Supporting information

FRET-based intracellular investigation of nanoprodrugs toward highly efficient anticancer drug delivery

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Experimental Section

1. Materials and Reagents

Cholesterol, 4-dimethylaminopyridine (DMAP), 12 molybdo(VI) phosphoric acid n-hydrate, anhydrous magnesium sulfate ($MgSO_4$), anhydrous sodium sulfate (Na_2SO_4), sodium chloride (NaCl), ammonium chloride (NH_4Cl), palladium-activated carbon (Pd 10%), hydrogen chloride (HCl), ethanol (EtOH), methanol (MeOH), acetonitrile (MeCN), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), tetrahydrofuran (THF), N,N-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), MES hydrate (titration, ≥99.5%), 0.4 w/v % Trypan Blue Solution, and formic acid were purchased from FUJIFILM Wako Pure Chemical Co. Esterase from porcine liver ammonium sulfate suspension, polyethyleneimine solution (PEI, 50% w/v in H_2O) and methyl (triphenylphosphoranylidene) acetate were purchased from Sigma-Aldrich Japan. SN-38, cholesterol hydrogen succinate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), Pyrrole-2-carboxaldehyde were purchased from Tokyo Chemical Industry Co. Chloroform-d ($CDCl_3$) was purchased from Acros Organics and Kanto Chemical Co., respectively. Silica gel plates 60F254 for thin layer chromatography (TLC) were purchased from Merck. Silica gel 60N (230–400 mesh) for flash chromatography was purchased from Kanto Chemical Co. Dulbecco's modified eagle medium (DMEM), Lysotracker Red® DND-99, Gentamicin, Dulbecco's phosphate buffered saline (PBS, no calcium, no magnesium), Hank's balanced salt solution (HBSS, no phenol red), GlutaMaxi supplement, fetal bovine serum (FBS, South America origin), Ethanol (absolute, 99.9%), Vybrant DiR cell-labeling solution were purchased from ThermoFisher Scientific. All the chemicals were used without further purifications.

2. Organic Synthesis

All reactions were carried out in flame-dried glassware, under an argon atmosphere, with dry solvents. Reactions were monitored by analytical thin layer chromatography (TLC) carried out on 0.25-mm silica gel plates. Visualization of the developed plate was performed using UV absorbance and aqueous cerium ammonium molybdate. Flash chromatography was performed on silica gel 60N (230–400 mesh) with the indicated solvent systems. NMR spectra were recorded on a Bruker AVANCE-400 spectrometer and calibrated using residual undeuterated solvent as an internal reference; $CDCl_3$ at δ 7.26 ppm for ^1H, and δ 77.16 ppm for ^{13}C NMR. High resolution mass spectrometry (HM-RS) was performed using micrOTOF-Q II-S1 (Bruker) using electrospray ionization (ESI) techniques.

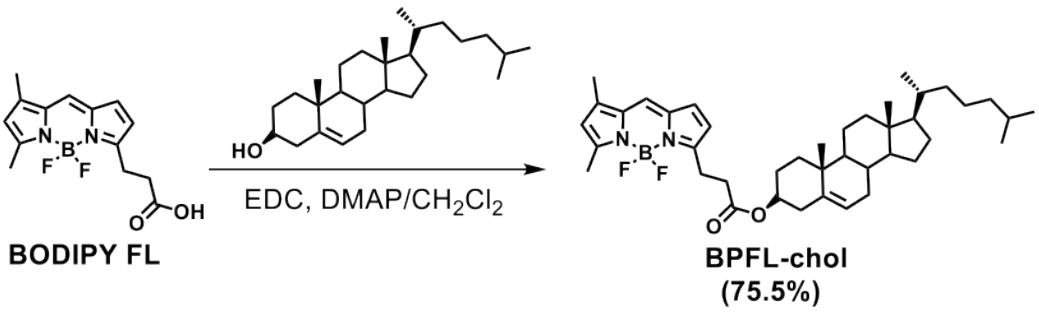
2.1. Synthesis of BPFL-chol molecules

BPFL-chol was synthesized by the esterification reaction between BODIPY FL and cholesterol, as shown in Scheme S1. BODIPY FL (10.9 mg, 37.3 μmol), cholesterol (14.4 mg, 37.3 μmol), EDC (14.3 mg, 74.6 μmol) and DMAP (0.9 mg, 7.5 μmol) were dissolved in CH_2Cl_2. After stirring overnight, the reaction mixture was diluted with CH_2Cl_2 and washed with saturated solution of NH_4Cl, water, and brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_3Cl = 100) to give BPFL-chol (18.6 mg, 28.2 μmol, 75.5%) as orange solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.08 (s, 1H), δ = 6.88 (d, J = 4.0 Hz, 1H), δ = 6.27 (d, J = 4.0 Hz, 1H), δ = 5.37 (d, J = 4.0 Hz, 1H), δ = 4.59 4.67 (m, 1H), δ = 3.28 (t, J = 7.6 Hz, 2H), δ = 2.73 (t, J = 7.6 Hz, 2H), δ = 2.56 (s, 3H), δ = 2.31 (d, J = 7.6 Hz, 2H), δ = 2.25 (s, 3H), δ = 0.85 2.22 (m, 39H), δ = 0.67 (s, 3H)

^{13}C NMR (100 MHz, $CDCl_3$): δ = 11.4, 12.0, 15.0, 18.8, 19.4, 21.1, 22.7, 23.0, 24.0, 24.1, 24.4, 27.9, 28.1, 28.4, 31.95, 32.01, 33.8, 35.9, 36.3, 36.7, 37.1, 38.2, 39.6, 39.8, 42.4, 50.1, 56.2, 56.8, 74.3, 116.9, 120.5, 122.7, 123.9, 128.2, 133.4, 135.2, 139.8, 143.8, 157.6, 160.4, 172.0

HR-MS (ESI-TOF): m/z calculated for $C_{41}H_{59}BF_2N_2O_2Na$ ($[M+Na]^+$) 683.4530, found 683.4522.



Scheme S1. Synthesis of BODIPY FL-chol.

3. NPDs preparation

All particles in this work were prepared by the reprecipitation method. SN-38-chol (9.5 mM) and BPFL-chol (0.5 mM) were dissolved in THF. 400 μL of the mixture was then rapidly injected to 10 mL

of vigorously stirred water, yielding FRET-NPDs. The fabricated FRET-NPDs were centrifuged at 15,000 rpm for 1 h and washed by distilled water. For the surface functionalization by PEI, a solution 0.75% PEI in H_2O was added to FRET-NPDs in water (1:1 v/v) and kept stirring. After 5 min, the solution was centrifuged at 15,000 rpm for 1 h and washed first with MES buffer pH 6 for 1 cycle and then with water for 2 cycles to remove the excess PEI. The resulted FRET-NPDs and PEI_FRET-NPDs were stored at 4 °C. For *in cellulo* cytotoxicity and *in vitro* drug release experiments, SN-38-NPDs and PEI_SN-38 NPDs without BPFL-chol were prepared in the same manner and applied.

4. Characterization of NPDs

After the preparation of a NPD dispersion in water, size and shape of NPDs were evaluated by field emission scanning electron microscopy (FE-SEM). The colloidal solution was deposited on polycarbonate membrane (Whatman® Nucleopore Track-Etched Membranes) by filtration and measured by FE-SEM (Hitashi S-4800). Size distribution and zeta potential was recorded at 25 °C by Malvern Zetasizer NanoZS, with He-Ne laser (3.0 mW, 633 nm). Excitation and emission spectra of the colloidal solutions were recorded by UV/Vis/NIR spectrophotometer (JASCO V-570) and fluorescence spectrophotometer (HITACHI F-7000) using a quartz cuvette with an optical path length of 1 cm.

4.1. Steady State Luminescent Spectroscopy

Emission and excitation spectra were recorded using an Edinburgh Instruments FLS 980 spectrometer on samples placed in a quartz cuvette (10 mm path length) and sealed by a Teflon stopper. The emission was collected in “right angle mode” through the quartz cuvette and sent to a monochromator and to a photomultiplier tube (PMT) detector. The spectra are corrected for the wavelength dependence of the excitation intensity and sensitivity of the detection channel.

4.2. Time-correlated Single Photon Counting (TC-SPC)

The fluorescence decay traces on the nanosecond time scale were determined by TC-SPC technique. The frequency-doubled output (375 nm, 8.18 MHz, 2 ps FWHM) of a mode-locked Ti:Sapphire laser (Tsunami, Spectra Physics) was used as excitation source. The linearly polarized excitation light was rotated to a vertical direction by the use of a Berek compensator (New Focus) in combination with a polarization filter and directed onto the samples. The sample colloidal solutions in water were placed in a quartz cuvette (10 mm path length), sealed by a Teflon stopper, and then mounted on the device. The emission was collected under 90° with respect to the incident light and guided through a polarization filter that was set at the magic angle (54.7°) with respect to the polarization of the excitation beam. The fluorescence was spectrally resolved by a monochromator (Sciencetech 9030, 100 nm focal length, wave- length accuracy 0.3 nm), and detected by a microchannel plate

photomultiplier tube (MCP-PMT, R3809U-51, Hamamatsu). A time-correlated single photon timing PC module (SPC 830, Becker & Hickl) was used to obtain the fluorescence decay histogram in 4096 channels. The decays were recorded with 10000 counts in the peak channel, in time windows of 12 ns corresponding to 3.62 ps per channel. The traces were then analyzed individually with a time-resolved fluorescence analysis (TRFA) software based on iterative reconvolution of the data with the instrumental response function (IRF). The full width at half-maximum (FWHM) of the IRF was typically in the order of 42 ps.

5. Cell culture

HepG2 (human liver carcinoma) and A549 (human lung carcinoma) cells were purchased from RIKEN cell bank and Sigma Aldrich, respectively. Both cell lines were cultured in 25 cm^2 cell culture flasks at 37 °C under 5% CO_2 atmosphere. The passage was performed via trypsinization when the confluency reached 80%. Cells were maintained in DMEM medium with 10% FBS, 1% L-glutammax and 0.1% gentamicin. For confocal imaging, cells were seeded into 35-mm glass bottom dishes (MatTeK). When the confluency reached about 60%, the medium was replaced with fresh medium and particles were added into the dishes, which were then incubated at 37 °C under 5% CO_2 for different time intervals.

6. Cellular uptake and degradation

HepG2 and A549 cells were incubated with FRET-NPDs and PEI_FRET-NPDs at the final concentration of 2 μM in an incubator with 5% CO_2 at 37° C. After 6 h, the dishes were washed 3 times with HBSS to remove the non-interacting particles. Before fluorescence imaging, the plasma membrane was stained with DiR (1 μM) in HBSS for 15 min and then washed 3 times with 1 mL of HBSS. Cells were kept in HBSS for visualization under a confocal fluorescence microscope (FV1000, Olympus). For the measurements after 24, 48, 72 and 96 h, HBSS was replaced with new culture medium and the cell dishes were placed back to the incubator. High magnification images were obtained with 100x oil objective (N.A. 1.40). Particles with SN-38 and BODIPY FL (FRET-NPDs) were visualized with 405 nm (3 μW) excitation wavelength, while DiR was imaged by using a 635 nm laser (5 μW). A DM 405/488/559/635 was chosen as the main dichroic mirror. The emissions of FRET donor (SN-38-chol) and of FRET acceptor (BPFL-chol) from the particles were detected through bandpass filters (BA) of 415-460 nm and 540-560 nm, respectively. Emission of DiR was detected through a BA 655-755. Images were processed using FV10-ASW Viewer and Igor Pro softwares.

7. Intracellular localization: fluorescence microscopy

SN-38-NPDs and PEI_SN-38-NPDs without BODIPY FL were used to track the intracellular localization of the particles over time. HepG2 and A549 cells were incubated with the particles and washed in

the same manner as for the cellular uptake experiments (see above). The images were taken at 6, 24, 48 and 72 h after the addition of the nanoparticles. The cells were stained with LysoTracker Red® (50 nM) in HBSS for 15 min, and then washed 3 times with 1 mL of HBSS right before the confocal images were taken. Confocal images were obtained with FV 1000 Olympus microscope, using 100x oil objective (N.A. 1.40). 405 nm (3 µW) and 561 nm (1 µW) excitation wavelengths were applied for detecting SN-38-chol from Sn-38-NPDs or PEI_SN-38-NPDs, and Lysotracker-stained lysosomes, respectively. A DM 405/488/559/635 was chosen as main dichroic mirror. Emission of SN-38-chol was detected through a BA 415-459 nm and emission of LysoTracker Red® was detected through BA 575-675 nm. Images were processed using FV10-ASW Viewer Software.

8. Cell viability

HepG2 and A549 cells were seeded in 12-well plates at a density of 2×10^5 cells/mL. After 24 h, culture medium was refreshed, and SN-38-NPDs were added to the cells. After incubating cells with SN-38-NPDs for 6 h, cells were washed with PBS 3 times to remove extracellular particles and fresh medium was added. After further incubation for 48, 72 or 96 h, cells were washed with PBS to remove dead cells. Viable cells were removed from the plate by trypsinization, stained with Trypan Blue (to exclude non-viable cells from the counting), and finally transferred to hemocytometer for counting under bright field microscopy. Cells without addition of NPDs were used as control. The experiment was performed in triplicate and the viability data was expressed as mean percentage of viable cells compared to control with error bars indicating the standard deviation (SD).

9. Drug release kinetics

5 µL of 2mM SN-38-NPDs in water were added to 495 µL of either water or esterase 150 unit in PBS, individually, and incubated at 4 °C or 37 °C. After 6, 24, 48, 72 or 96 h, 50 µL aliquots were transferred to new microtubes. To the tube, 450 µL of MeOH were added and the tube was vortexed. After centrifugation at 1000 g for 5 min, supernatant was transferred to a new microtube, diluted with MeCN and subjected to HPLC/MS-MS analysis. HPLC: 1260 Infinity (Agilent) and MS: HCT ultra-IMR (Bruker) were used for the quantitative determination of SN-38 release. The HPLC conditions were maintained constant for all samples as follows: column, reverse-phase column (Imtakt Cadenza UK-C8, 5 µm, ϕ 2×100 mm); column temperature, 30 °C; mobile phase, water with 0.1% formic acid/MeCN with 0.1% formic acid (v/v) = 90/10; flow rate, 0.3 mL/min; injection volume, 1 µL; retention times, SN-38 1.8 min. MS/MS was performed in $[M + H]^+$ as precursor ions and the major fragment ions from $[M + H]^+$ were detected. Precursor ions → fragment ions: SN-38 m/z 393.1 → m/z 349.1.

Abbreviation list

- NPDs: nanoprodrugs
- DDSs: drug delivery systems
- FDA: Food Drug Administration
- PEI: polyethylenimine
- SN-38: commercial name for 7-Ethyl-10-hydroxycamptothecin molecule (drug)
- SN-38-chol: cholesterol-modified SN-38 (prodrug)
- SN-38-NPDs: nanoprodrugs made of SN-38-chol
- Bodipy: boron-dipyrromethane
- BPFL: Bodipy FL
- BPFL-chol: cholesterol-modified Bodipy FL
- FRET: Förster Resonance Energy Transfer
- FRET-NPDs: Nanoprodrugs made of SN-38-chol+BPFL-chol
- PEI_SN-38-NPDs: polyethylenimine coated-nanoprodrugs made of SN-38-chol
- PEI_FRET-NPDs: polyethylenimine coated-nanoprodrugs made of SN-38-chol and BPFL-chol
- HBSS: Hank's balanced salt solution
- DMEM: Dulbecco's modified eagle medium
- FBS: Fetal bovine serum
- PBS: Dulbecco's phosphate buffered saline
- R: fluorescence intensity ratio of BPFL-chol:SN-38-chol
- THF: Tetrahydrofuran
- DMF: N,N-Dimethylformamide
- DMSO: dimethyl sulfoxide
- CLSM: confocal laser scanning microscopy
- DLS: dynamic light scattering
- FE-SEM: field-emission scanning electron microscopy
- HPLC/MS-MS: high performance liquid chromatography with tandem mass spectrometry
- λ_{det}: detection wavelength
- λ_{exc}: excitation wavelength
- Em: emission spectra
- Exc: excitation spectra
- SD: standard deviation

Supporting Figures

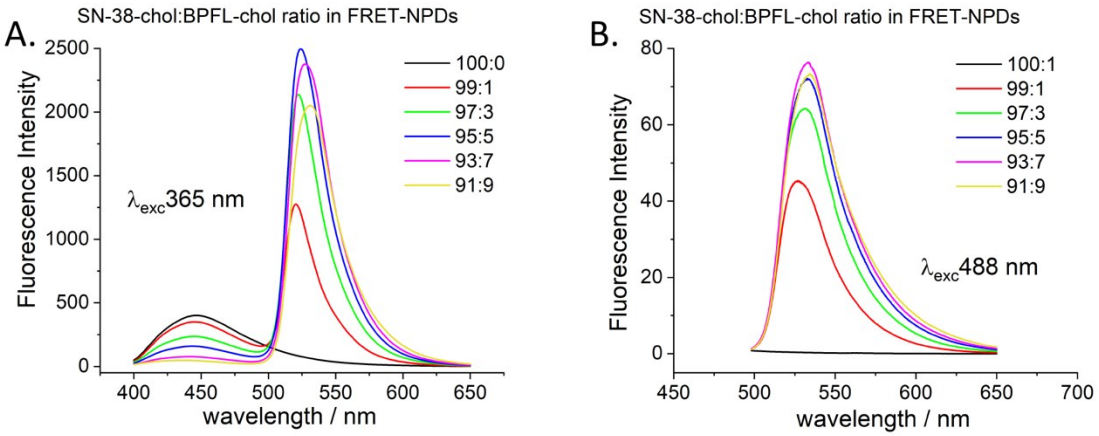


Figure S1. Optimization of SN-38-chol and BPFL-chol molar ratios in FRET-NPDs dispersed in water. Emission spectra of different SN-38-chol:BPFL-chol molar ratios: (A) λ_{exc} 365 nm; (B) λ_{exc} 488 nm.

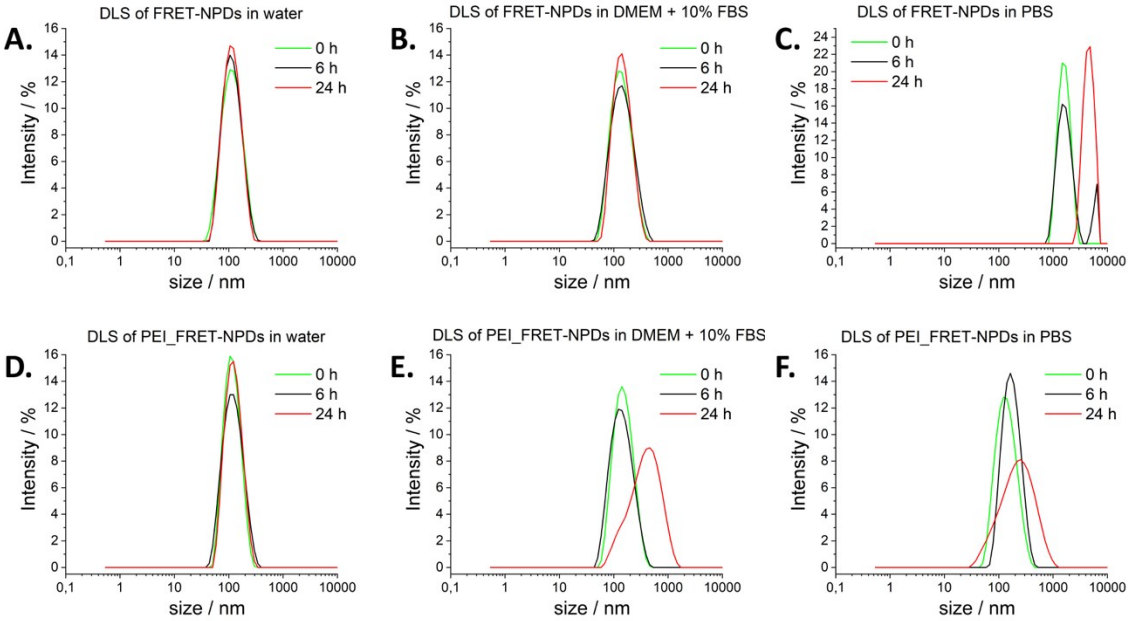


Figure S2. Nanoprodrug stability tests. DLS measurements of FRET-NPDs (A-C) and PEI_FRET-NPDs (D-F) in water (A,D), DMEM + 10% FBS (B,E) and PBS (C,F), respectively.

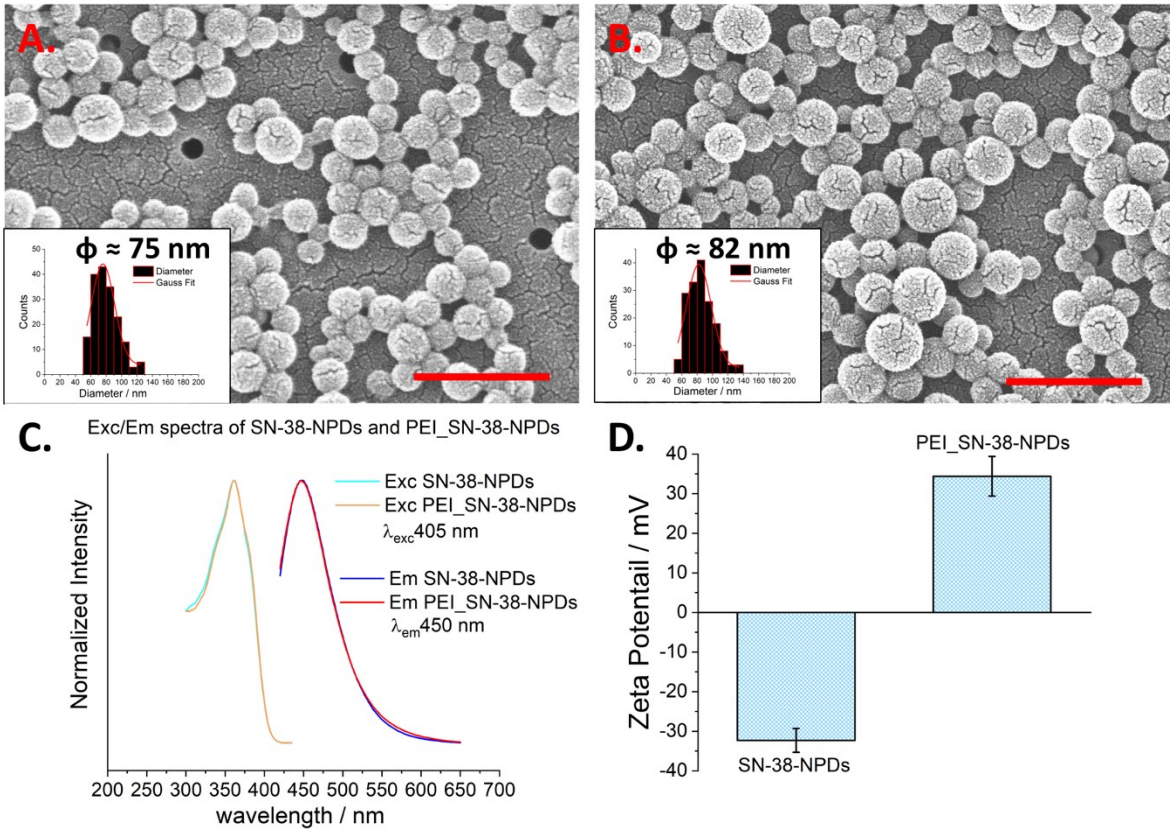


Figure S3. Characterization of SN-38-NPDs and PEI_SN-38-NPDs. FE-SEM images of SN-38-NPDs (A) and PEI_SN-38-NPDs (B), scale bar: 300 nm, inset: related diameter distribution; (C) excitation/emission spectra; (D) zeta potential.

	$\lambda_{excitation}$ (nm)	$\lambda_{detection}$ (nm)	τ_1 (ps)	A_1 (%)	τ_2 (ps)	A_2 (%)	τ_3 (ps)	A_3 (%)	χ^2
FRET-NPDs	375	525	**71**	-38.4	**1700**	47.8	**6200**	53.2	1.2
	375	470	-	-	**71**	56	**410**	44	1.9
SN-38-NPDs	375	470	-	-	**402**	44	**2000**	37.5	1.5

Table S1. Lifetimes (τ) and relative amplitudes (A) retrieved from the fit analysis of the decay traces reported in Figure 1C and D.

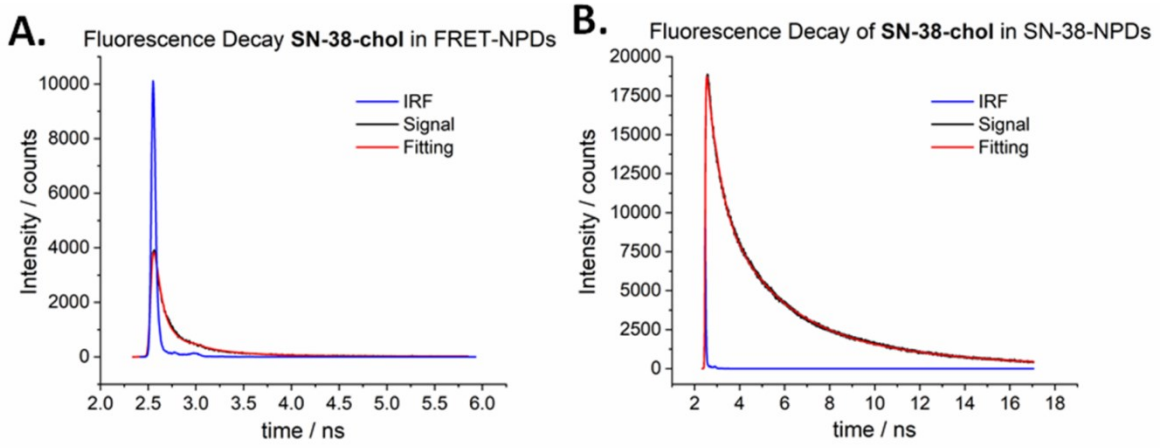


Figure S4. Fluorescence decay profiles of SN-38-chol in FRET-NPDs (A) (with BPFL-chol) and in SN-38-NPDs (B) (no BPFL-chol) (λ_{exc} 375 nm and λ_{det} 470 nm).

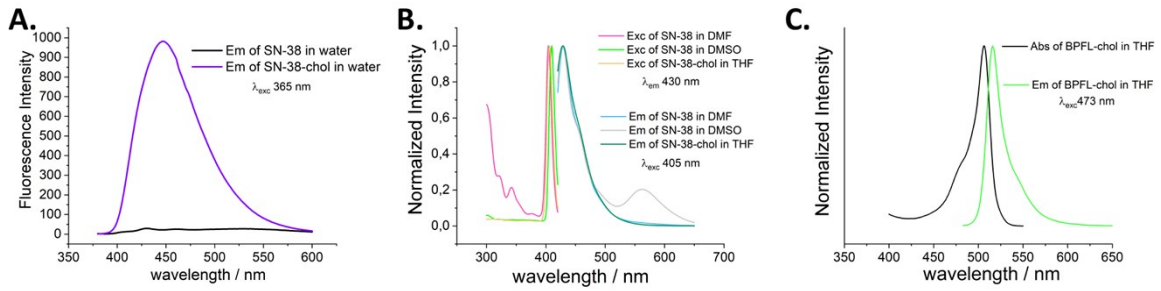


Figure S5. Fluorescence properties of SN-38-chol and BPFL-chol. (A) Emission spectra of SN-38 molecules in water and of SN-38-chol from SN-38-NPDs in water (λ_{exc} 365 nm), respectively; (B) excitation and emission spectra of SN-38 molecules dispersed in DMF and DMSO, and of SN-38-chol molecules dispersed in THF; (C) absorption and emission spectrum of BPFL-chol in THF.

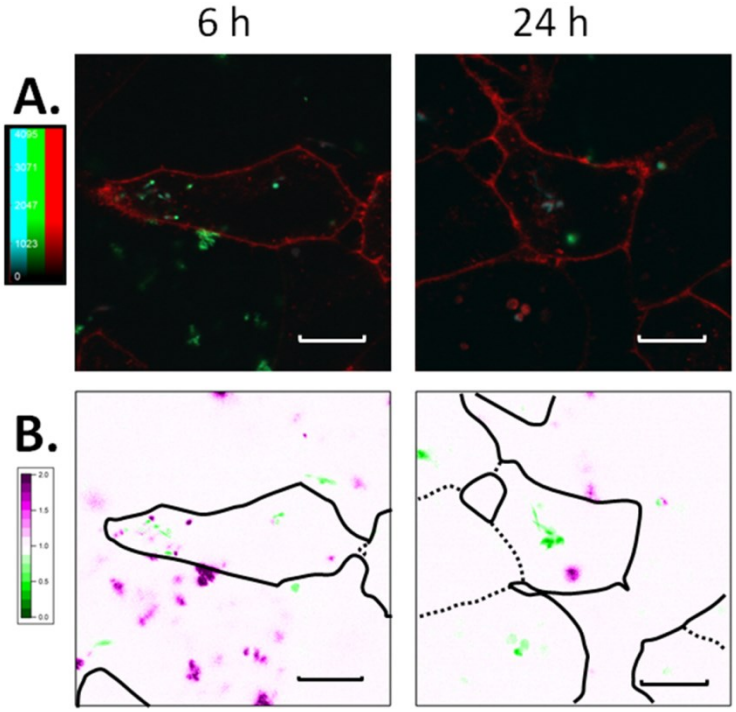


Figure S6. CLSM images of HepG2 cells (DiR staining in red) after 6 h of incubation with FRET-NPDs (A) (SN-38 chol in cyan and BPFL-chol in green): measurements taken at 6 and 24 h. BPFL-chol/SN-38-chol intensity ratio (R) images of FRET-NPDs (B) with $0 < R < 1$ (FRET OFF) in green and $R > 1$ in magenta (FRET ON). Scale bars: 10 μm. Images where entire (not degraded) FRET-NPDs were still observable (in green in A and magenta in B) were selected.

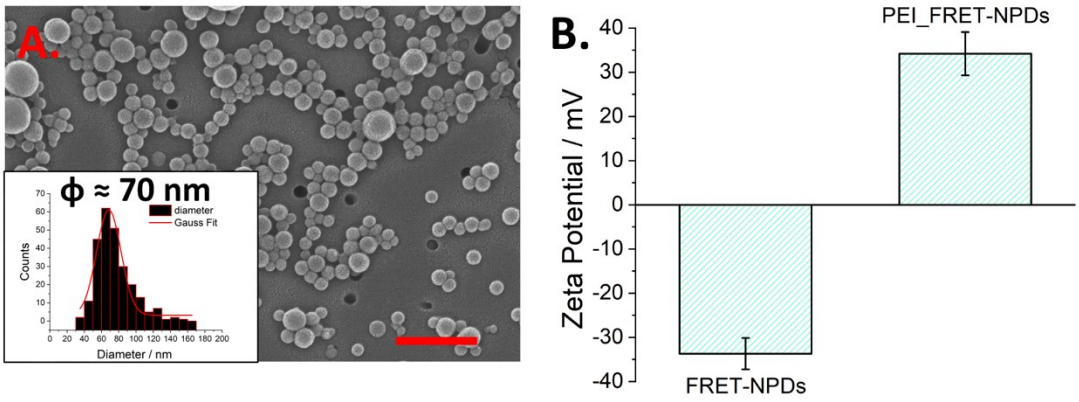


Figure S7. (A) FE-SEM images of PEI_FRET-NPDs (scale bar: 40 nm), inset: related diameter distribution; (B) zeta potential of FRET-NPDs, and PEI_FRET-NPDs.

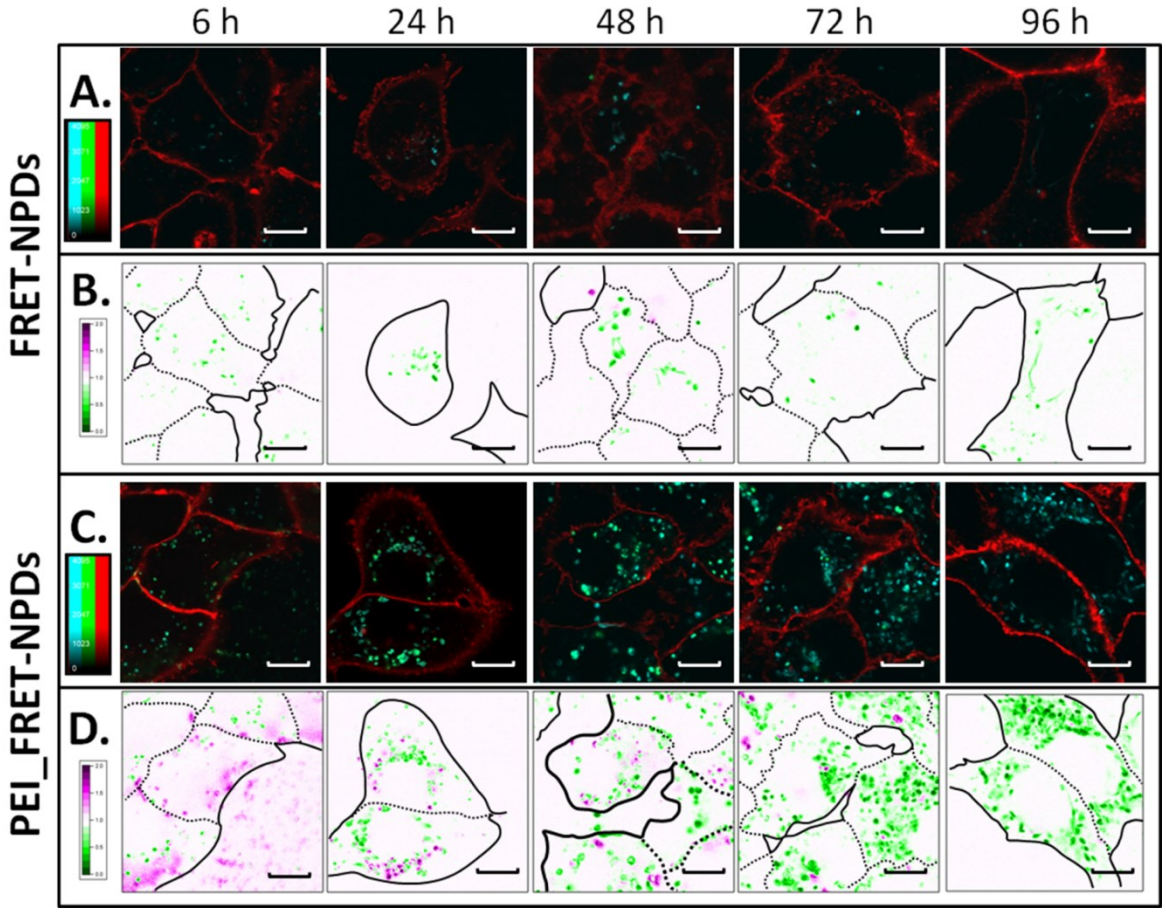


Figure S8. CLSM images of A549 cells (DiR staining in red) after 6 h of incubation with FRET-NPDs (A) and PEI_FRET-NPDS (C) (SN-38 chol in cyan and BPFL-chol in green): measurements were taken at 6, 24, 48, 72 and 96 h. BPFL-chol/SN-38-chol intensity ratio (R) images of FRET-NPDs (B) and PEI_FRET-NPDS (D) with $0 < R < 1$ (FRET OFF) in green and $R > 1$ in magenta (FRET ON). Scale bars: 10 μm.

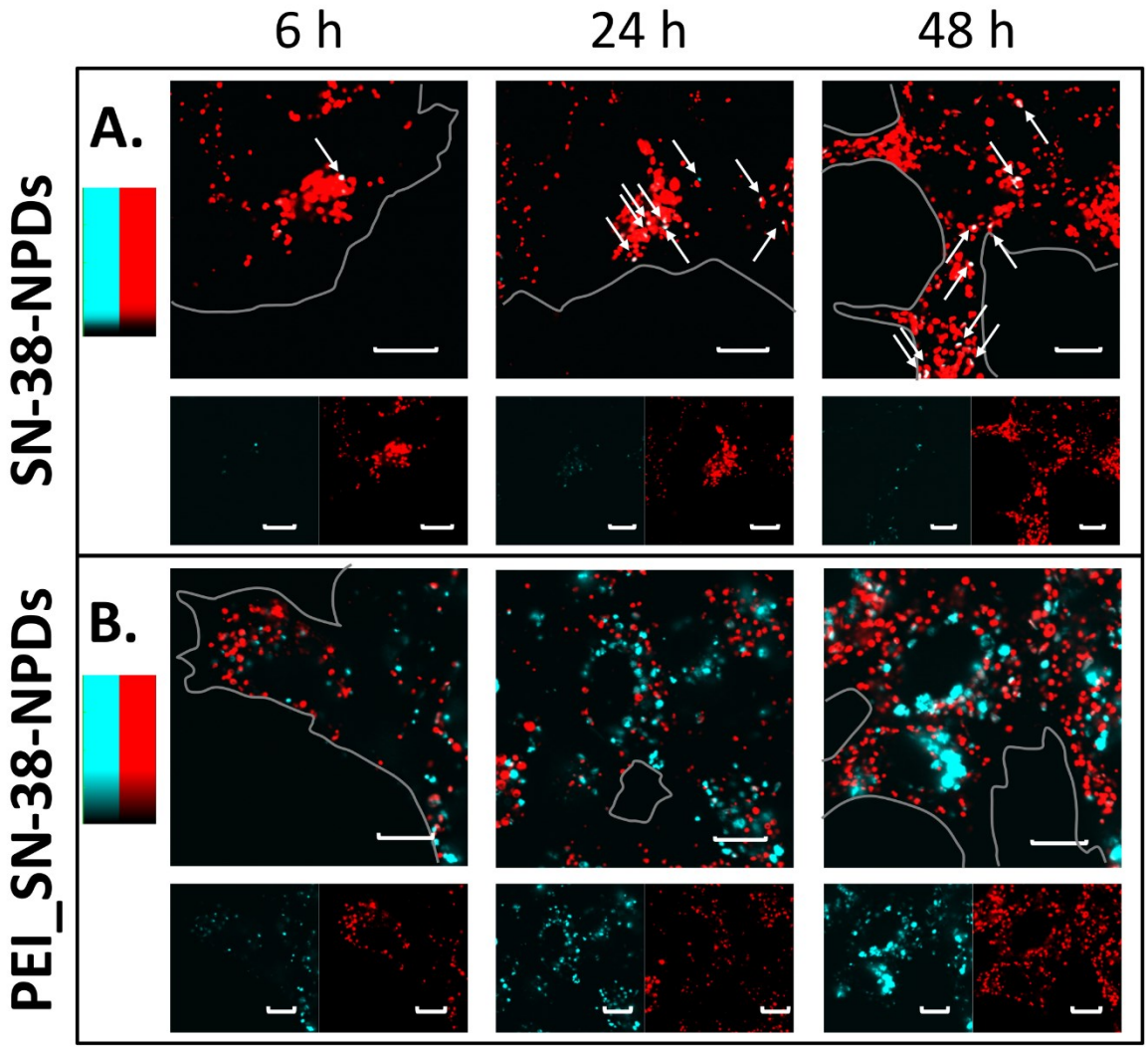


Figure S9. CLSM images of A549 cells stained with Lysotracker RED (in red) after 6 h of incubation with SN-38-NPDs (A) and PEI_SN-38-NPDs (B) (both in cyan): measurements were taken at 6, 24 and 48 h, scale bars: 10 μm.