

Electronic Supplementary Information

**Size-controlled long-circulating PICs as a ruler to measure critical
cut-off disposition size into normal and tumor tissues**

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Experimental Section

Materials

Block-anionomer, PEG-*b*-poly(α,β -aspartic acid) (PEG-P(Asp); M_n of PEG = 2,000, DP of P(Asp) = 75) and homo-cationomer, poly([5-aminopentyl]- α,β -aspartamide) (Homo-P(Asp-AP); DP of P(Asp-AP) = 82) were prepared as previously reported [1, 2]. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Cy5 mono-reactive dye pack was purchased from GE Healthcare (Tokyo, Japan). Preparation of Cy5-labeled PEG-P(Asp) (PEG-P(Asp)-Cy5) are shown in the Supporting Information. Murine colon 26 (C-26) adenocarcinoma cells were kindly supplied by the National Cancer Center (Tokyo, Japan). C-26 adenocarcinoma cells were maintained in RPMI 1640 medium (Sigma Chemical) containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37 °C. BALB/c nude mice (female; 3 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). All animal experiments were carried out in accordance with the guidelines for animal experiments at the University of Tokyo.

Preparation of Nano-PICsomes and PIC micelles [2]

PEG-P(Asp), PEG-P(Asp)-Cy5 and Homo-P(Asp-AP) solutions were prepared separately in 10 mM phosphate buffer (PB, pH 7.4). These solutions were filtered through a 0.22- μ m membrane filter to remove dust. The PEG-P(Asp) solution was mixed with the PEG-P(Asp)-Cy5 solution with the mixing ratio of 4/1. Resulted polyanion mixture was blended with the Homo-P(Asp-AP) solution in an equal unit ratio of $-\text{COO}^-$ and NH_3^+ , followed by vortex mixing to form the Cy5-Nano-PICsomes. PIC micelles were prepared using the same procedure as with the Nano-PICsomes, but PEG-P(Asp-AP) solutions was used instead of Homo-P(Asp-AP). Various sizes of Nano-PICsomes (102, 158, 197, 256, 298 nm) were prepared by using different polymer concentrations (total

polymer concentration = 1.0, 2.0, 3.0, 4.0 and 5.5 mg/mL) according to the previous report [2].

Preparation of cross-linked Nano-PICsomes [2]

EDC (10 mg) was dissolved in 10 mM PB solution (1 mL). Then, Nano-PICsome solution (960 μ L) was added to the EDC solution (670 μ L, 10 eqv. per $-\text{COOH}$ group in PEG-P(Asp)). After 12 h, the mixed solution was purified using a polyethersulfone ultrafiltration membrane (MWCO; 300,000).

Dynamic light scattering (DLS) measurements

The size of the PIC micelle and PICsomes was evaluated by DLS measurements at 25 °C using a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK) equipped with a He-Ne ion laser (λ 633 nm). A scattering angle of 173° was used in all measurements. In these measurements, the autocorrelation function, $g(\tau)$, was analyzed using the cumulant method [3,4], where

$$g(\tau) = \exp[-\bar{\Gamma}\tau + (\mu_2/2)\tau^2 - (\mu_3/3!)\tau^3 + \dots] \quad (1)$$

yields an average characteristic line width, $\bar{\Gamma}$. The z -averaged diffusion coefficient was obtained from the $\bar{\Gamma}$ based on the following equations:

$$\bar{\Gamma} = Dq^2 \quad (2)$$

$$q = (4\pi n/\lambda) \sin(\theta/2) \quad (3)$$

where q is the magnitude of the scattering vector, n is the refractive index of the solvent, and θ is the detection angle. The polydispersity index ($\text{PDI} = \mu_2/\bar{\Gamma}^2$) was derived from Equation 1.

Preparation of mice bearing murine colon 26 (C-26) adenocarcinoma s.c. tumor

Each mouse (BALB/c nude, female; 4 weeks old; $n = 3$) was subcutaneously inoculated with 100 μL of a suspension of C-26 adenocarcinoma cells (1×10^6 cells/mL). Tumors were allowed to grow for 14 days.

Evaluation of blood circulation

200 μL of phosphate buffer saline solution of all samples were administered by injection through the tail vein of each mouse. The concentration of the injecting samples was adjusted using the fluorescent intensity. Mice were sacrificed after defined time periods (1, 3, 6, 24, 48, 96 and 240 h). Blood was collected from the postcaval vein using heparinized syringes, and C-26 tumor tissue sample was also collected. After centrifugation of the collected blood at 2000g for 5 minutes at 4 °C, fluorescence of the resulting supernatant was measured by a fluorescence detector (Ex/Em=650/670).

Evaluation of biodistribution

Sample disposition in the lung, liver, spleen, kidney and C-26 tumor tissue was estimated from the intensity of the fluorescence signal of collected tissues with an IVIS Imaging System (Xenogen, Alameda, CA, USA) comprised of a highly sensitive, cooled CCD camera mounted in a light-tight specimen box. Because the liver has a high density of blood vessels, IVIS measurement was done after flushing saline solution into the sacrificing mice to avoid the signal from blood pool. The background fluorescence in each tissue was eliminated by subtracting the fluorescence from the organs of untreated animals. All animal experiments were carried out in accordance with the

guidelines for animal experiments at the University of Tokyo.

Pharmacokinetic data analysis

The time corresponding to removal of 50% of the injected dose ($T_{50\%}$) was calculated by linear interpolation.

AUC denotes the area under a concentration curve that is obtained from the pharmacokinetic study with time points at 1, 3, 6, 24, 48, 96 and 240 h. Values were calculated on the basis of the trapezoidal rule up to 240 h after intravenous injection as well as area under the first moment curve (AUMC) and mean residence time (MRT). The unit for AUC is defined as average radiance per plasma ($\text{p/s/cm}^2/\text{g}$) x h or average radiance per plasma organ x h for the blood or other tissues (tumor, kidney, liver, lung and spleen), respectively. AUMC and MRT were calculated from the following equations:

$$AUMC_{0 \rightarrow n} = \int_0^n t \cdot C \cdot dt = \sum_{i=0}^n \left(\frac{t_i \cdot C_i + t_{i+1} \cdot C_{i+1}}{2} \right) \cdot (t_{i+1} - t_i)$$

$$MRT_{0 \rightarrow n} = \frac{AUMC_{0 \rightarrow n}}{AUC_{0 \rightarrow n}}$$

In the case of PIC micelles and Nano-PICsomes with the diameter of 298 nm, data points at 240 h post injections were not exploited for calculation due to less reliability of values.

Size distribution determined by DLS

Table S1. Various sizes of PIC-nanoparticles injected into mouse

Combination of Polymers	Concentration (mg/mL)	Size (nm)*	Polydispersity Index*	d_w/d_n **
PEG-P(Asp)/PEG-P(Asp-AP)	1	38.0	0.023	1.092
	1	102	0.020	1.086
	2	158	0.035	1.104
PEG-P(Asp)/Homo-P(Asp-AP)	3	197	0.038	1.108
	4	256	0.054	1.091
	5.5	298	0.065	1.115

* Size and polydispersity index taken after cross-linking and removal of EDC.

** Using the histogram method, the polydispersity (d_w/d_n) defined as the ratio of the weight-averaged diameter and the number-averaged diameter was also determined.

Gel Permeation Chromatogram (GPC) of Various Sized Nano-PICsomes and PIC micelles

GPC measurements were carried out in pH 7.4 10 mM PB (150 mM NaCl) using a High performance liquid chromatography (HPLC) system (JASCO, Japan) equipped with Superose6TM 10/300 GL columns (Amersham, USA), fluorescent detectors (Ex/Em = 650/670 nm) and UV detector (absorbance at 220 nm). Columns were eluted with pH 7.4 10 mM PB (150 mM NaCl) at a flow rate of 0.5 mL/min at a temperature of 35 °C.

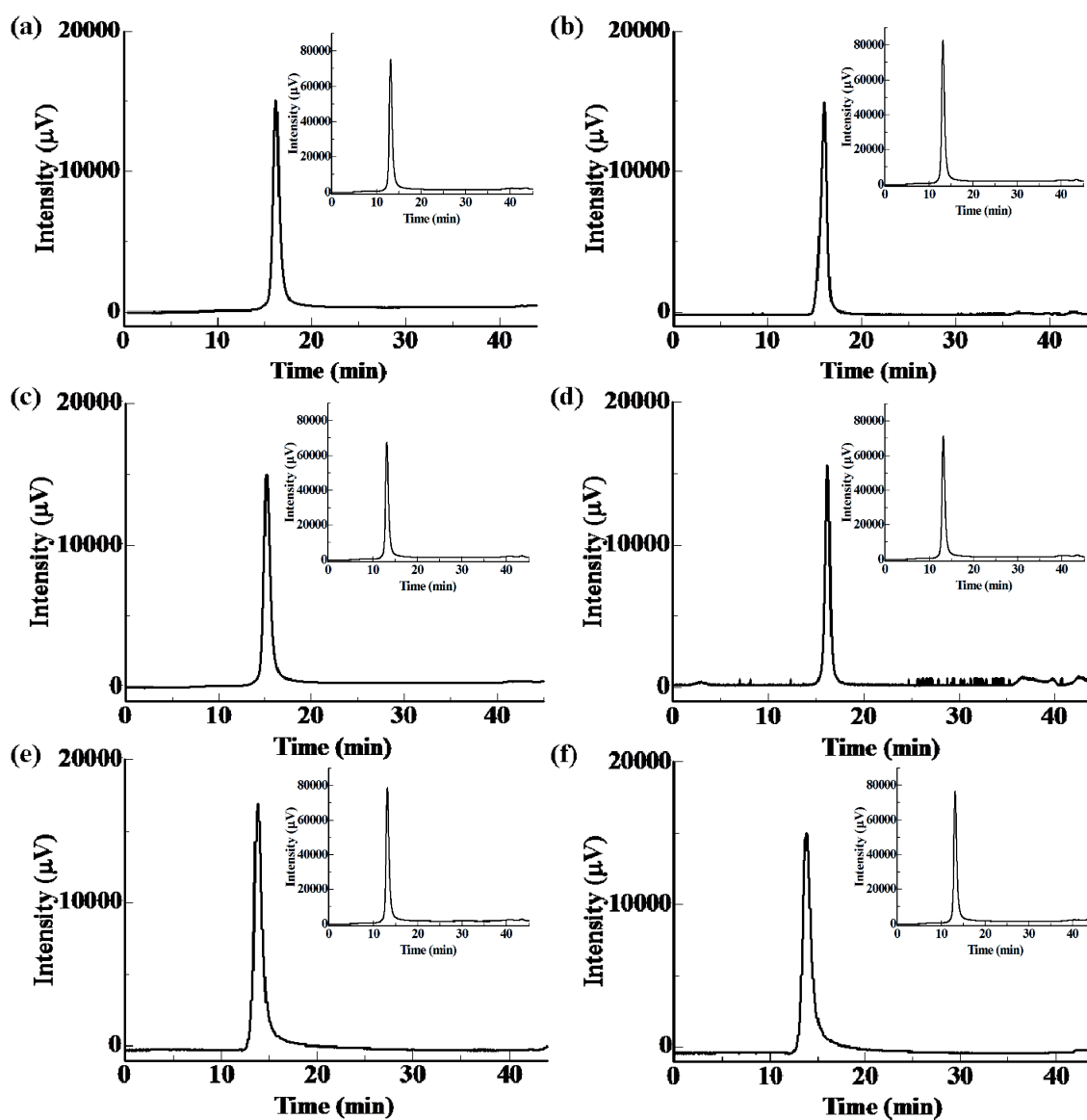


Figure S2. GPC trace of various sizes of cross-linked Cy5-Nano-PICsomes (b: 102 nm, c: 158 nm, d: 197 nm, e: 256 nm, f: 298 nm) and PIC micelles (a: 38.0 nm) determined by fluorescent detector (Ex/Em = 650/670 nm) and UV detector (inset, Absorbance at 220 nm).

Table S2. Pharmacokinetic parameters of various sizes of PIC nanoparticles.

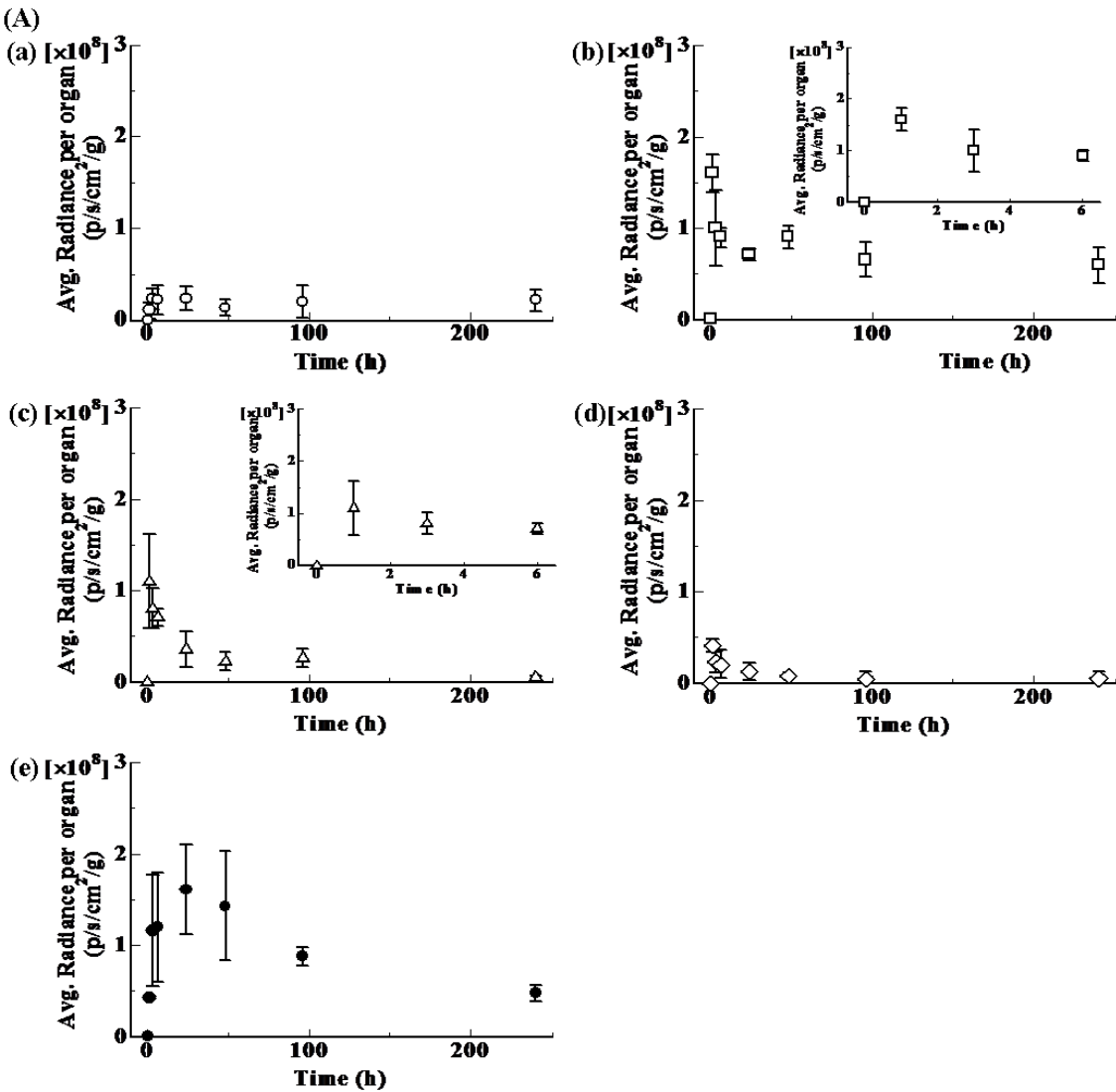
sample	blood	tumor	kidney	liver	lung	spleen
AUC (x 10 ⁹)						
PIC-38	210.0	22.0	2.3	19.0	4.8	5.5
PIC-102	260.0	21.0	0.9	13.0	5.0	6.1
PIC-158	309.0	5.9	0.4	8.8	1.5	11.0
PIC-197	308.2	0.5	0.3	7.7	0.4	21.0
PIC-256	238.3	0.2	6.4	11.0	0.3	24.0
PIC-298	230.7	0.2	4.6	7.1	0.3	27.0

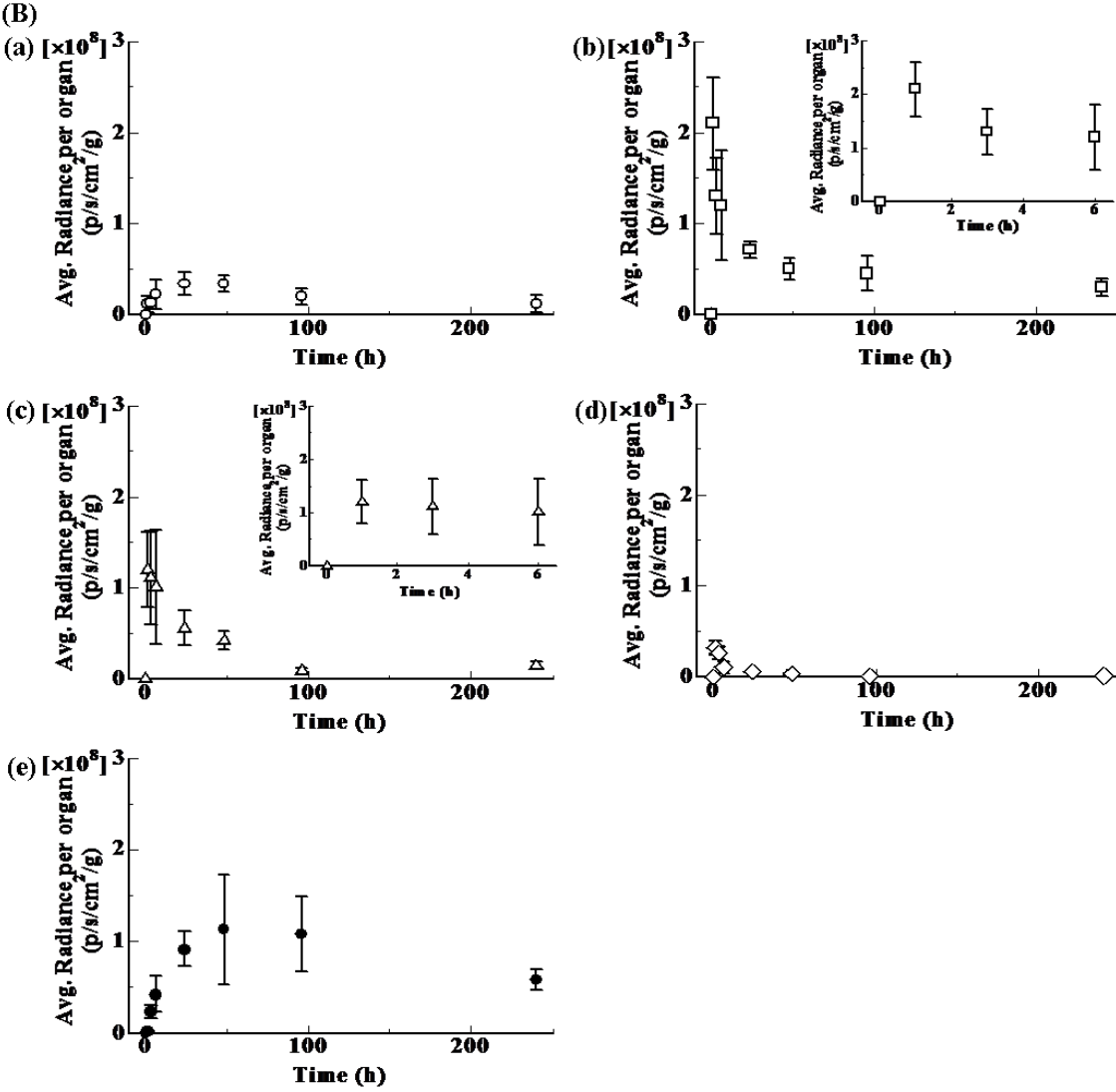
Table S3. Tumor selectivity of PIC nanoparticles.

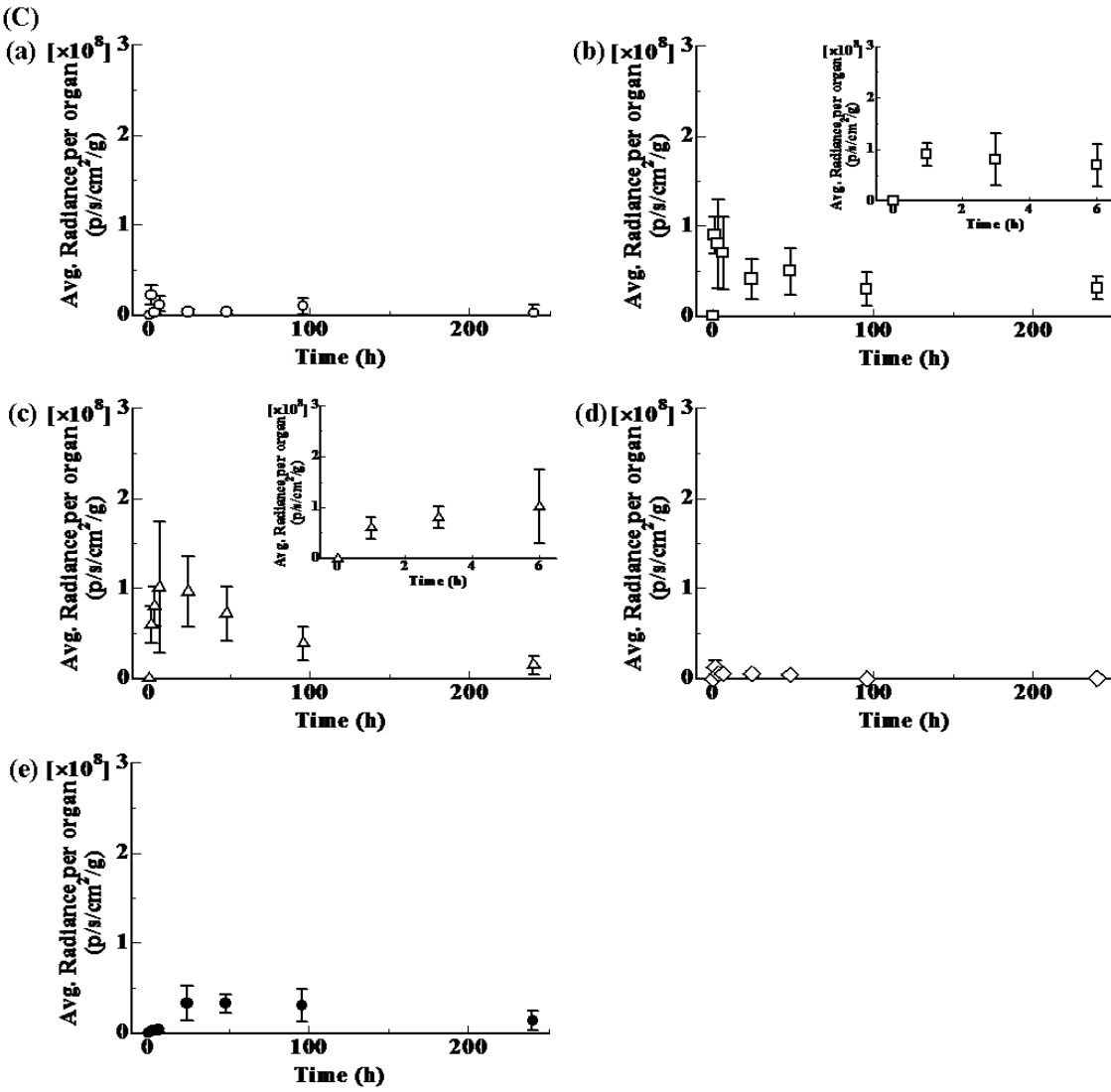
sample	blood	tumor	kidney	liver	lung	spleen
tumor to organ ratio* (AUC _{tumor} /AUC _{organ})						
PIC-38	-	1	8.0	1.1	3.8	3.4
PIC-102	-	1	25.0	1.8	4.2	3.4
PIC-158	-	1	15.0	0.7	3.9	0.5
PIC-197	-	1	1.2	0	0.8	0
PIC-256	-	1	0	0	0.5	0
PIC-298	-	1	0	0	0.5	0

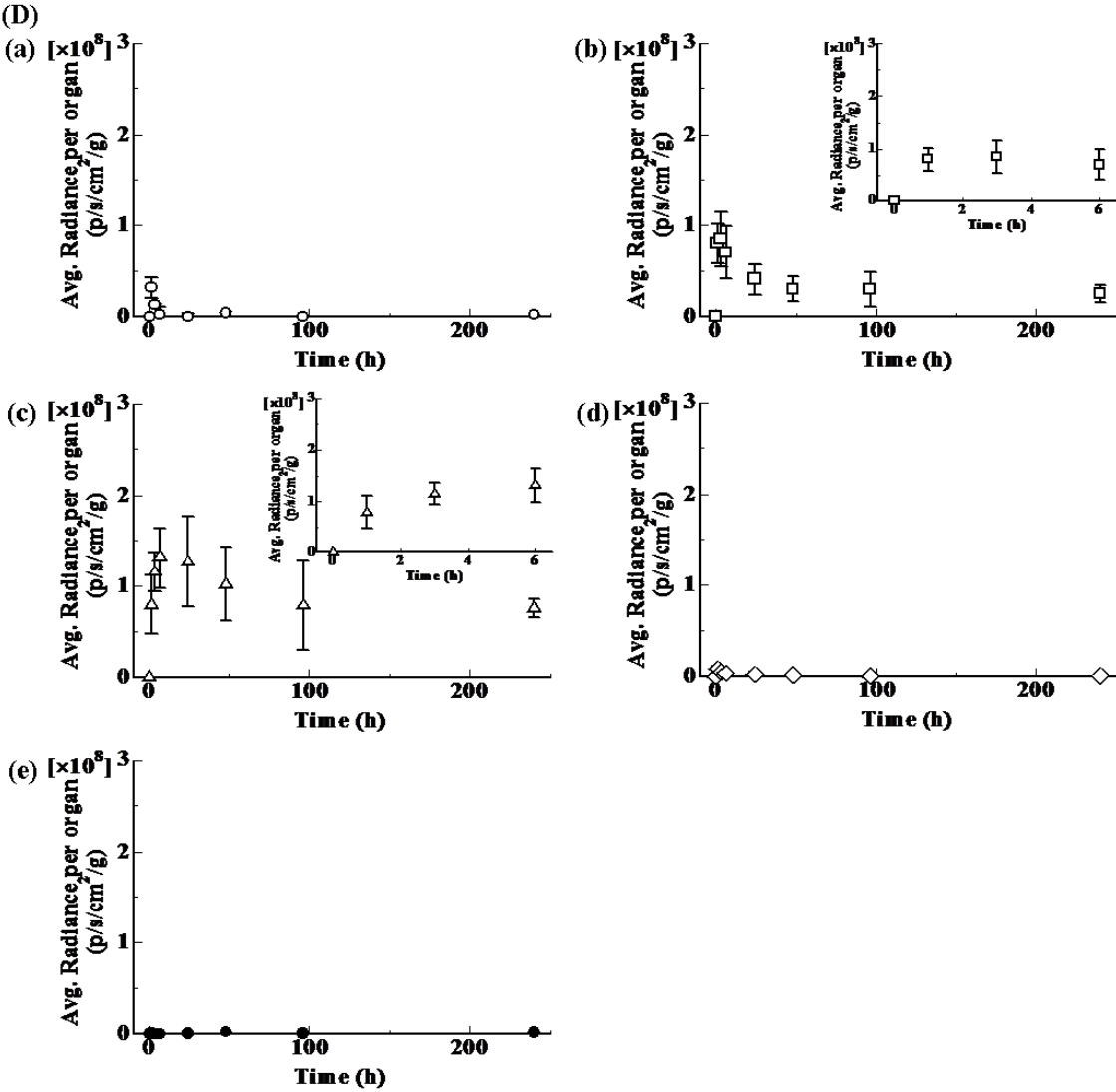
* Tumor selectivity of the particles was determined by calculating the relative accumulated concentrations between the tumor tissues and each organ (AUC_{tumor}/AUC_{organ}).

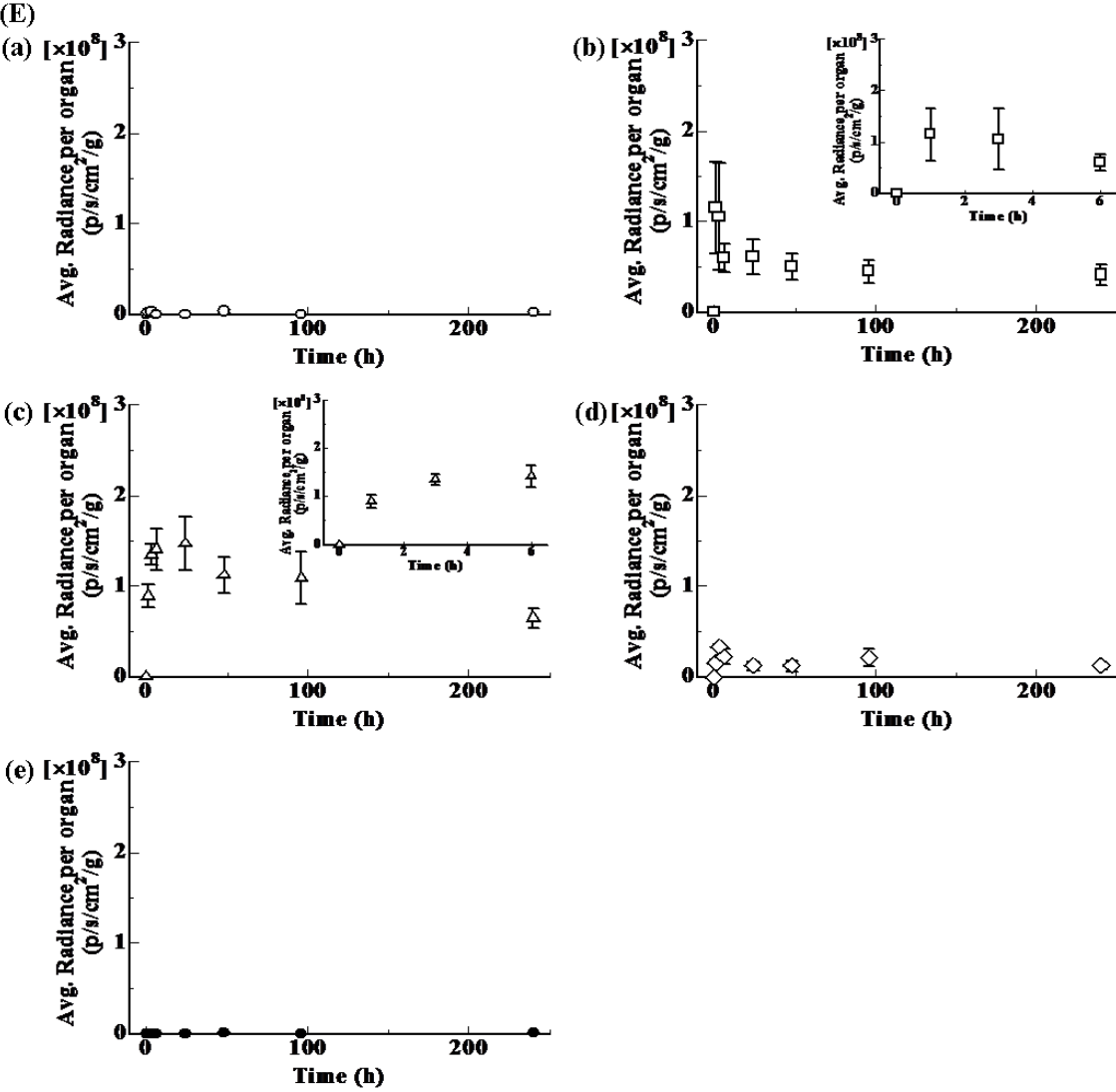
Detailed accumulation results for each organ











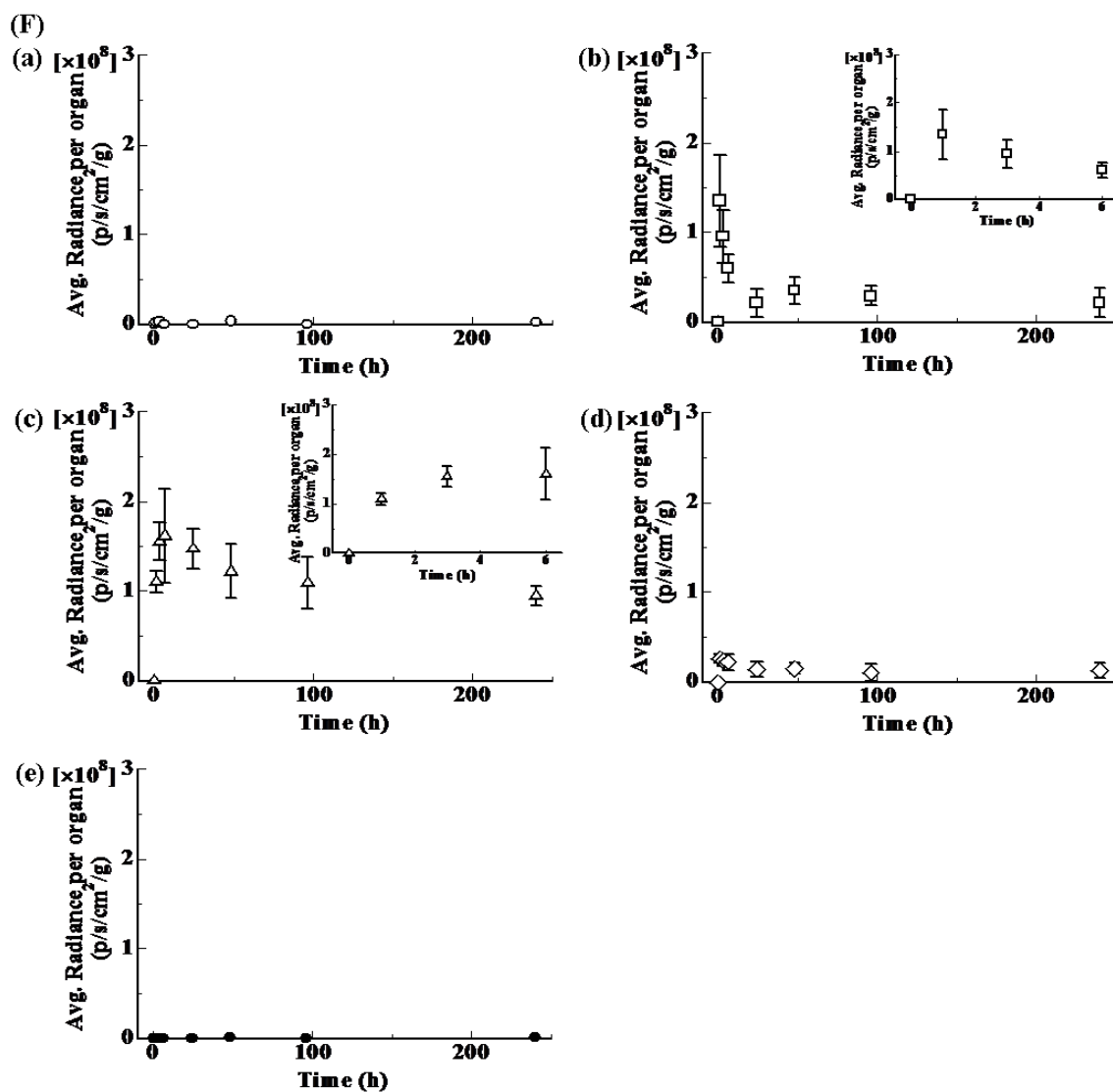


Figure S3. Accumulation of various sizes of cross-linked Cy5-Nano-PICsomes (B: 102 nm, C: 158 nm, D: 197 nm, E: 256 nm, F: 298 nm) and PIC micelles (A: 38.0 nm) in time as evaluated by fluorescence imaging. Open circles (a) lung, open square (b) liver, open triangle (c) spleen, diamond shape (d) kidney and closed circle (e) C-26 tumor.

References

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