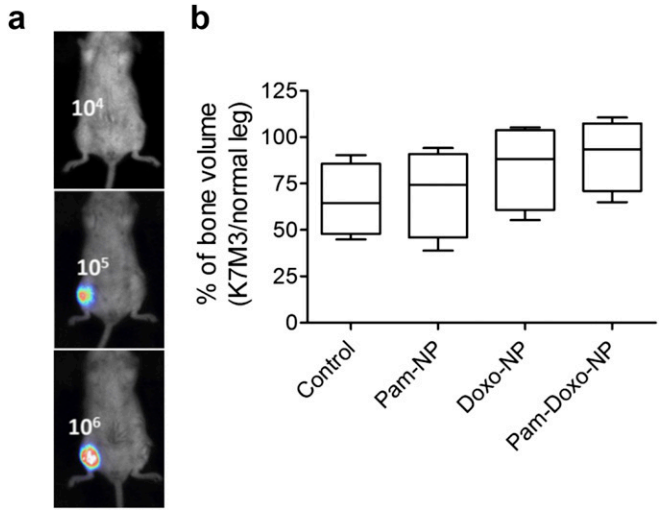


Fig. S6. In vivo efficacy of Pam-Doxo-NPs in orthotopic murine OS tumor model. (*A*) In vivo demonstration that K7M3 BLI correlates with absolute injected tumor cell density in BABL/c mice. (*B*) Percentage of bone volume (leg with K7M3/normal leg × 100%) among mice receiving different i.v. treatments of NPs.

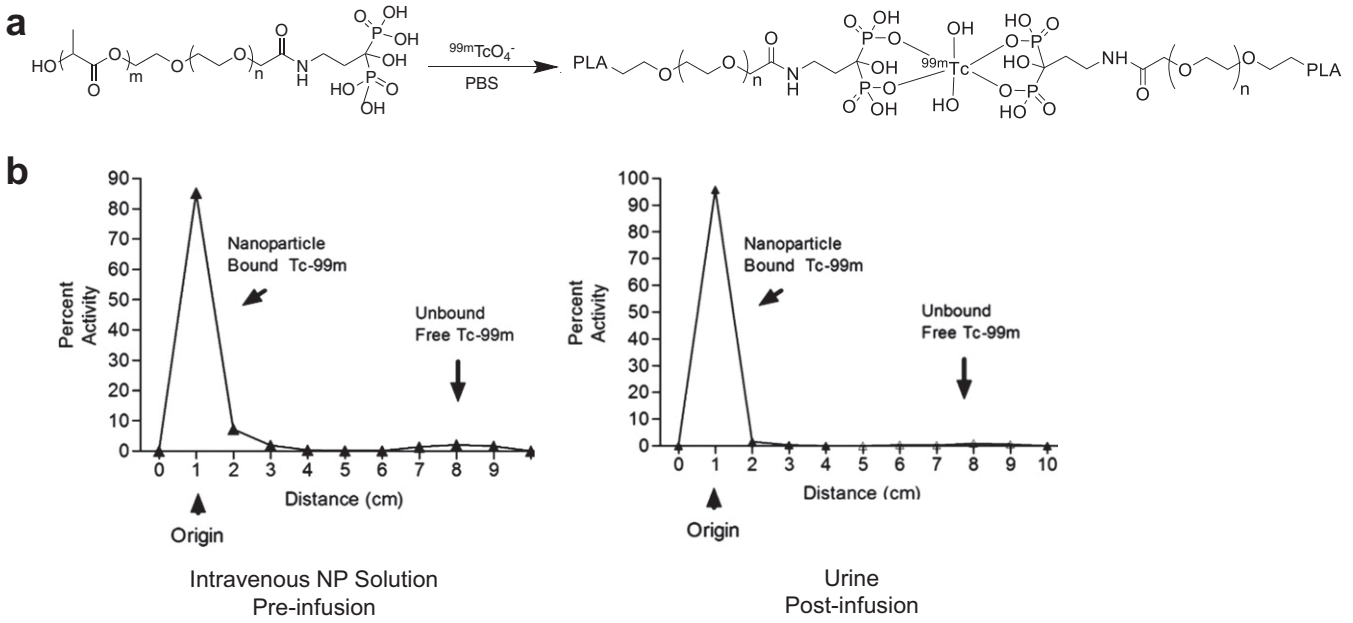


Fig. S7. In vivo biodistribution of Pam-Doxo-NPs in spontaneous canine OS tumor model. (*A*) Synthesis of ^{99m}Tc-labeled Pam-Doxo-NPs. (*B*) Radiochemical purity assessment of ^{99m}Tc-labeled Pam-Doxo-NPs immediately before i.v. infusion (*Left*) and 2 h postinfusion (*Right*).

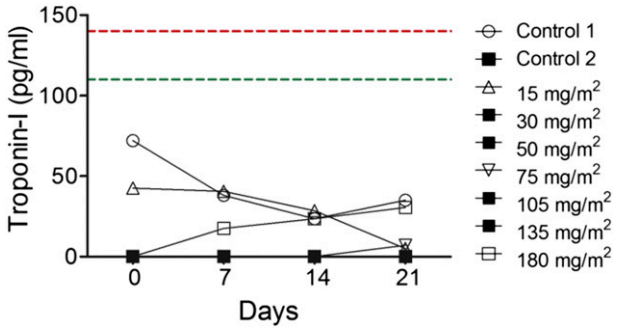
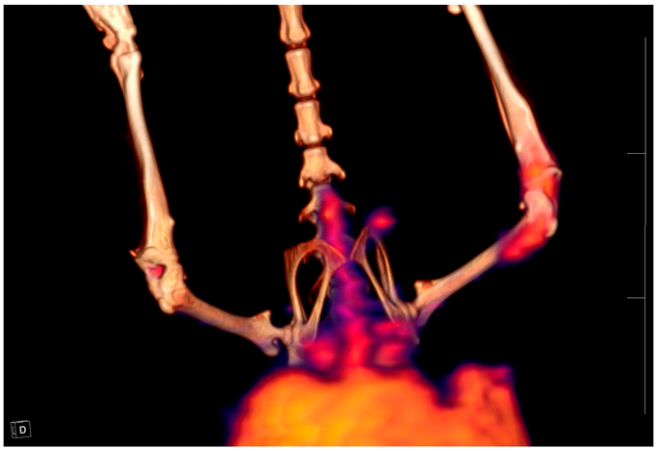
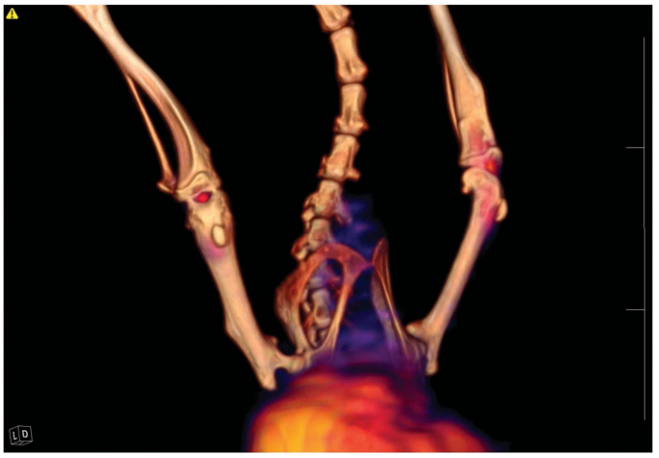


Fig. S8. Cardiac toxicity evaluation of Pam-Doxo-NPs. Serial cardiac troponin-I concentrations as a function of time postinfusion of Pam-Doxo-NPs in dogs receiving wide ranges of Doxo equivalent. Upper normal reference range (green dotted line) and lower reference range for cardiac insufficiency (red dotted line).



Movie S1. A 3D rendering of micro-PET/CT image taken 5 h p.i. showing accumulation of Pam-64Cu-NPs in the tumor-residing right hind leg and normal contralateral left hind leg.

Movie S1



Movie S2. A 3D rendering of micro-PET/CT image taken 5 h p.i. showing accumulation of 64Cu-NPs in the tumor-residing right hind leg and normal contralateral left hind leg.

Movie S2