Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Supporting information

Reassessment of long circulating *via* monitoring integral polymeric nanoparticles justifies more accurate understanding

Haisheng He[a†], Sifan Jiang[b†], Yunchang Xie[a], Yi Lu[a], Jianping Qi[a], Xiaochun Dong[a], Weili Zhao[ac], Zongning Yin[b] and Wei Wu*[a]

[a]*Key Laboratory of Smart Drug Delivery of MOE and PLA, School of Pharmacy, Fudan University, Shanghai 201203, China*

[b]*West China School of Pharmacy, Sichuan University, Chengdu 610041, China*

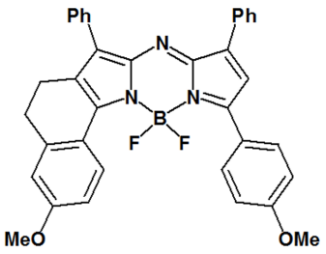
[c]*Key Laboratory for Special Functional Materials of the Ministry of Education, Henan University, Kaifeng 475001, China*

* E-mail: wuwei@shmu.edu.cn

[†] Contributed equally to this article.

Materials and Methods

Materials. Poly (ε-caprolactone) (PCL, Mn = 45000) and polyvinyl alcohol (PVA, Mn = 13000-23000, 87-89% alcoholised) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methoxy PEG-poly(*ε*-caprolactone) (mPEG-PCL) copolymers with different mPEG and PCL chain lengths ($mPEG_{5k}$-PCL_{45k}, $mPEG_{2k}$-PCL_{45k}) were synthesized, according to previous procedures,[1,2] and kindly provided by Professor Zhiyong Qian at Sichuan University, Chengdu, China. Paclitaxel (PTX) was purchased from Dalian Meilun Biotech Co., Ltd (Liaoning, China). Tween 80 was supplied by Sigma- Aldrich (St. Louis, MO, USA). The fluorescent probe P2 ($\lambda_{abs}/\lambda_{em}$ = 708/732 nm) was synthesized in our lab according to previous procedures.[3,4] Blank rat plasma was purchased from Fanke Biological Technology Co. Ltd (Shanghai, China). Dichloromethane (DCM) was purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Purified water was prepared using a Milli-Q purification system (Molsheim, France). All other reagents used in this study were of analytical grade and used as received.



The structural formula of P2

The Sprague-Dawley (SD) rats were supplied by Shanghai Laboratory Animal Center (Shanghai, China) and raised in the Laboratory Animal Holding Building of School of Pharmacy, Fudan University. Throughout the experiment, the animals were housed at a temperature of 24 ± 1 °C, a relative humidity of 55 ± 5%, and with 12 h light-dark cycles. If not specified otherwise, all animals were abstained from food for 12 h before experiment, but allowed free access to water. All animal care and experimental procedures were conducted according to the guidelines issued by the Ethical Committee of School of Pharmacy, Fudan University on use of experimental animals, following the principles outlined in the Declaration of Helsinki for all human and animal experimental investigations.

Preparation of fluorescently labeled blank mPEG-PCL nanoparticles. mPEG-PCL nanoparticles were prepared by an oil-in-water type emulsification/solvent evaporation method as reported previously.[5-7] Fluorescent labeling was achieved by dissolving a certain amount of the probe P2 (0.036-0.045%, w/w) in the oil phase first. Then, 200 mg PCL and a certain amount of mPEG-PCL, together with the fluorescent probes, were dissolved in 5.0 mL of DCM to form the organic phase. The aqueous phase was 20 mL 1% (w/w) PVA solution. The organic phase was instilled into the aqueous phase and emulsified by probe ultrasonication (Scientz Biotechnology Co., Ltd, Ningbo, China) for 3 min (540 W) in an ice bath to obtain a coarse emulsion. The coarse emulsion was stirred mechanically for 4 h to remove DCM and obtain mPEG-PCL nanoparticles (200 nm), whereas the 80-nm nanoparticles were prepared by disrupting the coarse emulsion at a pressure of 1000 bar for 3 min using a high-pressure homogenizer (Scientz Biotechnology Co., Ltd, Ningbo, China), followed by mechanical stirring for 4 h to remove DCM. The as-prepared nanoparticle suspensions were stored at 4 °C before analysis. To obtain different PEG coating chain length and coating density, different amount of mPEG-PCL with different mPEG chain lengths was incorporated into the PCL matrix. Blank PCL nanoparticles without mPEG coating was also prepared using pure PCL as matrix material.

Preparation of fluorescently labeled PTX-loaded mPEG-PCL nanoparticles. PTX-loaded mPEG-PCL nanoparticles were prepared by the same method as for blank nanoparticles. The same fluorescent probe (P2) was incorporated into nanoparticles following the same procedures in the same strength. Briefly, 20 mg of PTX, 200 mg of PCL and a certain amount of mPEG-PCL, together with 100 μg of P2, were dissolved in 5 mL of DCM to form the oil phase. The downstream procedures were just the same as for blank mPEG-PCL nanoparticles. After complete evaporation of DCM, the obtained PTX-P2-nanoparticles were filtered to remove non-encapsulated PTX. The amount of PTX encapsulated in nanoparticles was determined by high performance liquid chromatography (HPLC). The mobile phase was a mixture of water and acetonitrile (45/55, v/v). The elution rate was 1.0 mL/min and the detection wavelength was set at 227 nm. The encapsulation efficiency and drug loading of PTX was calculated by the method reported previously.[5]

Physicochemical characterization. The physicochemical properties of various nanoparticles were measured after dilution with purified water by 20 folds. Hydrodynamic diameter and zeta potential were determined by a Zetasizer Nano (Malvern Instruments, Malvern, UK) at 25 °C. Particle size was reported as the intensity-average mean. Fluorescence was measured by a Cary Eclipse Fluorescence Spectrophotometer (Agilent, USA) with excitation/emission wavelengths set to 708/732 nm.

The morphology of nanoparticles were observed using transmission electron microscopy (TEM) (JEM-1230 Electron microscope, JEOL, Japan). To prepare samples, a dilute suspension of the nanoparticles was dropped on copper grids, negatively stained with 1% (w/v) uranyl acetate and allowed to dry under ambient atmosphere.

Stability of fluorescence in buffers and plasma. Briefly, the nanoparticle suspensions were diluted by 15 folds with rat plasma or phosphate buffered saline (PBS) (pH 7.4), simulating *in vivo* dilution, and incubated in a water bath under continuous oscillatory shaking at 37 ℃. Samples (0.8 mL) were withdrawn at 0, 1, 2, 4, 8, 12, 24, 36, 48 h, and fluorescence was measured by a fluorospectrophotometer as described above.

Pharmacokinetics of nanoparticles. Male SD rats weighing 200 ± 20 g were used in the pharmacokinetic study. The rats were randomly divided into eight groups, three in each group, including seven groups of test formulations and one control group of quenched P2 solution. The formulations of all groups were injected *via* the tail vein. Blood samples were withdrawn at time intervals post injection. After that, the samples were deposited in a black 96-well plate with a pipette, 100 μL per cell. The fluorescence of the samples were measured by an IVIS spectrum live imaging system (PerkinElmer, USA) with excitation/emission wavelength set to 710/760 nm. Photographs were captured under an automatic exposure mode, regions of interest (ROI) were drawn over the fluorescent signals, and total radiant efficiency (TRE) within the ROIs were measured by vendor software for subsequent quantitative analysis.[8,9] By taking the fluorescence signal of the first measurement at 15 s as 100%, we calculated the relative fluorescence intensity of all time points.

Live imaging of the *in vivo* fate of mPEG-PCL nanoparticles. *In vivo* fate of nanoparticles was investigated by live imaging after intravenous administration. The experimental procedures were just the same as in the pharmacokinetic study except that live imaging was performed at time intervals instead of blood sampling. Blank images were taken before administration and live images were acquired post administration to as long as 48 h. During the image-capturing process, animals were narcotized by an on-line gas anesthesia system using isoflurane (Shandong Keyuan Pharmaceutical Co., Ltd., China). Fluorescence intensity of the whole body as an average and specific body regions (snout, four extremities and genitals) were measured following procedures similar to that specified in the pharmacokinetic study.

Real-time biodistribution in organs and tissues. To obtain images for quantification of organ distribution, the animals were sacrificed and dissected to visualize various organs such as liver, lung, spleen and so on. To curtail the total number of animals sacrificed, only one animal was used at each sampling point (5, 15 min, and 0.5, 1, 2, 4, 8, 12, 24, 36, 48 h). After cardiac perfusion with saline to exclude the interference of residual blood, the target organs were collected immediately and imaged *ex vivo* using the IVIS live imaging system. Fluorescence intensity of each organ was measured following procedures described above.

Protein adsorption assay. The *in vitro* protein adsorption on nanoparticles were evaluated according to previous methods with minor modifications.[10,11] Briefly, 100 μL of nanoparticles dispersion (5 mg/mL) was instilled into a bovine serum albumin (BSA) solution (1 mg/mL). After incubation at 37 °C with mild shaking (100 rpm) for 4 h, the nanoparticles were separated by centrifugation (40000 *g* × 20 min). The obtained pellet was carefully washed three times with PBS (pH 7.4), and afterwards resuspended in 200 μL of PBS (pH 7.4). The adsorbed proteins were quantified by Micro bicinchninic acid (BCA) protein assay using a UV-spectrometer with a microliter plate reader at a wavelength of 562 nm. Results were expressed as the amount of protein adsorbed (μg) divided by surface area of nanoparticles (m^2). The total surface area was calculated by a reported method.[12]

***In vitro* PTX release.** The *in vitro* PTX release from nanoparticles was first investigated in PBS (pH 7.4) containing 0.5% Tween 80 as reported previously.[13] Nanoparticles loading both PTX and P2 (0.5 mL, 200 μg/mL) were first filled into dialysis bags (MW cutoff = 12000-14000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), and then immersed into the release media and incubated at 37 °C under mild shaking. At each time point, 1 mL of the media were withdrawn and replaced with an equal volume of fresh media. Samples were analysed by HPLC as described above. Sink conditions were maintained throughout the sampling process. Experiments were conducted in triplicate. The PTX release profiles from nanoparticles in plasma was further evaluated following the method previously reported,[14] a certain amount of nanoparticles loading PTX and P2 or PTX solution was added to fresh rat plasma, with a final PTX concentration in plasma set to approximately 6 μg/mL. The samples was then incubated at 37 °C under mild shaking. At predetermined time points of 0.25, 1, 2 and 4 h, 200 μL of plasma was withdrawn. Plasma (50 μL) was diluted by 10 folds with deionized water to reduce viscosity, and then centrifuged (45000 *g* × 30 min) to separate nanoparticles. The released PTX in supernatant was extracted and determined by LC-MS/MS as described in the part of pharmacokinetic study. Results were expressed as mean ± standard deviation from three repeats.

Pharmacokinetics of PTX-loaded nanoparticles. Pharmacokinetics of PTX-loaded nanoparticles were evaluated by monitoring the fluorescence and PTX simultaneously. Briefly, PTX-loaded fluorescently labeled nanoparticles were administrated *via* the tail vein at a PTX dose of 4 mg/Kg. All groups of formulations were adjusted to similar fluorescence intensity. At predetermined time points of 0.0042, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h post administration, 500 μL blood samples were withdrawn. Immediately after sampling, 100 μL of blood was used to quantify the fluorescence by IVIS.

Meanwhile, the remained 400 μL of blood samples were immediately centrifuged at 4000 rpm for 5 min to separate plasma, which was frozen and stored at -20 °C until analysis. To determine PTX, 1 mL of *tert*-butylmethyl ether were added to 100 μL of plasma containing 10 μL of docetaxel (10 μg/mL) as an internal standard and vortex mixed for 5 min. After centrifugation at 12000 *g* for 10 min, the organic layer was collected and dried under mild nitrogen flow at 40 °C. The samples were then dissolved by 100 μL of a mixed solvent (acetonitrile/water = 70/30, v/v) and subjected to liquid LC-MS/MS analysis. Chromatography was performed utilizing an Agilent 1200 HPLC system equipped with a C18 column (2.1 mm × 10 mm, 3.5 μm, Agilent Eclipse, USA) at a temperature of 30 °C and a flow rate of 1.0 mL/min. The mobile phase was a mixture of acetonitrile and water (80/20, v/v) containing 0.1% formic acid and 5 μL of samples were injected for analysis. Mass spectrometric detection was conducted on an AB 4000 Q TRAP TM triple-quadrupole linear ion trap mass spectrometer (SCIEX, USA) in positive ionization mode (ESI). Detection of the ions was conducted in the multiple reaction monitoring (MRM) mode by monitoring the transition of the *m*/*z* 854.4 → 509.3 for paclitaxel and 808.4 → 226.1 for docetaxel, respectively. Pharmacokinetic parameters were calculated using the DAS (Drug and Statistic for Windows) software (version 2.0).

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Table S1 Compositions and physicochemical properties of mPEG-PCL nanoparticles

	Nanoparticle formulation code						
	$mPEG_{5k}$-29%-200 nm	$mPEG_{5k}$-29%-80 nm	$mPEG_{5k}$-17%-200 nm	$mPEG_{5k}$-9%-200 nm	$mPEG_{5k}$-9%-80 nm	$mPEG_{2k}$-29%-200 nm	PCL-200 nm
$mPEG_{5k}$-PCL_{45k} (mg)	80	80	40	20	20	/	/
$mPEG_{2k}$-PCL_{45k} (mg)	/	/	/	/	/	40	/
PCL (mg)	200	200	200	200	200	200	200
P2 (μg)	100	100	100	100	100	100	100
mPEG-PCL content (%)	28.57	28.57	16.67	9.09	9.09	28.57	0
mPEG-PCL/PCL	4/10	4/10	2/10	1/10	1/10	4/10	0/10
Particle size (nm)[a]	214	85	207	202	77	207	199
PDI[a]	0.03	0.15	0.05	0.05	0.12	0.06	0.03
Zeta potential (mV)[a]	-4	-3	-3	3	-2	-4	-3
Fluorescence intensity[a]	455.00±8.19	458.84±9.28	469.67±7.23	461.00±17.09	484.06±7.91	459.67±11.02	494.00±15.87

[a] Measured at 25 °C after dilution with purified water by 20 folds. All measurements were conducted in triplicate.

Table S2 Characterization of PTX encapsulated mPEG-PCL nanoparticles

Formulation code	Size (nm)	PDI	Entrapment efficiency (%)	Drug loading (%)
PTX-PCL-200 nm	200	0.093	95.2±2.1	8.69±0.19
PTX-PEG_{5k}-29%-200 nm	205	0.082	95.4±3.6	8.88±0.33
PTX-PEG_{5k}-29%-80 nm	88	0.138	70.2±4.9	6.65±0.46

Measurements were conducted in triplicate.

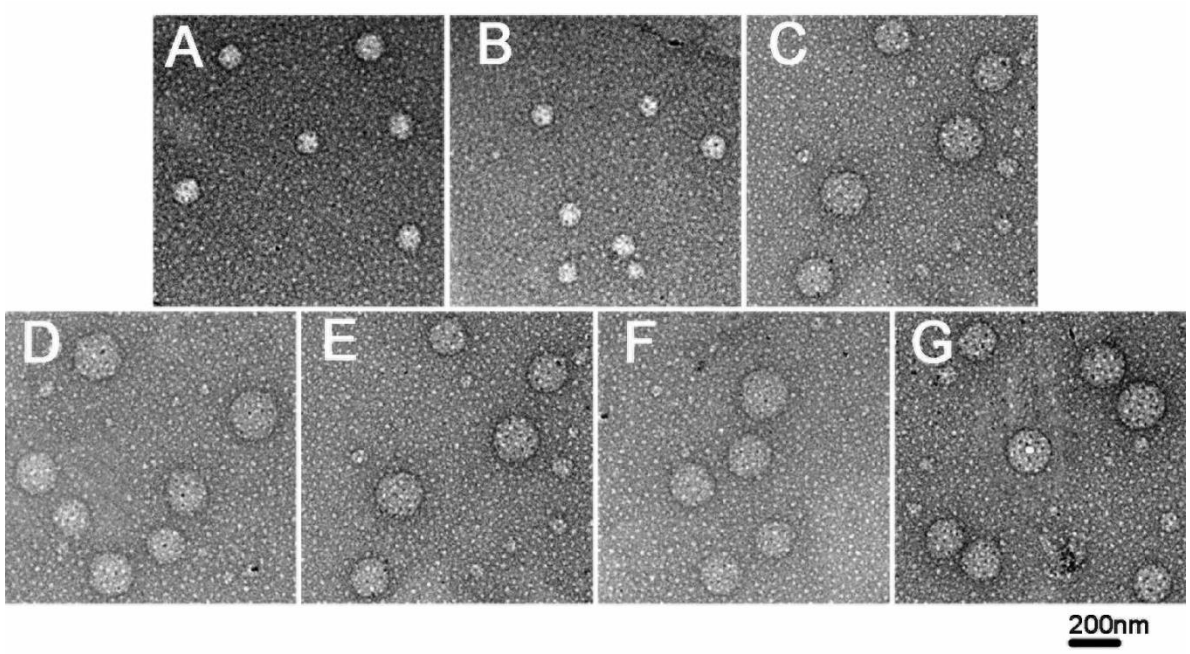


Figure S1. Representative TEM images of mPEG-PCL nanoparticles with various mPEG-PCL contents and different PEG chain lengths in correspondence to the formulation code as listed in Table S1 in the Supporting Information: mPEG$_{5k}$-29%-80 nm (A); mPEG$_{5k}$-9%-80 nm (B); mPEG$_{5k}$-29%-200 nm (C); mPEG$_{5k}$-17%-200 nm (D); mPEG$_{5k}$-9%-200 nm (E); mPEG$_{2k}$-29%-200 nm (F); PCL-200 nm (G).

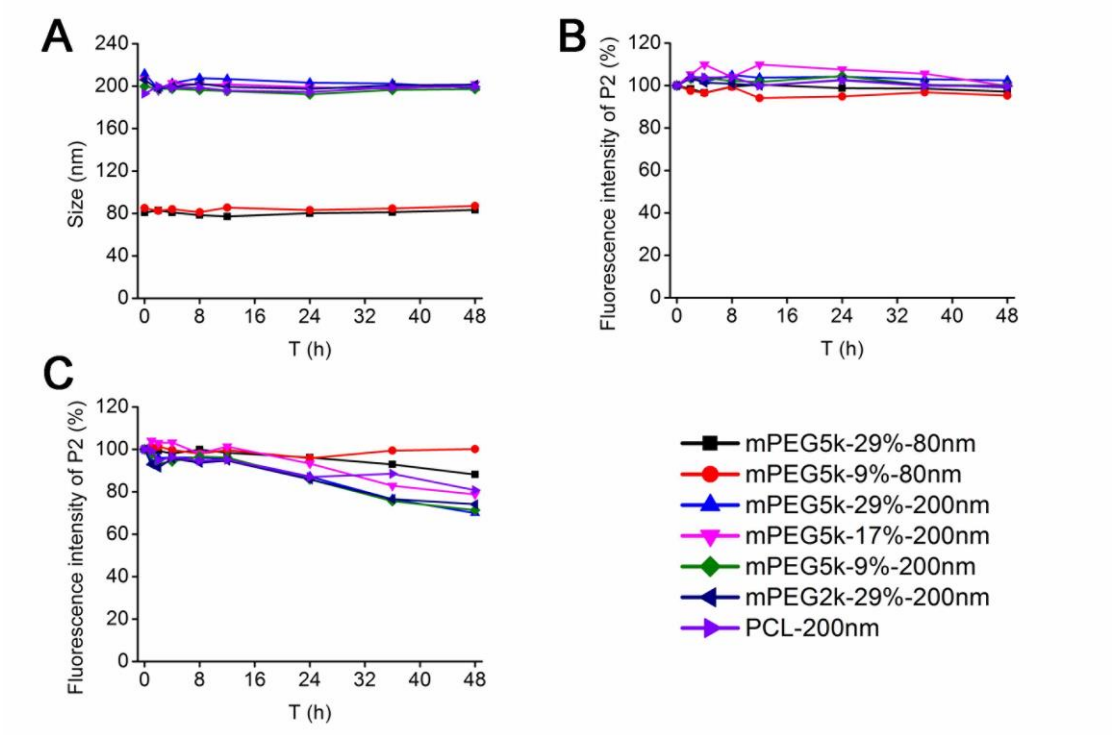


Figure S2. Physical stability of different formulations incubated in pH 7.4 phosphate buffered saline (A: particle size; B: fluorescence intensity) and in plasma (C: fluorescence intensity).

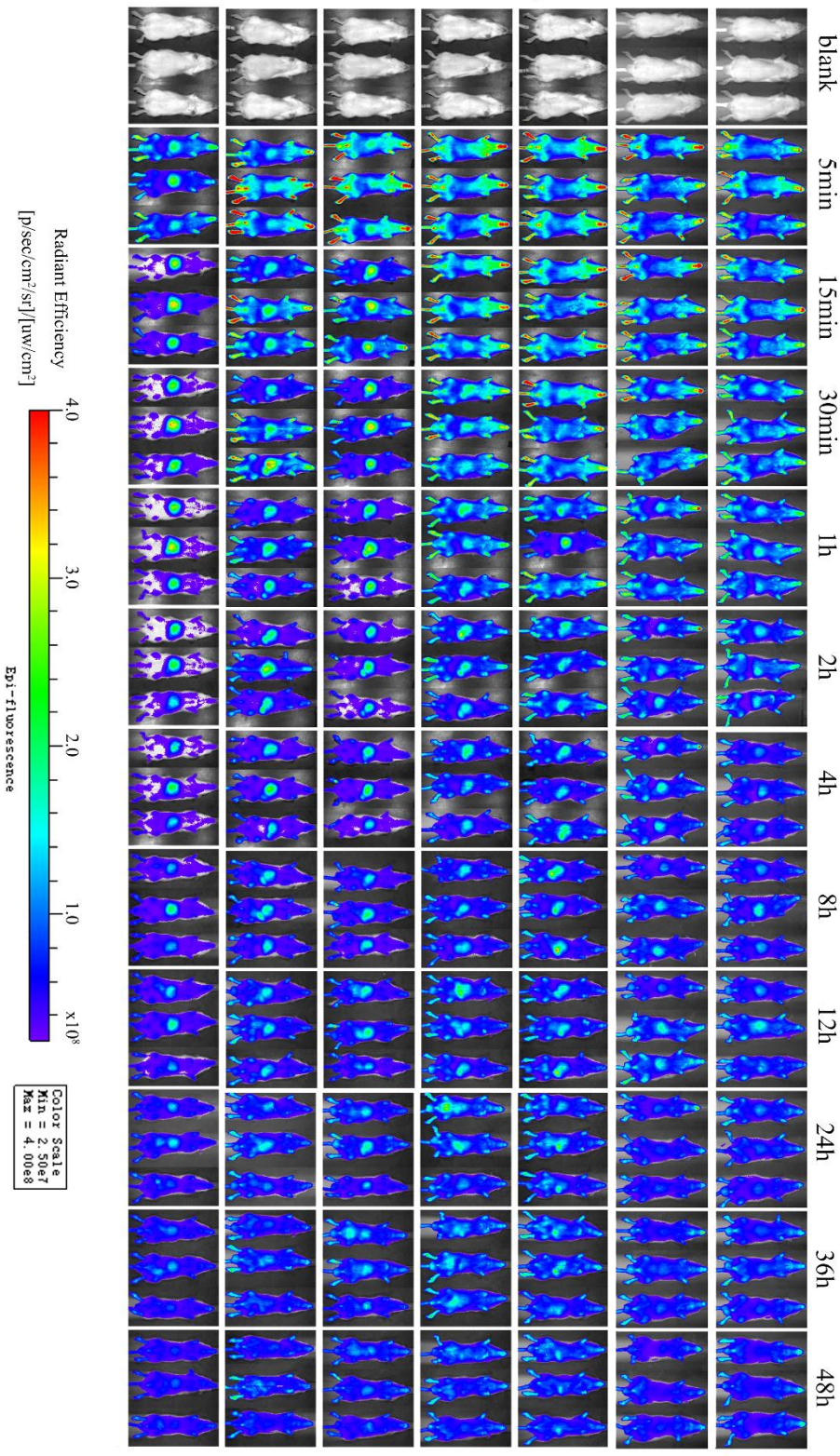


Figure S3. Live imaging of the distribution of P2-loaded nanoparticles after *i.v.* administration to rats

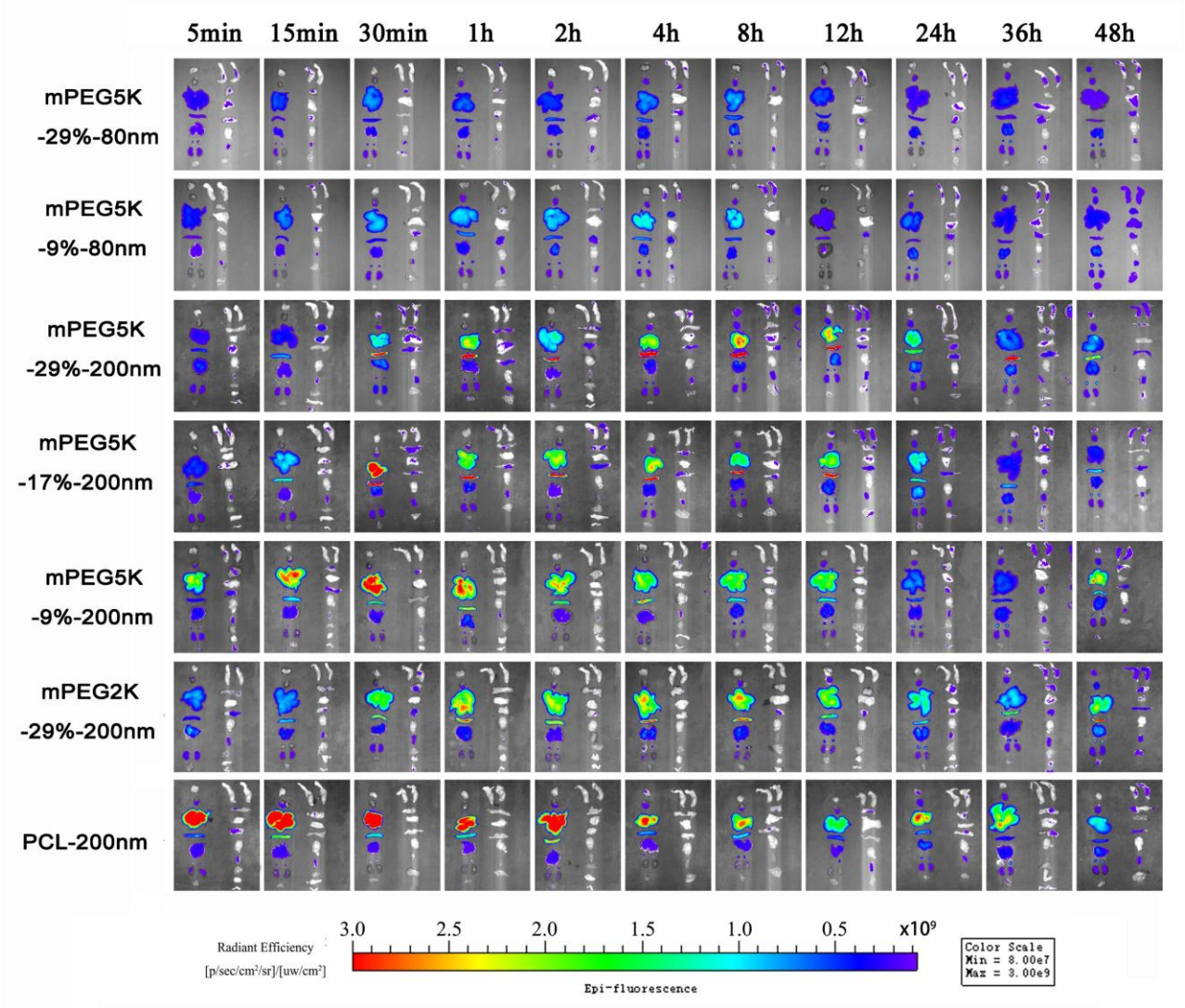
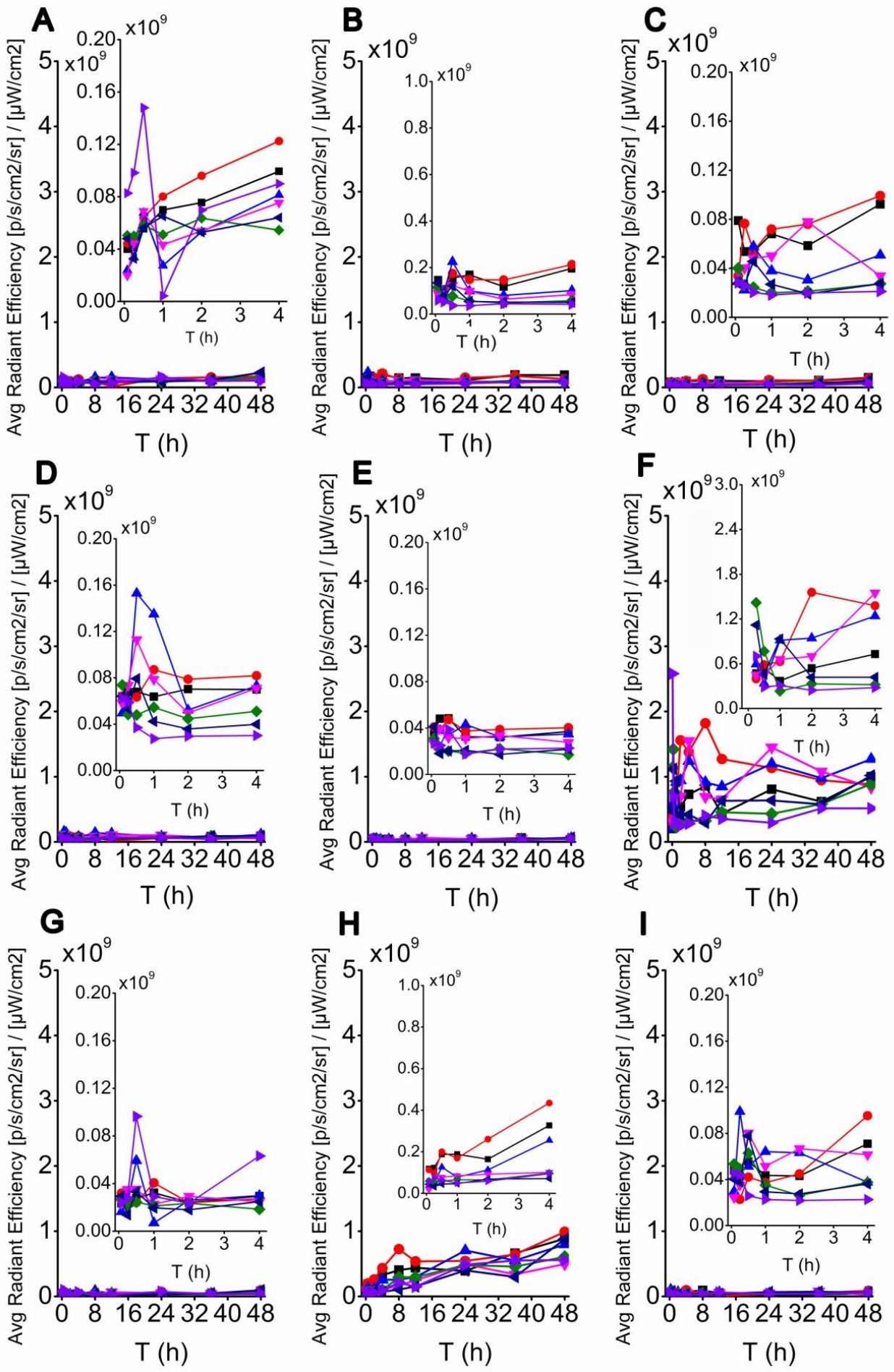


Figure S4. Live imaging of *ex vivo* organs and tissues dissected after oral administration of P2-loaded nanoparticles.


A
B
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Avg Radiant Efficiency [p/s/cm2/sr] / [µW/cm2]
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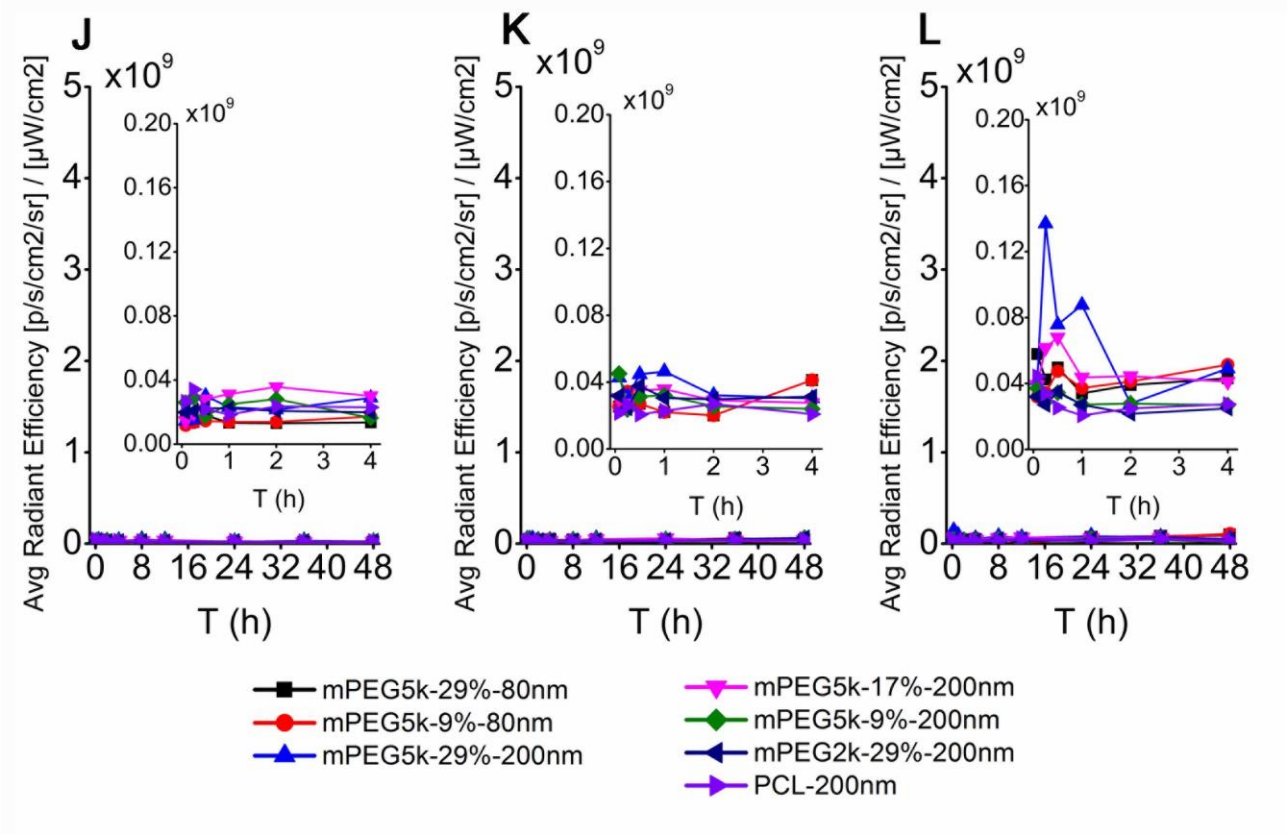

Figure S5. Semi-quantification of fluorescence expressed as average radiant efficiency (ARE) in various RES organs: heart (A), kidney (B), testicles (C), spine (D), sternum (E), mesentery (F), thymus (G), adrenal (H), pancreas (I), brain (J), muscle (K), skin (L).

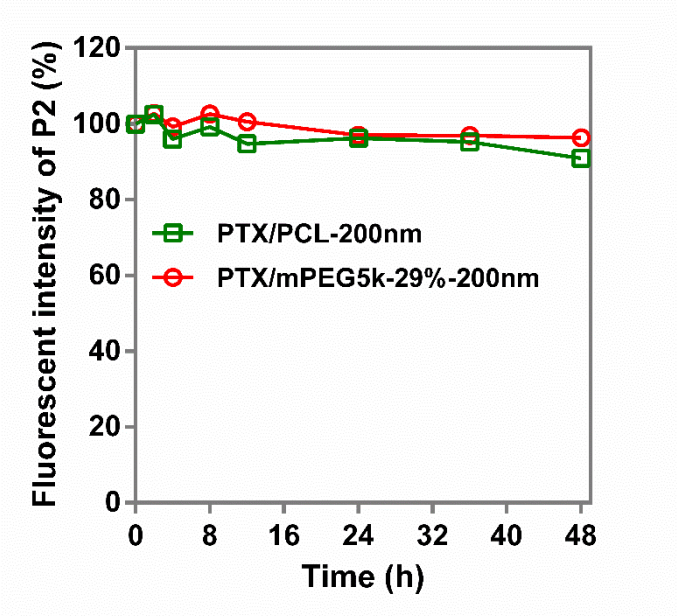

Figure S6. Fluorescence stability of P2-loaded nanoparticles in plasma.