SUPPORTING INFORMATION

High Efficiency Loading of Micellar Nanoparticles with a Light Switch for Enzyme-Induced Rapid Release of Cargo

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1. Materials and Methods

All reagents were obtained from Sigma Aldrich or Fisher Scientific and used without further purification. Polymerizations were performed under a dry nitrogen atmosphere using degassed dimethylformamide (DMF) as solvent. For the polymerization kinetics experiments, sealed ampules of DMF-d7 (Sigma Aldrich) were used. Modified second generation Grubbs' ruthenium initiator, (IMesH2)(C5H5N)2(Cl)2Ru=CHPh and *N*-(hexanoic acid)-*cis*-5-norbornene-exo-2,3-dicarboximide (Nor-Aha) were synthesized as previously reported.¹ Amino acids used for peptide synthesis were purchased from AAPPTEC, ChemPep or NovaBiochem. Thermolysin was obtained from Fisher Scientific.

NMR Spectroscopy: ¹H-NMR spectra were recorded on a Varian Inova 500 instrument at room temperature. Chemical shifts are reported relative to the residual proton signal of the deuterated solvents.

Mass Spectrometry (MS): LC-MS was performed using a Bruker amaZon X and MS spectra were detected using a amaZon SL. Analysis was performed at the Integrated Molecular Structure Education and Research Center (IMSERC) in the Department of Chemistry at Northwestern University.

Peptide Synthesizer: Peptide were synthesized using an AAPPTEC Focus XC automated synthesizer.

Reverse-phase High-Performance Liquid Chromatography (RP-HPLC): HPLC analyses of products and peptides were performed on a Jupiter 4μ m Proteo 90Å Phenomenex column (150 x 4.60 mm) with a binary gradient, using a HitachiElite LaChrom 2130 pump that was equipped with a Hitachi-Elite LaChrom L-2420 UV-Vis detector. Products and peptides were purified on a Armen Glider CPC preparatory HPLC. The solvent system for both HPLC instruments consists of (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile.

Liquid Chromatography–Mass Spectrometry (LC-MS): LC-MS analysis was performed using a Bruker amaZon X. Analysis was performed at the Integrated Molecular Structure Education and Research Center (IMSERC) in the Department of Chemistry at Northwestern University.

Size Exclusion Chromatography (SEC-MALS): Polymer molecular weights and polydispersity were determined by size-exclusion chromatography (Phenomenex Phenogel $5\mu 10^3$ Å, 1K-75K, 300 x 7.8 mm in series with a Phenomenex Phenogel $5\mu 10^3$ Å, 10K-100K, 300 x 7.8 mm) at 65 °C in 0.05 M LiBr in DMF, using a ChromTech Series 1500 pump equipped with a multi-angle light scattering detector (DAWN-HELIOS II, Wyatt Technology) and a refractive index detector (Wyatt Optilab T-rEX) normalized to a 30,000 MW polystyrene standard.

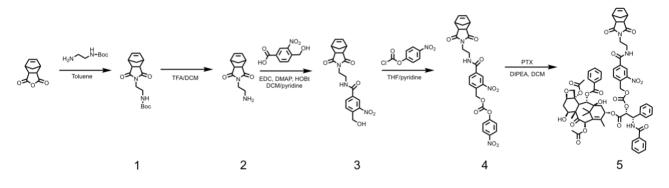
Dynamic Light Scattering (DLS): Nanoparticle hydrodynamic radii (R_h) were determined by dynamic light scattering (DLS) using a Wyatt DynaPro NanoStar.

Transmission Electron Microscopy (TEM): Nanoparticles were characterized using a Hitachi HD-2300 STEM. Grids were prepared by dropcasting the nanoparticle solutions followed by staining using a 1 % uranyl acetate solution.

Fluorescence Microscopy: Fluorescence microscopy imaging was performed on a Leica SP5 II laser scanning confocal microscope.

2. Experimental Section

2.1 Synthesis of (*N*-aminoethyl-o-nitrobenzyl)-5-norborene-exo-2,3-dicarboximide (NorENB) conjugated paclitaxel (NorENB-PTX)



Scheme S1. Synthetic scheme for NorENB-PTX.

(*N*-4-nitrophenyl ester-4-(hydroxymethyl)-3-nitrobenzoic acid-aminoethyl)-5-norborene-exo-2,3dicarboximide (**4**).

(*N*-4-(hydroxymethyl)-3-nitrobenzoic acid-aminoethyl)-5-norborene-exo-2,3-dicarboximide, **3**, was prepared as previously reported.² 4-nitrophenyl chloroformate (125 mg, 0.62 mmol) was slowly added to a solution of **3** (200 mg, 0.52 mmol) in 50 mL THF/pyridine co-solvent in a 100 mL round bottom flask at 0°C in an ice bath. After stirring for 10 minutes at 0 °C, the reaction was stirred for 12 hr at room temperature. Reaction progress was monitored via TLC (5:1 toleuene:diethyl ether, Rf =0.6). Purification was achieved through extraction with water (1 x 30 mL), followed by 0.5 M HCl (3 x 10 mL), and finally saturated NaHCO₃ (3 x 10 mL). The organic phase was dried over MgSO₄ and solvent was removed via rotary evaporation followed by purifying with column chromatography (5:1, toleuene:diethyl ether) to give the desired product as a yellow solid.

(*N*-Paclitaxel ester-4-(hydroxymethyl)-3-nitrobenzyl-aminoethyl)-5-norborene-exo-2,3dicarboximide (**5**, NorENB-PTX).

Paclitaxel (225 mg ,0.264 mmol) was added to a solution of **4** (120 mg, 0.22 mmol) and *N*,*N*-diisopropylethylamine (46 ul ,0.264 mmol) in 50 mL dry DCM in a 100 mL round bottom flask. The reaction was stirred overnight under N₂. Reaction progress was monitored via TLC (1:1 hexane:ethyl acetate, Rf =0.3). Purification was achieved through extraction with water (1 x 30 mL), followed by 0.5 M HCl (3 x 10 mL), and finally saturated NaHCO₃ (3 x 10 mL). The organic phase was dried over MgSO₄ and solvent was removed via rotary evaporation followed by purifying with column chromatography (1:1, hexane:ethyl acetate) to give the desired product as a yellow solid. (Yield = 54%) ¹H NMR (400MHz, DMF) : δ (ppm) 1.13-1.16 (s, 6H, CH₃) 1.67 (s, 3H, CH₃) 1.82 (m, 1H, CH₂) 1.92 (m, 1H, CH₂) 1.97 (s, 3H, CH₃) 2.14 (m, 1H, CH) 2.22 (s, 3H, CH₃) 2.42 (s, 2H, CH₂) 2.48 (m, 1H, CH) 2.72 (s, 2H, CH₂) 3.12 (s, 2H, CH) 3.74 (m, 2H, 2xCH₂) 3.8 (m, 1H, CH₂) 4.18 (m, 2H, CH₂) 4.37 (m, 1H, CH₂) 5.03 (t, 2H, CH₂) 5.65 (t, 1H, CH) 5.75 (s, 2H, CH₂) 5.91 (t, 1H, CH) 6.11 (t, 1H, CH) 6.32 (s, 2H, 2xCH) 6.48 (s, 1H, CH) 7.3-8.64 (m, 18H, Ar) 8.98 (s, 1H, NH).

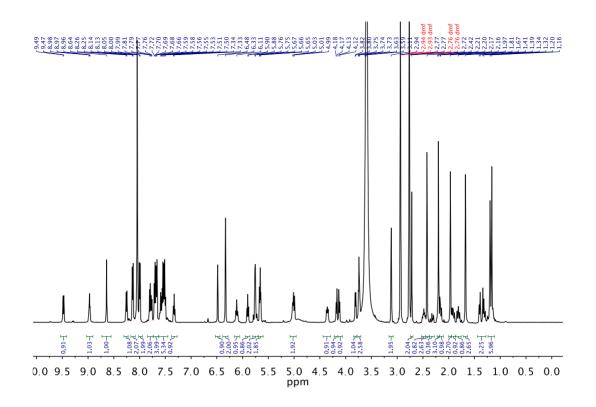


Figure S1. ¹H-NMR of NorENB-PTX in DMF.

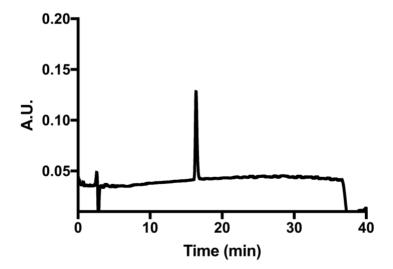


Figure S2. RP-HPLC trace of NorENB-PTX. Gradient: 15 to 65 % B over 30 minutes.

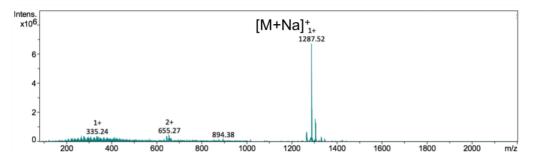


Figure S3. ESI-MS of NorENB-PTX, [M+Na]⁺ = 1287.52.

2.2 Synthesis of NorAha conjugated MMP-9 responsive peptide (NorAha-GPLGLAGGERDG)

MMP-9 responsive peptide (GPLGLAGGERDG), was synthesized on rink amide resin (loading 0.67 mmol/g) via standard Fmoc-based solid phase peptide synthesis. Fmoc deprotection was performed by agitating the resin in 20 % 4-methylpiperidine in DMF for 5 min, draining, and repeating this procedure for another 15 min. Amino acid couplings were carried out for 45 min per amino acid using N,N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) and N,N-diisopropylethylamine (DIPEA) (resin/amino acid/HATU/DIPEA 1:3:3:6). Nor-Aha was incorporated at the peptide *N*-terminus on the resin. Final peptides were cleaved from resin by treatment with trifluoracetic acid (TFA), triisopropyl silane (TIPS), and water (TFA/TIPS/ H₂O 9.5% v/v : 2.5% v/v : 2.5% v/v) for 2 hr. Peptides were then precipitated in cold ethyl ether and centrifuged, this procedure was repeated twice. The precipitated peptide products were evaporated in vacuo to give a white crude solid. Peptides were purified by RP-HPLC (20 % to 40 % buffer B over 50 min, retention time = 37 min) and lyophilized to afford a pure white solid. ESI MS (mass calculated [M+H]⁺ =1357.31 m/z; mass observed [M+H]⁺ = 1356.86 m/z).

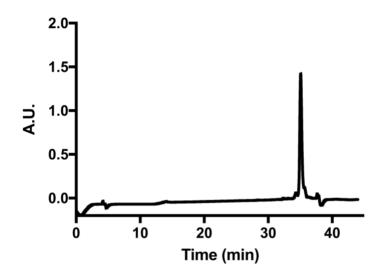


Figure S4. RP-HPLC trace of MMP-cleavable peptide. Gradient: 0 to 65 % B over 40 minutes.

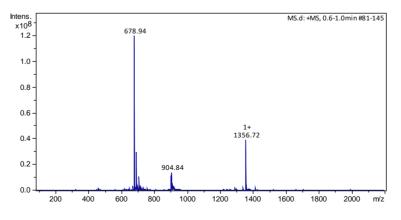
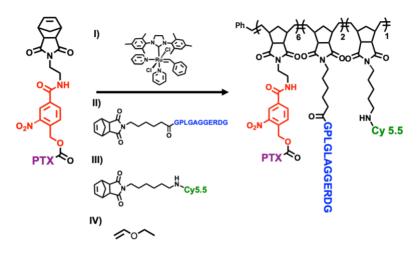


Figure S5. ESI-MS of MMP-cleavable peptide, $[M+H]^+ = 1357.31$.

2.3 Block copolymer synthesis – Light activable polymers (LAPs)



Scheme S2. Synthetic scheme of LAPs.

To a stirred solution of NorENB-PTX (25 mg, 19.8 μ mol) in dry DMF (2.0 mL), 1.45 mg (2.0 μ mol) of catalyst ((IMesH2)(C5H5N)2(Cl)2Ru=CHPh) was added under N₂ atmosphere. The reaction was left to stir under nitrogen for 45 min, after which an aliquot (20 μ L) was removed and quenched with ethyl vinyl ether. After 15 min the quenched polymer was precipitated in diethyl ether to give the homopolymer P-(NorENB-PTX)₆ as a white solid which was characterize by SEC-MALS. To the remaining reaction solutions, 8.1 mg (6.0 μ mol) of NorAha-GPLGLAGGERDG monomer was added. The mixtures were left to stir under nitrogen for 75 min. After this time, each reaction mixture was split into two portions, half was reacted with ethyl vinyl ether (50 μ L) and precipitated in diethyl ether to give the block copolymers P-(NorENB-PTX)₆-(NorAha-GPLGLAGGERDG)₂ as off-white solid. The second solutions were reacted with Nor-Cy5.5 (1.9 mg, 2.0 μ mol) for 2 hrs. After this time, ethyl vinyl ether (50 μ L) was added to quench the catalyst. After 15 min the solutions were precipitated by dropwise addition to cold anhydrous ethyl ether to give the P-(NorENB-PTX)₆-(NorAha-GPLGLAGGERDG)₂-(Nor-Cy5.5)₁.

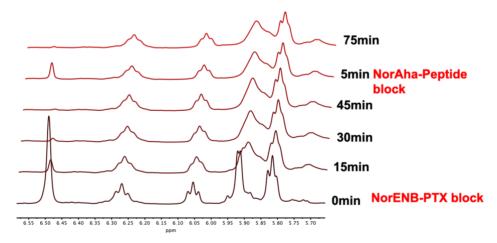
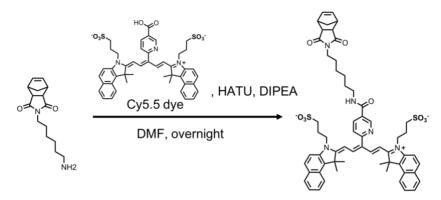


Figure S6. Polymerization kinetics of LAPs as determined by ¹H-NMR. Note the disappearance of the resonance at $\delta = 6.5$ ppm corresponding to the olefin protons of the monomer and the coincident appearance of resonances at $\delta = 5.5-6.3$ ppm, which correspond to the cis and trans olefin protons of the polymer backbone.

Table S1. Number average molecular weights (M_n) and polydispersity (PDI) of the light-activable block copolymers (LAPs).

| Block Copolymers | SEC-MALS |
|-------------------------------------|--------------------------------------|
| | M _n PDI (g/mol) (-) |
| (NorENB-PTX) ₆ | 6968 1.029 |
| (NorENB-PTX)6-(NorAha-GPLGLAGGERDG) | ⁾ ² 9821 1.044 |

2.4 Synthesis of (N-Cy5.5)-5-norborene-exo-2,3-dicarboximide (Nor-Cy5.5)



Scheme S3. Synthetic scheme of Nor-Cy5.5.

25 mg (0.030 mmol) Cy5.5, 11 mg (0.030 mmol) HATU and 62 μ L (0.36 mmol) DIPEA were dissolved in 2 mL anhydrous DMF under nitrogen. After 10 minutes, 32 mg (0.12 mmol) *N*-(hexanediamine)-*cis*-5-norbornene-exo-2,*3*-dicarboximide were added and the reaction was stirred overnight. The product was purified by RP-HPLC using a gradient from 40 to 70 % acetonitrile over 30 minutes (retention time = 30 min). ESI MS (mass calculated [M+H]⁺ =1063.42 m/z; mass observed [M+H]⁺ = 1062.99 m/z) and lyophilized to afford a pure white solid.

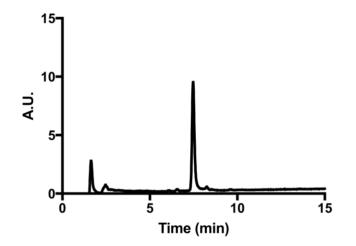


Figure S7. RP-HPLC trace of Nor-Cy5.5. Gradient: 50 to 85 % B over 15 minutes.

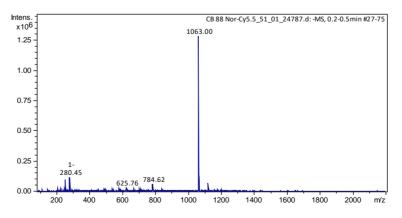


Figure S8. ESI-MS of Nor-Cy5.5, [M+H]⁺ = 1063.42.

2.5 Nanoparticle formulation – Light activable micelles (LAMs)

Nanoparticles were prepared by sonication method from DMF into DPBS. To this end, a probe sonicator (Fisher scientific) was used at 500 W. 4.2 mg (0.453 μ mol) of (NorENB-PTX)₆-(NorAha-GPLGLAGGERDG)₂ and 3.5 mg (0.339 μ mol) of (NorENB-PTX)₆-(NorAha-GPLGLAGGERDG)₂-(Nor-Cy5.5)₁, were dissolved in DMF (7.7 mL, 1 mg/mL). In the case of fluorescent nanoparticles, 60 % (mol) block copolymer without dye and 40 % (mol) block copolymer containing the Cy5.5 dye were dissolved together in DMF. DPBS (7.7 mL) was added dropwise to the polymer solution under sonication over a period of a minute. The solution was left stirring overnight and subsequently dialyzed against DPBS 1X using 3,500 molecular weight cut off (MWCO) SnakeSkin dialysis tubing. The buffer was changed two times per day for 3 days. The nanoparticles were concentrated using EMD Millipore Amiccon Ultra-15 centrifugal filters (10K Nominal Molecular Weight Limit, NMWL) when necessary and filtered through 0.2 μ m EMD Millipore sterile filters prior in vitro cell experiments.

2.6 Enzymatic cleavage of LAMs

LAMs (100 μ M, with respect to total peptide) were treated with thermolysin (1 μ M) in a total volume of 100 μ L for up to 24 hr in DPBS 1X. Experiments were performed at 40 °C and at 65 °C (optimal reaction temperature for thermolysin). Variation of the nanoparticles size was determined by DLS measurements and the morphological shift was monitored *via* TEM imaging (Figure 3 of the manuscript). LC-MS studies were performed to detect the cleaved peptide fragment (LAGGERDG) upon enzymatic digestion of the responsive nanoparticles.¹

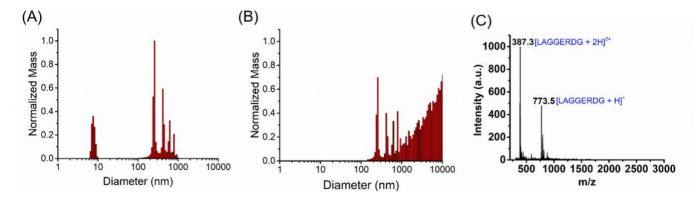
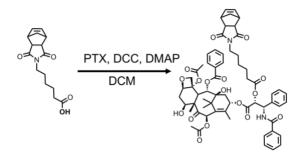


Figure S9. A) DLS of the LAM after 24 h enzymatic digestion at 40 °C and at B) 65 °C. C) Example of mass spectra of the peptide fragment (LAGGERDG) cleaved from LAM and eluted at 1.7 min retention time (RP-HPLC gradient). Control experiments determining the mass of the fragments deriving from Nor-GPLGLAGGERDG monomer cleavage: LAGGERDG and Nor-Aha-GPLG (retention time: 2 min and 7 minutes, respectively), have been previously reported using both thermolysin and MMP-9.¹

2.7 Synthesis of NorAha conjugated paclitaxel (NorAha-PTX)



Scheme S4. Synthetic scheme of NorAha-PTX.

To a solution of paclitaxel (100 mg, 117 µmol) and Nor-Aha (38.8 mg, 140 µmol) in 50 mL dry DMF in a 100 mL round bottom flask under N₂, was added 4-(dimethylamino)pyridine (1.43 mg, 11.7 µmol). After stirring for 5 minutes at 0°C in an ice bath, *N*,*N*'-dicyclohexylcarbodiimide (26.5 mg, 128.7 µmol) was dripped into the reaction mixture and allowed to stir for 7 hours. Reaction progress was monitored via TLC (1:1 hexane:ethyl acetate, Rf =0.3). The precipitated urea was removed via filtration, and the filter cake was washed with DCM. The solvent was removed via rotary evaporation and the resulting crude product was dissolved in 30 mL CHCl₃. Purification was achieved through extraction with water (1 x 30 mL), followed by 0.5 M HCl (3 x 10 mL), and finally saturated NaHCO₃ (3 x 10 mL). The organic phase was dried over MgSO₄ and solvent removed via rotary evaporation to afford the purified product in 68 % yield solid. ¹H NMR (400MHz, DMF) : δ (ppm) 1.30-2.40 (m, 22H, 12xCH₃, 5xCH₂) 2.65 (m, 4H, 1xCH, 3xCH₃) 2.91 (s, 2H, CH₂) 3.31 (s, 2H, CH₂) 3.54 (t, 2H, CH₂) 3.97 (s, 2H, CH₂) 4.35 (m, 2H, CH₂) 5.15 (s, 2H, CH₂) 5.76 (d, 1H, CH) 5.81 (d, 1H, CH) 6.04 (t, 1H, CH) 6.25 (t, 1H, CH) 6.66 (s, 1H, CH) 7.5-8.33 (m, 15H, Ar-H).

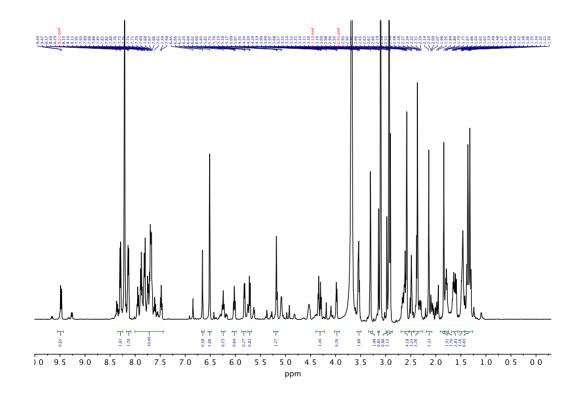


Figure S10. ¹H-NMR of NorAha-PTX in DMF.

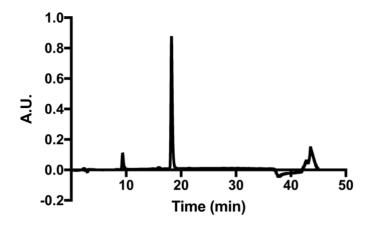


Figure S11. RP-HPLC of NorAha-PTX. Gradient: 50 to 80 % B over 30 minutes.

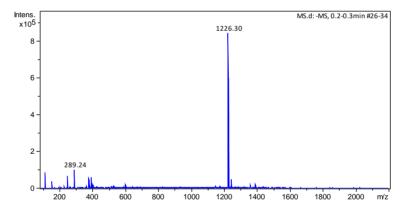
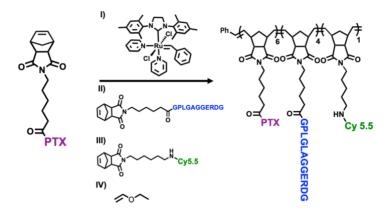


Figure S12. ESI-MS of NorAha-PTX, [M+TFA]⁺ = 1226.30.

2.8 Block copolymer synthesis – Ester Polymers (EPs)



Scheme S5. Synthetic scheme of EPs.

To a stirred solution of NorAha-PTX (25 mg, 22.5 μ mol) in dry DMF (2.25 mL), 1.64 mg (2.3 μ mol) of catalyst ((IMesH2)(C5H5N)2(Cl)2Ru=CHPh) was added under N₂ atmosphere. The reaction was left to stir under nitrogen for 30 min, after which an aliquot (20 μ L) was removed and quenched with ethyl vinyl ether. After 15 min the quenched polymer was precipitated in diethyl ether to give the homopolymer P-(NorAha-PTX)₆ as a solid which was characterize by SEC-MALS. To the remaining reaction solutions, 9.2 mg (6.75 μ mol) of NorAha-GPLGLAGGERDG monomer was added. The mixtures were left to stir under nitrogen for 75 min. After this time, each reaction mixture was split into two portions, half was reacted with ethyl vinyl ether (50 μ L) and precipitated in diethyl ether to give the block copolymers P-(NorAha-PTX)₆-(NorAha-GPLGLAGGERDG)₄ as off-white solid. The second solutions were reacted with Nor-Cy5.5 (2.2 mg, 2.3 μ mol) for 2 hrs. After this time, ethyl vinyl ether (50 μ L) was added to quench the catalyst. After 15 min the solutions were precipitated by dropwise addition to cold anhydrous ethyl ether to give the P-(NorAha-PTX)₆-(NorAha-GPLGLAGGERDG)₄-(Nor-Cy5.5)₁.

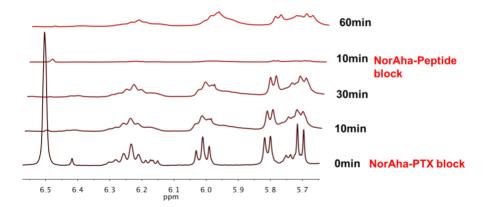


Figure S13. Polymerization kinetics of EPs as determined by ¹H-NMR. Note the disappearance of the resonance at $\delta = 6.5$ ppm corresponding to the olefin protons of the monomer and the coincident appearance of resonances at $\delta = 5.5-6.3$ ppm, which correspond to the cis and trans olefin protons of the polymer backbone.

Table S2. Number average molecular weights (M_n) and polydispersity (PDI) of the ester-containing block copolymers (EPs).

| Block Copolymers | SEC-MALS |
|------------------|-----------------------------------|
| | M _n PDI (g/mol) (-) |
| (NorAha-PTX)6 | 6817 1.047 |

(NorAha-PTX)6-(NorAha-GPLGLAGGERDG)4 12890 1.038

2.9 Nanoparticle formulation – Ester micelles (EMs)

Nanoparticles were prepared by sonication method from DMF into DPBS. A probe sonicator (Fisher scientific) was used at 500W. In a model experiment, 3.1 mg (0.240 μ mol) of (NorAha-PTX)₆-(NorAha-GPLGLAGGERDG)₄ was dissolved in DMF (3.1 mL, 1 mg/mL). DPBS (3.1 mL) was added dropwise to the polymer solution under sonication over a period of a minute. The solution was left stirring overnight and subsequently dialyzed against DPBS 1X using 3,500 molecular weight cut off (MWCO) SnakeSkin dialysis tubing. The buffer was changed two times per day for 3 days. The nanoparticles were concentrated using EMD Millipore Amicon Ultra-15 centrifugal filters (10K Nominal Molecular Weight Limit, NMWL) when necessary and filtered through 0.2 μ m EMD Millipore sterile filters prior in vitro cell experiments.

2.10 Light-induced PTX release from monomers and nanoparticles

1) Cleavage kinetics of monomers (NorAha-PTX and NorENB-PTX).

Monomer solutions (1 mM) were irradiated by UV light (365 nm) for 1hr. Each data was collected at each time points (0min, 10min, 20min, 30min, 60min).

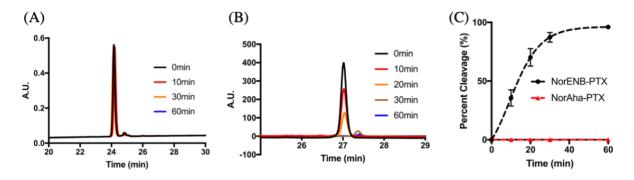


Figure S14. (A) RP-HPLC trace of NorAha-PTX under UV irradiation. (B) RP-HPLC trace of NorENB-PTX under UV irradiation. (C) Percent cleavage (%) of monomers under UV irradiation. RP-HPLC Gradient: 30 to 90 % B over 40 minutes.

2) Drug release profile from nanoparticles (EMs and LAMs).

Nanoparticles solutions were irradiated by UV light (365 nm) for 2 hr. The data were collected at different time points. After the irradiation, the solutions were analyzed by RP-HPLC and the resulting peak was compared to that of free PTX (at 18.7 min). Drug release percentage was calculated based on the free drug calibration curve and on the drug concentration in the micelle solutions.

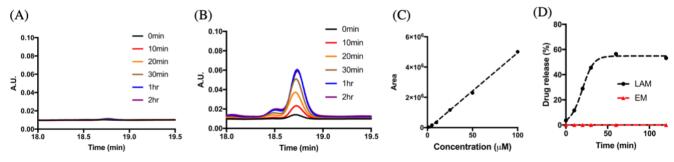


Figure S15. (A) RP-HPLC trace of EM upon UV irradiation. (B) RP-HPLC trace of LAM upon UV irradiation. (C) Calibration curve of free PTX drug. (D) Drug release (%) profile of EM and LAM upon UV irradiation. RP-HPLC Gradient: 30 to 90 % B over 40 minutes.

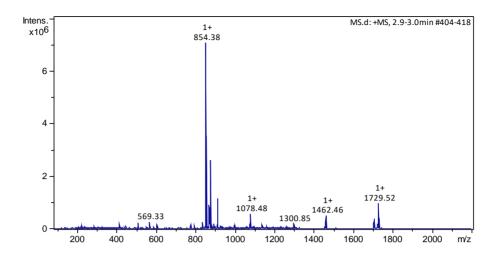


Figure S16. ESI-MS of the LAMs after UV irradiation for 30 min. The RP-HPLC is shown in Figure 4D. The observed mass corresponds to that of PTX cleaved from the polymer backbone: $[M+H]^+ = 854$.

2.11 In vitro testing

Cell culture

HT1080 cells were purchased from ATCC. Cells were cultured in Minimum Essential Media (MEM, ThermoFisher Scientific) supplemented with 10 % fetal bovine serum (FBS) (Omega Scientific, cat. #11140-050) and 1 % penicillin/streptomycin (Corning Cellgro, cat. #30-002-C1). Cells were maintained in a humidified atmosphere containing 5 % (v/v) of CO₂ at 37 °C. Cells were grown in T75 culture flasks and subcultured at ~75-80 % confluency.

Cell viability assay

The cytotoxicity of materials was assessed using the CellTilter-Blue assay. HT1080 cells were plated at a density of 10000 cells per well (100 μ L suspension) in a 96 well plate 24 hours prior to treatment. 100 μ L solution containing the compounds to be evaluated (free PTX or the micelle formulations) in DPBS at the desired concentration were added to the wells along with a 10 % DMSO positive control. Cells were incubated for 72 hours at 37 °C. Note that all concentrations were calculated with respect to paclitaxel to ensure that all polymers are compared with respect to their therapeutic components. Subsequently, the media was removed and 80 μ L of new media without phenol red was added followed by 20 μ L of CellTilter-Blue reagent. The cells were incubated for 3 hours at 37 °C. The fluorescence was measured at 560 nm excitation and 590 nm emission wavelength.

Fluorescence Microscopy.

HT-1080 cells (500 μ L) were seeded on a 4-well glass-bottom chamber at a density of 50 000 cells per well and incubated in medium for 24 h. After that, the medium was replaced with a solution of Cy5.5-labeled LAMs and EMs in medium without phenol red. The lowest concentration used for viability studies was employed to maintain high cell viability during imaging. Cells were incubated for additional 24 h. Cells nuclei were stained with Hoechst solution and imaged. All settings were kept constant between images.

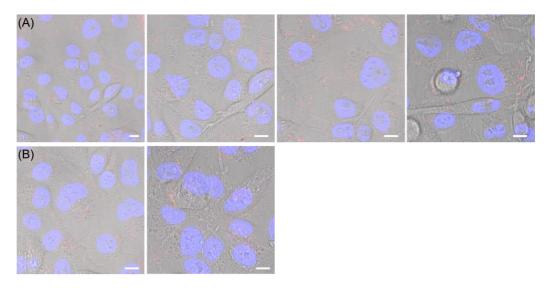


Figure S17. A) Live fluorescence imaging of A) Cy 5.5-labeled EMs and B) Cy 5.5-labeled LAMs in cell medium after 24 h incubation with HT-1080 cells. Micelle aggregates are indicated in red (Cy5.5) and the cell nuclei are stained in blue (Hoechst). Scale bars 10 μ m.

References

- 1. C. Battistella, C. E. Callmann, M. P. Thompson, S. Yao, A. V. Yeldandi, T. Hayashi, D. A. Carson and N. C. Gianneschi, *Adv Healthc Mater*, 2019, **8**, e1901105.
- 2. Y. K. Kim, Y. Huang, M. Tsuei, X. Wang, N. C. Gianneschi and N. L. Abbott, *Chemphyschem*, 2018, **19**, 2037-2045.