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Supplementary Information

Anticancer Camptothecin-*N*-Poly(lactic acid) Nanoconjugates with Facile Hydrolysable Linker

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Experimental section

Materials. 20(*S*)-camptothecin (CPT) was purchased from LC Laboratories (Woburn, MA, USA) and stored at −30 °C in a glovebox prior to use. *β*-Diimine (BDI) ligands and the corresponding metal complex (BDI-EI)ZnN(TMS)$_2$ were prepared by following the published procedure[1] and stored at −30 °C in a glovebox. All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and used as received unless otherwise noted. Anhydrous tetrahydrofuran (THF), dimethylformamide (DMF), and dichloromethane (DCM) were purified by alumina columns and kept anhydrous by using molecular sieves.

Instrumentation. The molecular weights (MWs) of PLA$_n$ were determined by gel permeation chromatography (GPC, also known as size exclusion chromatography (SEC)) equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS 18-angle laser light scattering detector and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). 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, Torrance, CA, USA) 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, Santa Barbara, CA, USA). HPLC analyses were performed on a System Gold system equipped with a 126P solvent module and a System Gold 128 UV detector (Beckman Coulter, Fullerton, CA, USA) 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, Torrance, CA, USA). The NMR studies were conducted on a Varian UI500NB

system (500 MHz) or a Varian U400 (400 MHz). Infrared spectra were recorded on a PerkinElmer 100 serial FTIR spectrophotometer (PerkinElmer, Waltham, MA, USA). The sizes and the size distributions of the PLA_n NCs were determined on a ZetaPALS dynamic light scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). Lyophilization of the NCs was carried out on a benchtop lyophilizer (FreeZone 2.5, Fisher Scientific, USA).

Synthesis of CPT-Br. CPT (104 mg, 0.30 mmol) was suspended in anhydrous dichloromethane (5 mL) followed by the addition of 4-dimethylaminopyridine (4 mg, 0.03 mmol), bromoacetic acid (300 mg, 1.8 mmol) and diisopropylcarbodiimide (260 μL, 1.8 mmol). The mixture was stirred at rt for 24 h. The reaction was monitored by HPLC. After the solvent was evaporated in vacuum, the crude product was purified by silica column (CH_2Cl_2/EtOAc=4/1) to give CPT-Br (370 mg, 91% yield).[2] ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.40 (s, 1H, Ar*H*), 8.23 (d, 1H, Ar*H*), 7.93 (d, 1H, Ar*H*), 7.84 (t, 1H, Ar*H*), 7.69 (t, 1H, Ar*H*), 7.27 (s, 1H, –N–C=C*H*–C–), 5.71 (d, 1H, –N–CO–C–CH_2–O–), 5.43 (d, 1H, –N–CO–C–CH_2–O–), 5.30 (s, 2H, Ar–CH_2–N–), 3.83 (m, 2H, –O–CO–CH_2Br), 2.32 (q, 1H, –CH_2–CH_3), 2.20 (q, 1H, –CH_2–CH_3), 1.00 (t, 3H, –CH_2–CH_3). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 167.0, 166.2, 157.5, 152.5, 149.2, 146.8, 145.1, 131.4, 130.9, 130.0, 128.7, 128.5, 128.4, 128.3, 120.6, 96.0, 67.5, 50.2, 42.4, 32.1, 25.2, 7.8. HPLC purity: >95%. MALDI (m/z): calcd for $C_{22}H_{17}BrN_2O_5$, 470.3, [M]; found, 471.0 $[M+H]^+$.

Synthesis of CPT-*N*-OH. The mixture of CPT-Br (114 mg, 0.24 mmol), ethanol amine (22 μL, 1.5 equiv), and TEA (33 μL, 3 equiv) was dissolved in anhydrous DMF (3 mL). The mixture was stirred at rt. HPLC traced the progress of reaction. The crude product was purified by prep TLC

(CH_2Cl_2/DMF=5/1) to give CPT-*N*-OH (48 mg, 35% yield). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.48 (s, 1H, Ar*H*), 8.14 (d, 1H, Ar*H*), 8.10 (d, 1H, Ar*H*), 7.84 (t, 1H, Ar*H*), 7.69 (t, 1H, Ar*H*), 7.32 (s, 1H, –N–C=C*H*–C–), 5.52 (d, 1H, –N–CO–C–CH_2–O–), 5.40 (d, 1H, –N–CO–C–CH_2–O–), 5.26 (s, 2H, Ar–CH_2–N–), 3.58 (m, 2H, –O–CO–CH_2–NH–), 3.20 (m, 2H, –NH–CH_2–CH_2–OH), 2.80 (m, 2H, –NH–CH_2–CH_2–OH), 1.97(q, 1H, –CH_2–CH_3), 1.83 (q, 1H, –CH_2–CH_3), 0.96 (t, 3H, –CH_2–CH_3). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 178.1, 174.0, 167.9, 166.0, 158.5, 153.4, 149.0, 137.0, 135.8, 135.1, 134.4, 134.0, 133.3, 132.9, 132.5, 103.6, 83.3, 63.0, 60.5, 46.7, 41.4, 38.5, 36.2, 12.9. HPLC purity: >95%. MALDI (m/z): calcd for $C_{24}H_{23}N_3O_6$, 449.3, [M]; found, 450.3 $[M+H]^+$.

Synthesis of CPT-*N*-PLA$_n$. CPT-*N*-OH was utilized as the initiator for the polymerization of lactide (LA), in the presence of (BDI-EI)ZnN(TMS)$_2$. A typical procedure is given here. In a glovebox, CPT-*N*-OH (4.49 mg, 0.01 mmol) solution in anhydrous tetrahydrofuran (THF, 300 μL) was mixed with a 100 μL THF solution (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 THF at 50 mg/mL concentration and added to above solution under stirring. The reaction proceeded in the glovebox overnight. The conversion of LA was determined by FT-IR (by monitoring the disappearance of anhydride band at 1772 cm^{-1}) (Figure S1). After LA was completely consumed, the reaction was stopped by quenched with cold methanol solution (300 μL). The polymer was then precipitated with ether (20 mL) and dried by vacuum line. Molecular weight and PDI was accessed by GPC according to general experimental condition.

Synthesis of mPEG$_{5k}$-PLA. mPEG$_{5k}$-OH was utilized as the initiator for the polymerization of LA, in the presence of (BDI-EI)ZnN(TMS)$_2$. A typical procedure is given here. In a glovebox,

$mPEG_{5k}$-OH (50 mg, 0.01 mmol) solution in anhydrous dichloromethane (DCM, 3 mL) was mixed with a 100 μL DCM solution (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 above solution under stirring. The reaction proceeded in the glovebox overnight. The conversion of LA was determined by FT-IR (by monitoring the disappearance of anhydride band at 1772 cm^{-1}). After LA was completely consumed, the reaction was stopped by quenched with cold methanol solution (300 μL). The polymer was then precipitated with ether (50 mL) and dried by vacuum line. Molecular weight and PDI was accessed by GPC according to general experimental condition.

CPT-*N*-polylactide nanoconjugatess (NCs) formulation. CPT-*N*-PLA_n conjugate were prepared by following the procedures as described above. A DMF solution of the CPT-*N*-PLA_n conjugate (100 μL, 10 mg/mL) was added dropwise to nanopure water (2 mL). The resulting NCs were collected by ultrafiltration (5 min, 3000 × *g*, Ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA, USA), washed with water and then characterized by DLS for particle sizes and size distributions.

PEGylated CPT-*N*-polylactide nanoconjugatess (NCs) formulation. A DMF solution of the mixture of CPT-*N*-PLA_n conjugate (100 μL, 20 mg/mL) and $mPEG_{5k}$-PLA (100 μL, 20 mg/mL) was added dropwise to fast stirring nanopure water (4 mL). The resulting NCs were collected by ultrafiltration (5 min, 3000 × *g*, Ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA, USA), washed with water and then characterized by DLS for particle sizes and size distributions.

Extraction of CPT from NCs. PEGylated CPT-*N*-PLA_{10} NCs (1 mg) in water (1 mL) was treated with the NaOH solution (1 M, 1 mL) for 12 h at rt. The pH value of CPT-*N*-PLA_{10} suspension was then tuned to 2 by phosphoric acid. The solution color turned yellow. The resulting solution was concentrated, and the released CPT was collected and purified through semi-prep HPLC column (Jupitor, 250 × 21.20 mm, 10 μ, Phenomenex, Torrance, CA, USA). The mobile phase of HPLC was acetonitrile:water:TFA (25:75:0.75, v/v/v). The collected yellow oily compound after removal of the solvents was dissolved in trace of phosphoric acid:methanol (1:1, v/v) solution (500 μL). The pH of the solution was tuned to 3-4 by NaOH (0.1 M). The resulting solution was extracted with chloroform (5 × 10 mL). The organic phase was then dried by magnesium sulfate. After the organic solvent was evaporated, a slight yellow solid was obtained. The solid was analyzed by HPLC and its spectrum was compared with the authentic CPT, showing that the released compound has identical elution time as to the authentic CPT.

Stability of PEGylated CPT-*N*-PLA_{10} NCs. PEGylated CPT-*N*-PLA_n NCs were prepared by following the procedures as described above. The obtained NCs were dispersed in PBS, cell culture medium, and human serum buffer (human serum:PBS = 1:1, v/v), respectively. The particle sizes were measured by DLS and followed over 60 min.

Lyophilization of NCs with bovine serum albumin (BSA). PEGylated CPT-*N*-PLA_n NCs were prepared by following the procedures as described above. The resulting NCs were analyzed by DLS. Bovine serum albumin (BSA) was then added into the NC 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 rt. The resulting NC solution was analyzed by DLS to assess NC size and size distribution.

Release kinetic study of NCs. PEGylated CPT-*N*-PLA_{10} NCs and PEGylated CPT-PLA_{10} NCs were prepared by following the procedures as described above. The NCs were collected and purified by ultrafiltration (5 min, 3000 × g, ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA, USA). The collected NCs were dispersed in human serum buffer (human serum:PBS=1:1, v/v, 5 mL), divided into 10 equal-volume portions, and added to 10 eppendorf microcentrifuge tubes (500 μL NC solution in each tube). The tubes were incubated at 37°C. Complete release of CPT from NC was predetermined by the hydrolysis mentioned above. At scheduled time points, two tubes were taken out, to which methanol (500 μL) was added to precipitate proteins. The precipitates were removed by centrifugation (5 min, 15000 × g). The supernatant then was injected into HPLC for analysis of the released CPT. For the HPLC analysis, acetonitrile:water (containing 1% TFA) was used as mobile phase. The gradient for acetonitrile:water (containing 1% TFA) was changed linearly from 25:75 to 75:25 over 30 min and then kept at 75:25 for 30 min and changed back to 25:75 over 5 min. the flow rate was 1.0 mL/min. Analytical C18 column (Luna C18, 250 × 4.6 mm, 5 μ, Phenomenex, Torrance, CA, USA) was used to perform the analysis. The integrated area of the CPT peak was documented and compared with that of 100% released CPT to determine the released CPT.

Cell viability. MCF-7 cells (ATCC, Manassas, VA, USA) used in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 1000 units/mL aqueous penicillin G, and 100 μg/mL streptomycin. MCF-7 cells were placed in a 96-well plate for 24 h (10 000 cells per well). Cells were washed with 100 μL of prewarmed PBS. Freshly prepared PEGylated CPT-PLA_{10} NCs, PEGylated CPT-*N*-PLA_{10} NCs, and free CPT (prepared in 1× PBS,

100 μL) were added to the cells. CPT was used as a positive control. Untreated cells without CPT were used as negative controls in this MTT assay. The cells were incubated for 72 h in a 5% CO_2 incubator at 37 °C. The standard MTT assay protocols were followed thereafter.

Tumor reduction study. C57BL/6 mice (female, 6-8 weeks) were purchased from Charles River (Wilmington, MA, USA). *Ad libitum* feeding was provided. Light was provided in a 12/12-hour light/dark cycle. The study protocol was reviewed and approved by the Illinois Institutional Animal Care and Use Committee (IACUC) of University of Illinois at Urbana–Champaign. These mice were anesthetized, shaved, and prepared for implantation of the tumor cells. Lewis lung carcinoma (LLC) cells were collected from culture, and 1×10^5 cells suspended in a 1:1 mixture of hank's balanced salt solution (HBSS) buffer and matrigel were then injected subcutaneously into left and right flank of a mouse. After two weeks when tumors had reached ~200 mm^3, mice were divided into five groups of six mice, minimizing weight and tumor size differences. Four groups of mice were treated by intravenous injection of PBS (1×), mPEG-PLA NC, PEGylated CPT-PLA NC (50 mg/kg), and PEGylated CPT-*N*-PLA NC (50 mg/kg). The remaining one group of mice was treated with free irinotecan (100 mg/kg) intraperitoneally. After dosing, the animals were monitored closely, and measurements of the tumor size for each animal were performed at regular intervals using calipers without knowledge of which injection each animal had received. The tumor volume for each time point was calculated according to the formula (length) × $(\text{width})^2/2$, where the long axis is the length, the short axis is the width. If body weight loss is beyond 20% of predosing weight, the animals were euthanized. When the tumor load reached 1200 mm^3 or the animal had become moribund, the mouse was sacrificed. The statistical analysis was undertaken using Mann-Whitney U test and p-values < 0.05 were considered statistically significant. When an animal exited the study due to tumor size or

treatment related death, the final tumor size recorded for the animal was included with the data used to calculate the mean size at subsequent time point. At the end of study, the mice were euthanized, and the LLC tumors were collected and freshly frozen with optimum cutting temperature (O.C.T.) compound (Sakura Finetek USA, Torrance, CA, USA). Tumor sections of 5 μm thickness were collected by cryostat (Leica CM3050S). Cell apoptosis in tumors was analyzed using in situ cell death detection kit (Roche Diagnostics GmbH, Mennheim, Germany) and deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end (TUNEL) staining was performed following the manufacture's procedure. Positive cells were red fluorescent and background cells were blue fluorescent (stained with 4',6-diamidino-2-phenylindole (DAPI)). The tumor sections were imaged with the confocal laser scanning microscopy (LSM 700, Zeiss) at 20 × magnification. The total number of cells (blue) and apoptotic cells (red) was counted in multiple sections (> 10) per tumor by ImageJ to quantify the apoptotic index (ratio of apoptotic cell number (TUNEL, red) to the total cell number (DAPI, blue).

Statistical analysis. Student's *t*-test comparisons at 95% confidence interval were used for statistical analysis of release kinetics and cell viability. The results were deemed significant at $0.01 < *p \leq 0.05$, highly significant at $0.001 < **p \leq 0.01$, and extremely significant at $***p \leq 0.001$.

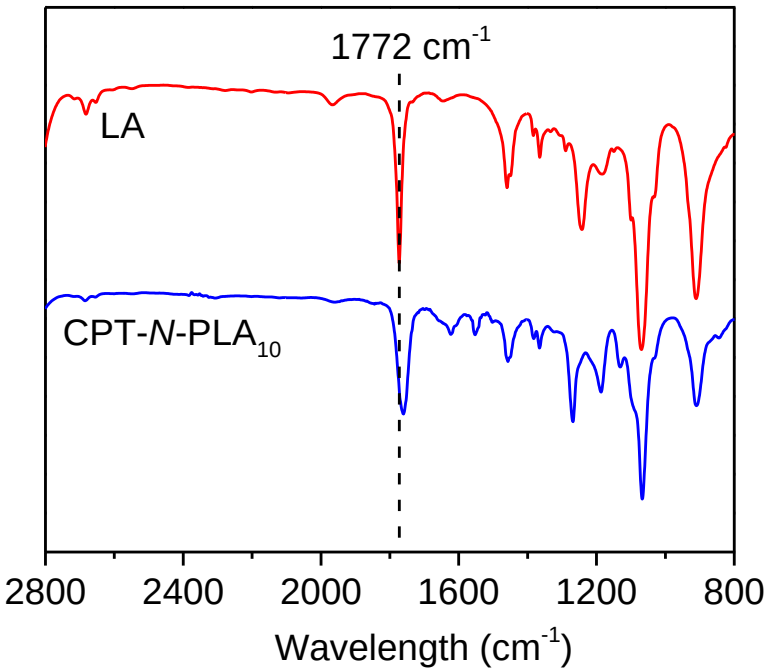
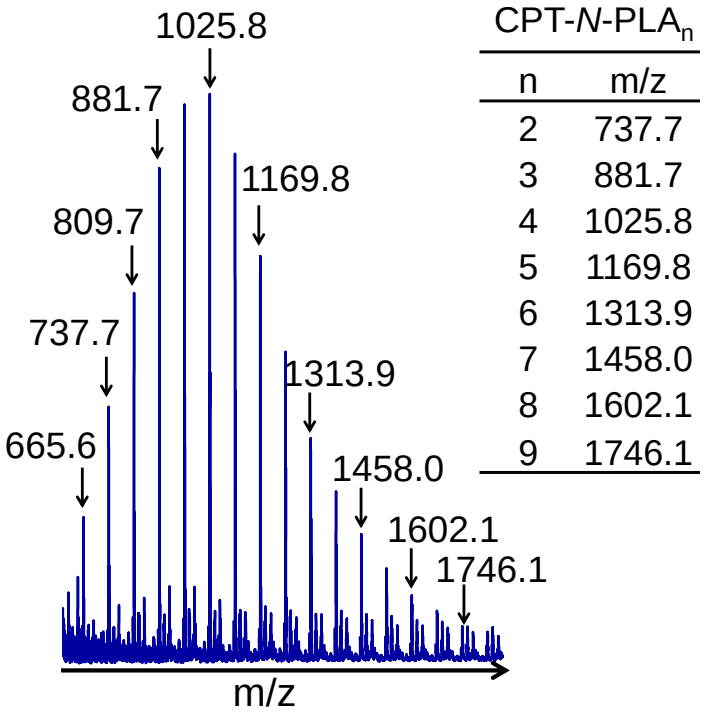


Figure S1: FT-IR spectra of lactide (LA, red) and CPT-*N*-PLA_{10} conjugates (blue).



CPT-*N*-PLA$_n$

n	m/z
2	737.7
3	881.7
4	1025.8
5	1169.8
6	1313.9
7	1458.0
8	1602.1
9	1746.1

Figure S2: MALDI-TOF MS analysis of CPT-*N*-PLA$_{10}$. The obtained m/z is identical to the calculated m/z of CPT-*N*-PLA$_n$ (449.4 + 144.13× n).

References:

1 B. M. Chamberlain, M. Cheng, D. R. Moore, T. M. Ovitt, E. B. Lobkovsky and G. W. Coates, *J Am Chem Soc*, **2001**, *123*, 3229-3238.
2 L. Tang, T. M. Fan, L. B. Borst and J. J. Cheng, *Acs Nano*, **2012**, *6*, 3954-3966.