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Supporting Information

69451 Weinheim, Germany

Self-Assembly of Thermally Responsive Nanoparticles of a Genetically Encoded Peptide Polymer by Drug Conjugation**

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Supporting Information

Materials and Methods

Synthesis of chimeric polypeptides. The ELP segment of the CP selected for conjugation to the series of small molecule maleimide derivatives and the therapeutics gemcitabine, oxycodone, and paclitaxel– was comprised of the sequence SKGPG-$(XGVPG)_{160}$-WPC$(GGC)_7$, where the guest residue X = Val_1, Gly_7, Ala_8, and is hereafter referred to as ELP_1. This specific guest residue composition of $V_1A_8G_7$ and 160 VPGXG pentapeptide repeats was chosen to ensure that the CP has a T_t above body temperature in the concentration range of 1-100 μM, a concentration range that is useful for systemic delivery of drugs. This construct was synthesized using recursive directional ligation, as described elsewhere.[1, 2] The ELP segment of the CP nanoparticle that displays a transition temperature in the range of 37-42 °C in 90% FBS was comprised of the sequence SKGPG-$(XGVPG)_{160}$-$(CGG)_8$-WP, where X = Val_1, Ala_9, and will be referred to as ELP_2. This construct was synthesized using plasmid reconstruction recursive directional ligation.[3]

Expression and purification of CP. The ELP was expressed using a previously published hyperexpression protocol, which relies on the leakiness of the T7 promoter. [4] 50 mL cultures grown for 16 h were used to inoculate six 1 L flasks of TBDry supplemented with 100 μg/mL ampicillin (ELP_1) or 45 μg/mL kanamycin (ELP_2). Each 1 L flask was then incubated at 37 °C for 24 hrs and 210 rpm, after which the cell suspension was centrifuged at 3,000 rpm for 10 min at 4 °C. Each ELP was purified using Inverse Transition Cycling, which has been described elsewhere.[5] Briefly, the cell pellet was resuspended in PBS and lysed via sonication on ice for 3 mins (10 s on, 40 s off) (Masonix S-4000; Farmingdale, NY). Polyethyleneimine (PEI) 0.7% w/v was added to the lysate to precipitate nucleic acid contaminants. The supernatant was then subjected to multiple rounds of ITC as follows. The solution was heated to 37 °C in the presence of 3 M NaCl. The coacervate was centrifuged for 10 min at 14,000 g and 20 °C, and resuspended in 20 mM TCEP in water, pH 7. This suspension was cooled to 4 °C, and then centrifuged for 10 min at 14,000 and 4 °C to remove any insoluble contaminants. Typically, 3-5 rounds of ITC generated a sufficiently pure product (>95% by SDS-PAGE).

Purity Analysis. Following CP purification, SDS-PAGE was performed on Biorad ReadyGels with a 4-20% Tris gradient. The gels were visualized by copper staining (0.5 M $CuCl_2$).

Light Scattering characterization. Dynamic and Static light scattering measurements were performed using the ALV/CGS-3 goniometer system (Germany). Samples for the ALV/CGS-3 goniometer system were prepared in PBS at 20 μM CP and filtered through 0.22um Millex-GV filters into a 10 mm disposable borosilicate glass tube (Fischer). The tube was precleaned by washing three times with filtered ethanol (0.2 μM cellulose acetate filter). Simultaneous SLS/DLS measurements were obtained at 25°C for angles between 30°-150° at 5° increments, with each angle consisting of 3 runs for 10 seconds. Results were analyzed by partial Zimm plot analysis using ALV/Dynamic and Static FIT and PLOT software.

Cryogenic Transmission Electron Microscopy. Cryogenic Transmission Electron Microscopy was performed at the University of Pennsylvania in the Penn Regional Nanotechnology Facility (Philadelphia, PA). Lacey formvar/carbon grids (Ted Pella) were washed in chloroform to remove the formvar template and carbon coated with a Quorum Q150T ES carbon coater (Quorum Technologies, United Kingdom). Grids were cleaned with hydrogen/oxygen plasma for 15 seconds using the Solarus Advanced Plasma System 950 (Gatan, Pleasanton, CA). A sample in the form of a 2 μl drop was deposited onto the grid and added to a Gatan Cp3 cryoplunger (Gatan, Pleasanton, CA). The samples were blotted by hand and plunged into liquid ethane. Grids were transferred to a Gatan CT3500TR cryoholder (Gatan, Pleasanton, CA) and immediately inserted into a JEOL 2010 TEM (JEOL, Tokyo, Japan) operating at 80 keV. Micrographs were imaged with an Orius SC200 digital camera.

Absorbance spectroscopy. The transition temperature (T_t) of each sample was calculated by recording the optical density at 350 nm as a function of temperature (1 °C/min ramp) on a temperature controlled

UV-Vis spectrophotometer (Cary 300 Bio; Varian Instruments, Palo Alto, CA). The T_t was defined as the inflection point of the turbidity plot, or the maximum of the first derivative. All samples were analyzed in PBS, and 3-5 different concentrations ranging from 1 μM to 100 μM were used for each sample.

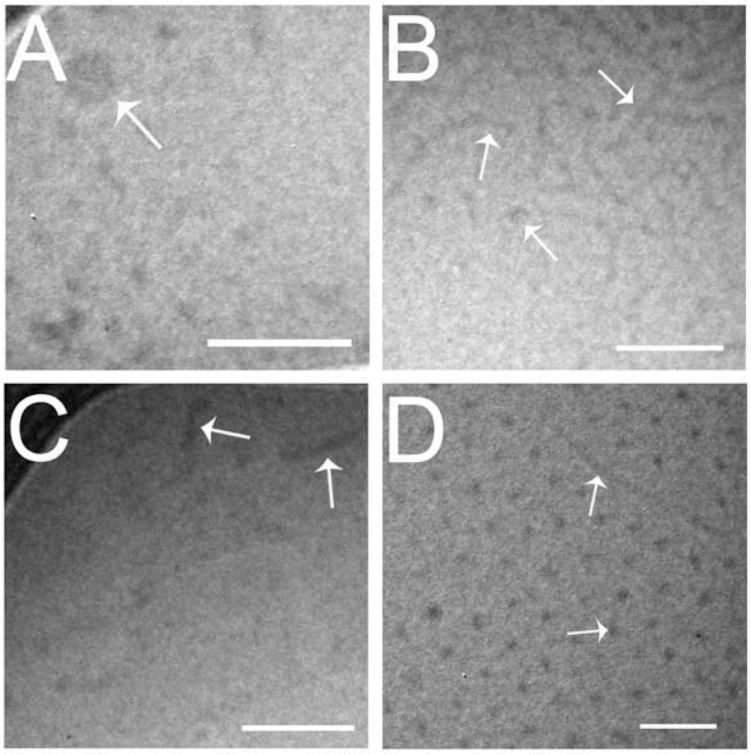
Fluorescence spectroscopy. An assay to determine the critical aggregation concentration (CAC) using pyrene as a probe was performed using a Cary Eclipse spectrophotometer equipped with a Xenon flash lamp (Varian Instruments; Palo Alto, CA). 1 μL of a stock solution of 12 mM pyrene (Fluka/Sigma-Aldrich; St. Louis, MO) in ethanol was diluted into 20 mL of PBS. Both the stock solution and the PBS mixture were sonicated for 10 minutes prior to use. This solution was used to resuspend 1-5 mg of lyophilized sample, which was then used to create a dilution series. Each sample was placed in a reduced volume cuvette, and scanned (Ex: 334; Em: 360-380, Ex slit 10 nm; Em slit 2.5 nm). Pyrene fluorescence displays four peaks; the intensity at the first (I_1; 370-373 nm) and third peak (I_3; 381-384 nm) were recorded. The ratio I_1/I_3 was plotted as a function of CP concentration. The CAC was defined as the inflection point of the sigmoid of best fit.

Conjugation of the maleimide derivatives. 15 mg of lyophilized CP was resuspended in 800 μL of 100 mM phosphate buffer, pH 7.4, and spiked with an additional 100 μL of 100 mM TCEP in water, pH 7.4. Finally 100 μL of a 50 mM solution of each maleimide derivative in DMSO was added to the CP solution dropwise, and allowed to mix for 3 hours (additional DMSO was added for compounds that were not soluble in 10% DMSO). Following conjugation, the CP was purified by passage through a PD10 column (SEC) and dialysis in ddH20 overnight. The solution was centrifuged at 13,000 g and 4 °C for 10 minutes, lyophilized, and stored at -20 °C for future use.

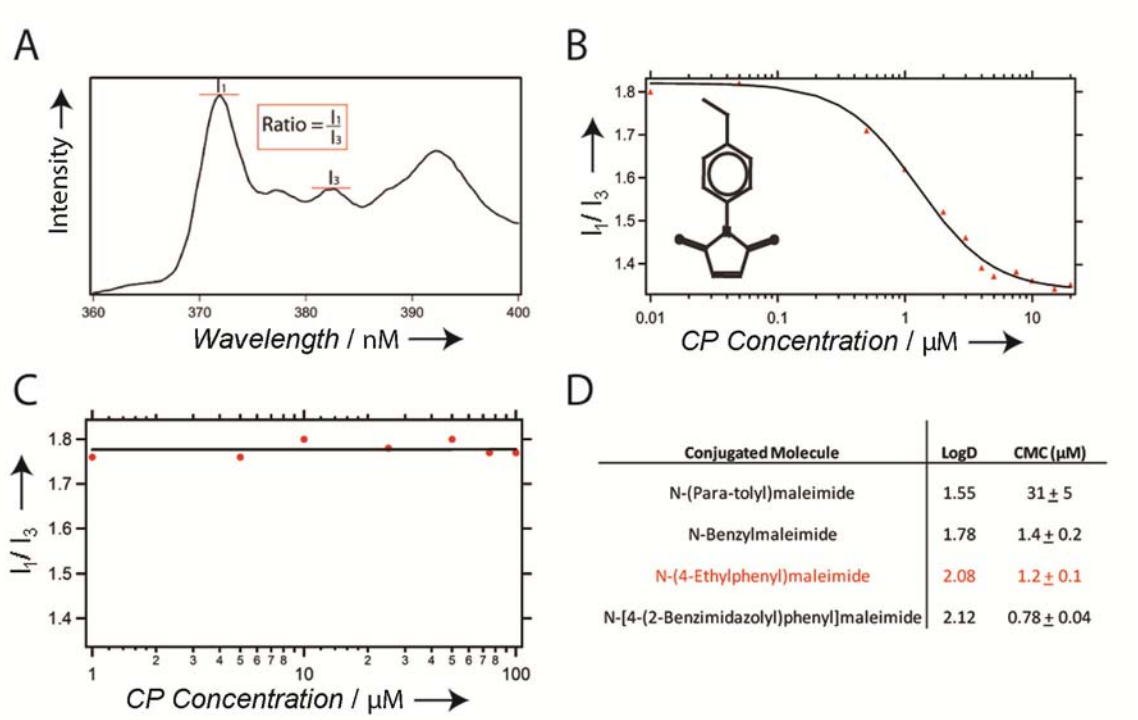
Definition of assembly. The determination of whether a construct assembled into a nanoparticle or remained in its unimer form was based on a combination of the R_h and the transition behavior (Fig 2A in manuscript). A few samples showed a combination of unimer and nanoparticle at 25 μM by DLS. This could be due to the following factors: 1) micelle theory indicates that nanoparticles assemble above a specific threshold concentration of unimer (CMC). As the bulk concentration increases past this threshold, the concentration of nanoparticles increases while the unimer concentration remains constant. If the concentration (25 μM) is near the CMC, then the unimer population will be visible via light scattering. 2) Since assembly is induced through the conjugation of chemicals, a polydisperse conjugation may result in some chains that are unconjugated or below the hydrophobicity threshold for assembly (*i.e.* only 1 molecule/chain), resulting in a substantial unimer population in addition to a nanoparticle population (3-4 molecules/chain). All samples reported as unimer were >80% unimer by mass, displayed a T_t concentration dependence of approximately -5 °C/Log(concentration), and had an average T_t of 65 ± 8 °C (over all concentrations). Samples considered nanoparticles were >50% nanoparticle by mass, displayed a T_t concentration dependence of approximately -1 °C/Log(concentration), and had an average T_t of 50 ± 1 °C.

Calculation of conjugation ratio. Following purification, 2-3 mg of lyophilized ELP was dissolved in 1 mL ddH_20. In order to break any pre-existing disulfide bonds between unconjugated cysteine residues, 100 μL of the conjugate was incubated with 100 μL of Immobilized TCEP Reducing Gel (Thermo Fisher Scientific; Rockford, IL) for 1 hr at 25 °C. Included spin columns were utilized to separate the TCEP beads from the CP conjugates. The solution was then split for use in two parallel assays. To determine the CP concentration, a 96-well bicinchoninic acid assay (Thermo Fisher Scientific) was used on a Victor^{3TM} microplate reader (Perkin Elmer; Waltham, MA) at an absorbance of 560 nm. 10 μL of CP solution was mixed with 200 μL of BCA working reagent, incubated for 30 min at 37 °C, and was compared to an ELP standard curve (100, 75, 50, 40, 33, 30, 25, 20, 16.7, 15, 13.3, 10, 6.7, 5, 3.3, 1.7, 0 uM) fit to a 2nd order polynomial in order to estimate the CP concentration. Each conjugate was measured in triplicate. To determine the concentration of free thiols, a 96-well Ellman's assay was developed for use on the Victor^{3TM} microplate reader at an absorbance of 405 nm. 40 μL of an ELP solution was mixed with 200 μL of a working reagent (25 μM Ellman's reagent in 100 mM phosphate buffer, 1mM EDTA, pH 8.0),

incubated for 2 hrs at 25 °C, and was compared to a standard curve of the ELP prior to conjugation (33, 30, 25, 20, 16.7, 15, 13.3, 10, 6.7, 5, 3.3, μM). The available cysteines in each sample could then be calculated by determining the ratio between the Ellman's assay standard curve (assumed to have 8 free cysteines / CP) and the Ellman's assay sample measurement at the concentration determined by the BCA assay. The conjugation ratio was the difference between the number of cysteines (8 / CP) and the calculated number of free cysteines.



SI Figure 1: Cryo-TEM of trace amounts of non-spherical morphologies. **A-B)** Arrows indicate both spherical (large and small) and worm-like nanoparticles present within the n-acridinylmaleimide sample. **C)** The 2-maleimidofluorene sample contained rod-like morphologies. **D)** Even within the close-packed grid of the Paclitaxel samples, worm-like micelles were present. The scale bars represent 100 nm.

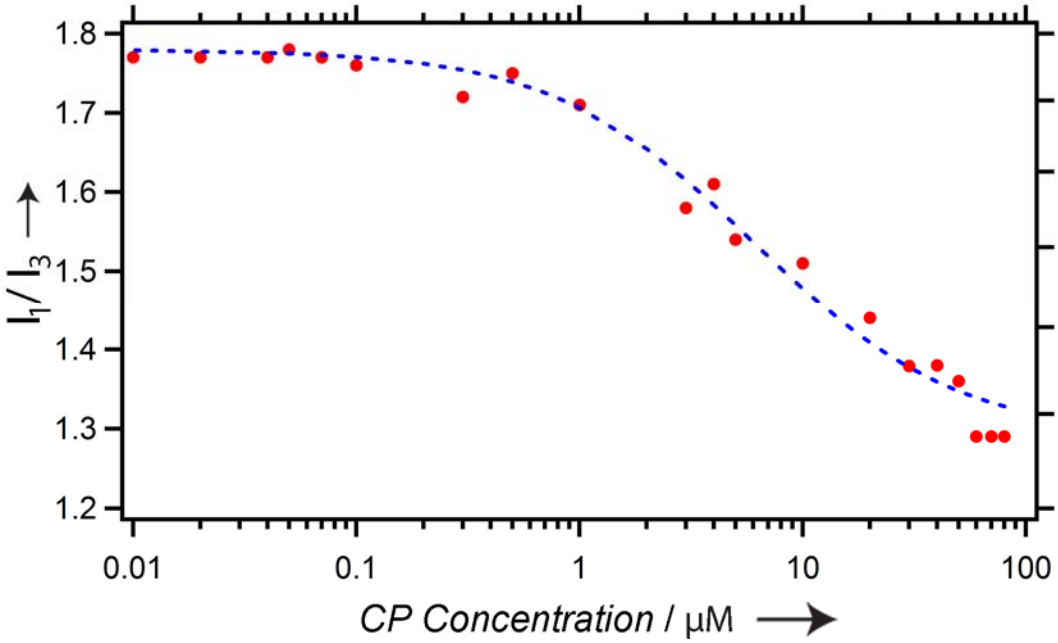


D

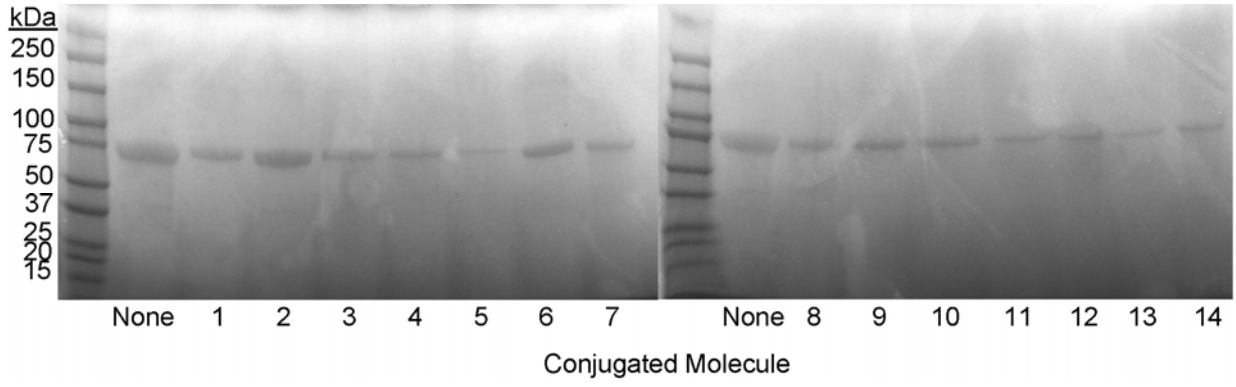
Conjugated Molecule	LogD	CMC (µM)
N-(Para-tolyl)maleimide	1.55	31 ± 5
N-Benzylmaleimide	1.78	1.4 ± 0.2
N-(4-Ethylphenyl)maleimide	2.08	1.2 ± 0.1
N-[4-(2-Benzimidazolyl)phenyl]maleimide	2.12	0.78 ± 0.04

SI Figure 2. **Fluorescence spectroscopy**. **A)** Pyrene fluorescence in water. **B)** Pyrene fluorescence of a CP conjugate of N-(4-ethylphenyl)maleimide (compound 8) as a function of CP concentration in water. **C)** Pyrene fluorescence as a function of concentration of unconjugated CP (control) in water. **D)** Table showing the Log(D) of the conjugated molecule and the critical aggregation concentration (CAC) of the CP nanoparticle conjugates calculated from the pyrene fluorescence assay for each conjugate.

Four of the conjugates that formed nanoparticles were further characterized by fluorescence spectroscopy using pyrene as a probe of the local hydrophobicity, which enables measurement of the critical aggregation concentration (CAC) of self-assembled nanoparticles.[6] For each conjugate, the ratio of the first fluorescence emission peak ($I_{370\text{-}373}$) and the third peak ($I_{381\text{-}384}$) were plotted over a range of CP concentrations (SI Figure 2A). The ratio smoothly transitioned from a low value indicating a lipophilic environment into which the pyrene can partition (I_1/I_3 = 1.35 – 1.45) at higher polymer concentrations to ~1.87 at lower polymer concentrations, a ratio indicative of disassembly and the release of pyrene into water (SI Figure 2B). This two-state transition indicates the disassembly of the nanoparticle upon reducing the concentration below the CAC. The sigmoid of best fit was used to calculate the CAC, defined as the inflection point of the curve. For an unmodified CP, the ratio remained independent of polymer concentration consistent with the absence of a lipophilic compartment, indicating the lack of self-assembly even at higher concentrations of the CP (SI Figure 2C). The CAC ranged from ~1-30 µM for the different nanoparticles, and higher Log(D) values were associated with a lower CAC (SI Figure 2D), and thus higher stability, which qualitatively scales with their hydrophobicity.



SI Figure 3: CAC of CP-Paclitaxel. The pyrene fluorescence was measured for CP-PTX as a function of CP concentration in PBS. The CAC was defined as the inflection point of the curve; CAC = 6.1 µM ± 0.6. The dashed line represents the sigmoid of best fit.



SI Figure 4: SDS-PAGE Analysis. Each of the CP conjugates (1-14) was examined by SDS-PAGE and visualized with $CuCl_2$ to ensure that the product was sufficiently pure (>95%).The values for BioRad's Precision Plus Protein Kaleidoscope standard are shown on the left.

Synthesis of CP–GEM conjugate

Synthesis of 3′, 5′-O-Bis (tert-Butoxycarbonyl)gemcitabine (1): Selective protection of 3′-OH and 5′-OH of gemcitabine was performed as described previously [7]. Briefly, to a stirred solution of gemcitabine hydrochloride (240 mg, 0.8 mmol) in 16 mL of 1M aqueous KOH, di-*tert*-butyl dicarbonate (DBDC) (1.75 g, 8 mmol) in 16 mL of dioxane was added drop wise. The reaction mixture was then stirred at 25 °C for an additional 40 min and extracted with ethyl acetate, washed with brine, dried over Na_2SO_4 and concentrated to dryness under reduced pressure.

At 25 °C, additional DBDC (1.74g) in 16 mL of dioxane was added to a stirred clear solution of the above residue, followed by the addition of 40 mL of 1 M aqueous KOH. The reaction mixture was stirred at 25 °C for another 40 min and subsequently extracted by a similar procedure as described before. The product was purified by column chromatography, and synthesis was confirmed via TLC (chloroform-acetone-EtOH 5:4:1) and LC/MS (Agilent Series 1100 LC/MSD Trap; Agilent Technologies). R_f: 0.6 in CH_2Cl_2-acetone-EtOH 5:4:1. ESI/MS: 464 [M+H].

Synthesis of 3′,5′-O-Bis(tert-Butoxycarbonyl)-4-N-(ß-Maleimidopropiocarbonyl) gemcitabine (2): To a stirred solution of BMPA (0.036 g, 0.2 mmol) and EDCI (0.041 g, 0.2 mmol) in anhydrous DCM at 0 ^oC, 1 (0.05 g, 0.1 mmol) was added, along with a catalytic amount of triethylamine. The reaction mixture was stirred for an additional 16 h at r.t and subsequently extracted with ethyl acetate, washed with brine, dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The product was purified by column chromatography, and synthesis of 2 was confirmed via TLC (chloroform-acetone-EtOH 5:4:1) and LC/MS. R_f: 0.8 in CH_2Cl_2-acetone-EtOH 5:4:1. ESI/MS: 615 [M+H].

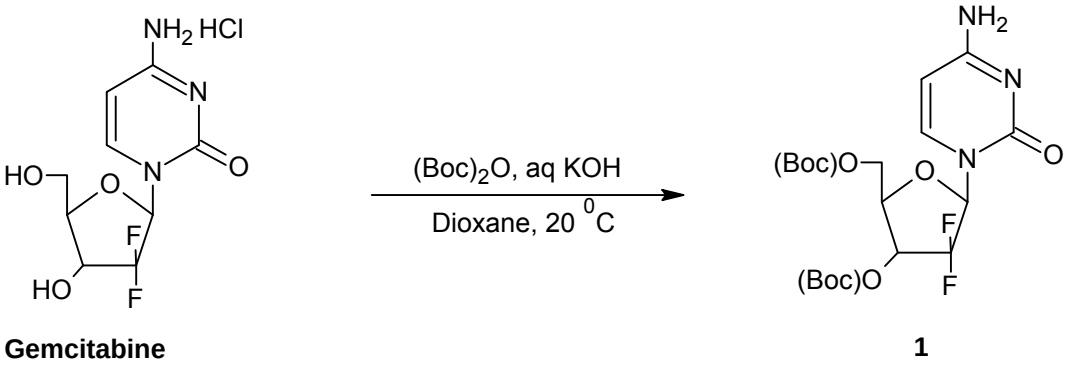
Synthesis of activated gemcitabine and Conjugation of activated gemcitabine and CP: Prior to conjugation to CP, gemcitabine-BMPA was activated via removal of *t*-BOC groups. Trifluoroacetic acid (500 µL) was added to a stirred solution of 2 (0.05 g, 0.1 mmol) in anhydrous DCM (500 µL) at 0 ^oC, and the reaction mixture was stirred at 0 ^oC for 3 hours. TFA was subsequently removed under reduced pressure and the mixture was triturated with ether to yield the desired product 3. Activated gemcitabine 3 was immediately used for the next step.

Prior to reaction, purified CP was suspended in reaction buffer (0.1 M NaPO4, 1 mM EDTA, pH 7.0) and reduced with 1 mL of TCEP at neutral pH (100 mM, pH 7.0) at ~5x excess to thiol. Excess TCEP was removed from the solution by initiating the phase transition with sodium chloride (2.5 M) and centrifugation at 4,000 rpm at 25 °C for 10 minutes. The CP pellet obtained by centrifugation was re-suspended in 5 mL of reaction buffer. Activated gemcitabine (0.03 g, 0.07 mmol) was re-suspended in 2 mL of methanol and slowly transferred to the stirring CP solution. After mixing, 1 mL of pH neutral TCEP (100 mM) was added and the reactants were stirred for 16 hours at 20 °C in the dark. After reaction, the methanol was evaporated under a nitrogen stream, and unreacted gemcitabine precipitates were removed via centrifugation at 13,000 rpm at 4 °C for 10 minutes. The supernatant contained CP-gemcitabine conjugate, which was further purified by initiating the CP phase transition with sodium chloride (2.5 M) to aggregate the CP-Gem conjugate followed by centrifugation at 4,000 rpm at 25 °C for 10 minutes. The CP-gemcitabine conjugate pellet obtained by centrifugation was re-suspended in phosphate buffered saline (PBS) (pH 7.4) at a concentration of 10 mg / mL and purified using size exclusion PD-10 chromatography (GE Healthcare Life Sciences).

Determination of gemcitabine conjugation ratio

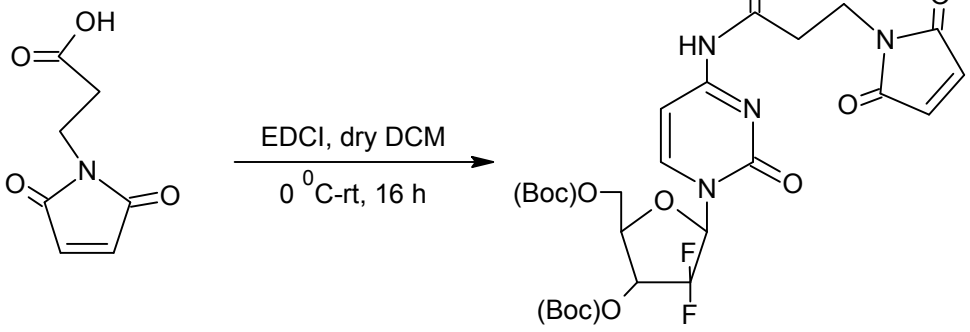
The conjugation ratio of gemcitabine to CP was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the CP-gemcitabine conjugates and free CP using a Voyager DE-Pro Maldi-MS (Applied Biosystems) instrument equipped with a nitrogen laser (337 nm). The MALDI-TOF-MS samples were prepared in an aqueous 50% acetonitrile solution containing 0.1% TFA, using a sinapinic acid matrix. The conjugation ratio was determined by examining the increase in mass of the conjugate relative to free ELP.

Scheme 1. Synthesis of ELP-GEM conjugate.



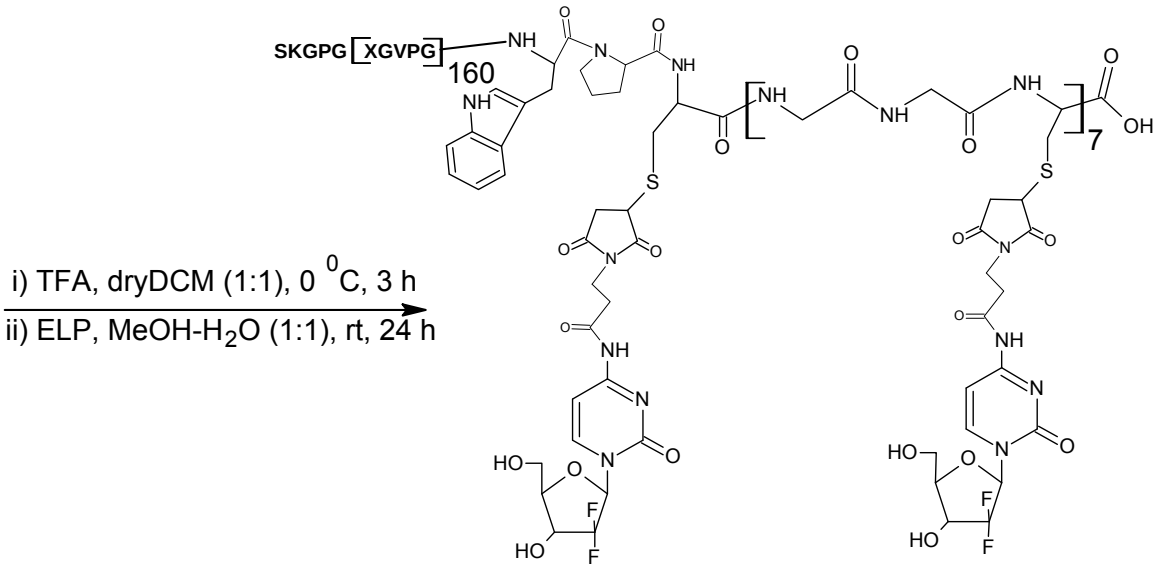
Gemcitabine **1**

1 +



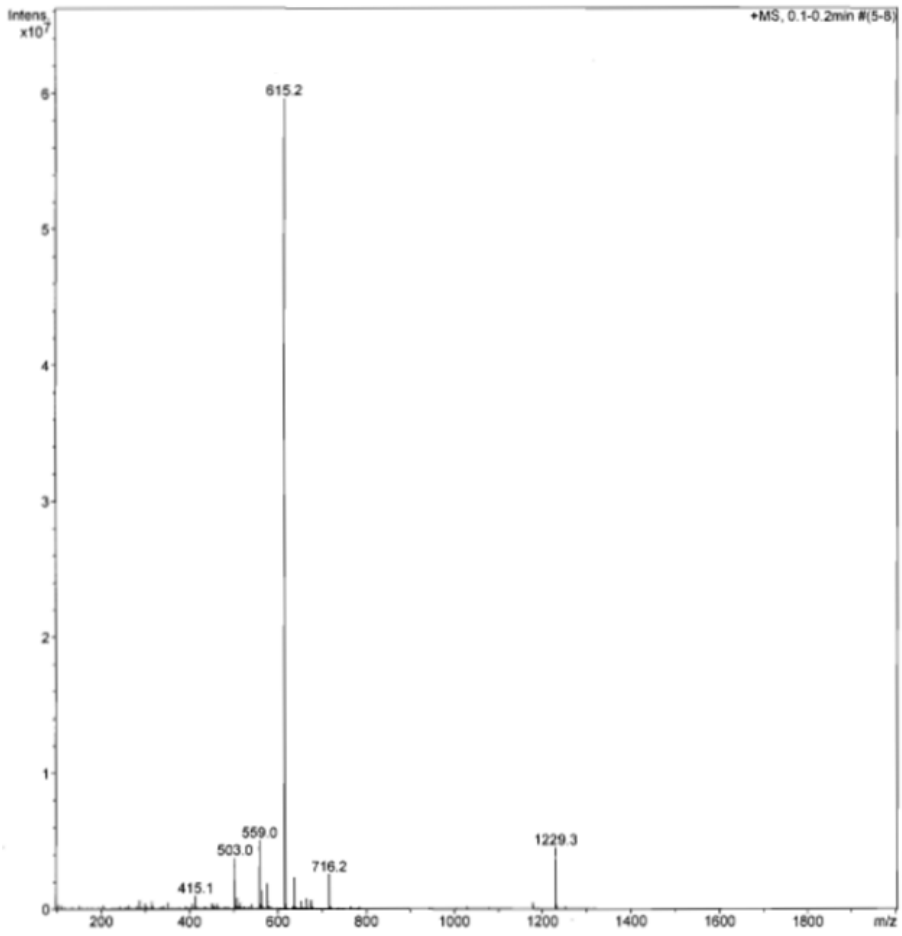
BMPA **2**

2

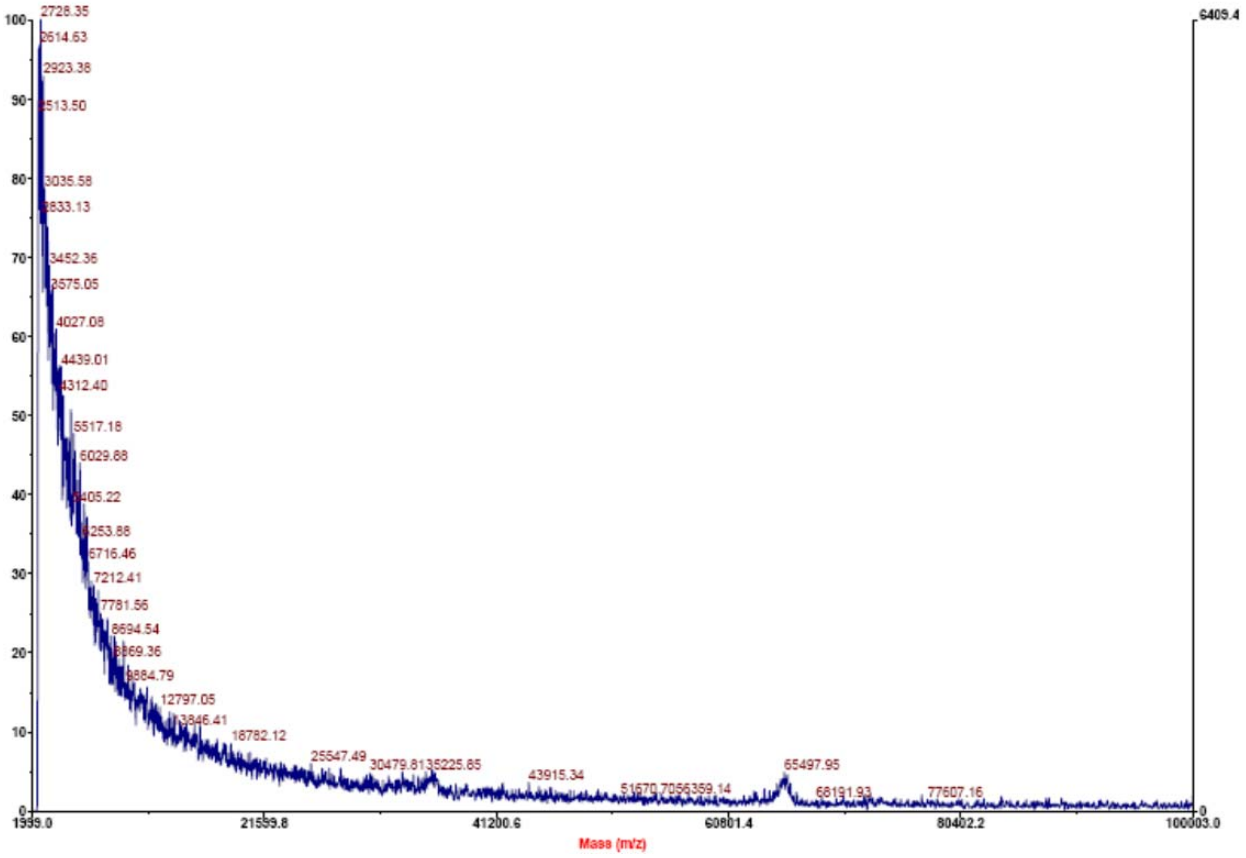


ELP-Gemcitabine Conjugate

ESI-MS of 3′,5′-O-Bis(tert-Butoxycarbonyl)-4-N-(ß-Maleimidopropiocarbonyl) gemcitabine (2):



MALDI-MS of CP-Gemcitabine Conjugate:



Synthesis of ELP-Paclitaxel Conjugate:

Synthesis of paclitaxel-levulinic acid (I) conjugate: Paclitaxel-levulinic acid was prepared as described previously. [8] Briefly, levulinic acid (0.08 g, 0.7 mmol) and DCC (0.145 g, 0.7 mmol) was dissolved in dry DMF and mixed to each other, stirred for 30 mins at -20 ^{0}C. Paclitaxel (0.5 g, 0.58 mmol) and DMAP (0.5 g, 0.58 mmol) were dissolved in dry DMF and were added to the above mixture. The reaction mixture left stirred for 24 h at 4 ^{0}C. The reaction mixture was filtered and the DMF was evaporated to dryness. The compound I was purified with column chromatography with silica gel and 1.5% MeOH in chloroform as eluent. R_f: 0.48 in EtOAc/Hexane=2:1.

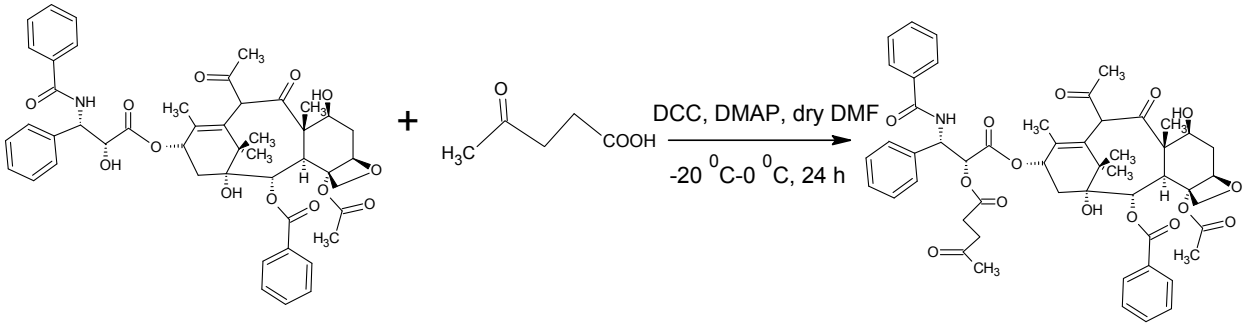
Synthesis of activated paclitaxel (II) and Conjugation of paclitaxel with CP: Compound I (0.05 g, 0.05 mmol) and BMPH (0.018 g, 0.06 mmol) was dissolved in dry MeOH and left stirred in dark for 48 h at 45 ^{0}C. After that the MeOH was evaporated to dryness and compound II was purified with column chromatography with silica gel and 2-2.5 % MeOH in chloroform as eluent. Compound II was immediately used for next step. R_f: 0.6 in 10 % MeOH in $CHCl_3$. ESI-MS: 1117 [M+H], 1139 [M+Na].

Prior to conjugation with activated paclitaxel, purified CP was suspended in reaction buffer (0.1 M NaPO4, 1 mM EDTA, pH 7.0) and reduced with 1 mL of TCEP at neutral pH (100 mM, pH 7.0) at ~5x excess to thiol. Excess TCEP was removed from the solution by initiating the phase transition with sodium chloride (2.5 M) and centrifugation at 4,000 rpm at 25 °C for 10 minutes. The CP pellet obtained by centrifugation was re-suspended in ~2 mL of reaction buffer. Compound IV was re-suspended in ~2 mL of methanol and slowly transferred to the stirring CP solution. 1 mL of pH neutral TCEP (100 mM) was added and the reactants were stirred for 16 hrs at 20 °C in the dark. After reaction, the methanol was evaporated under a nitrogen stream to a volume ~20 mL. Unreacted paclitaxel (PTX) precipitates were removed via centrifugation at 13,000 rpm at 10 °C for 10 minutes. The supernatant was further purified by initiating the CP phase transition with sodium chloride (2.5 M) to aggregate the CP-PTX conjugate followed by centrifugation at 4,000 rpm at 25 °C for 10 minutes. The CP-PTX pellet obtained by centrifugation was re-suspended in phosphate buffered saline (PBS) (pH 7.4) at a concentration of ~10 mg mL^{-1} and dialyzed using Spectra/Por 2 membrane (Spectrum labs, MWCO 12-14 kD) in 20 % acetonitrile-water, followed by 100% DI water.

Determination of paclitaxel conjugation ratio

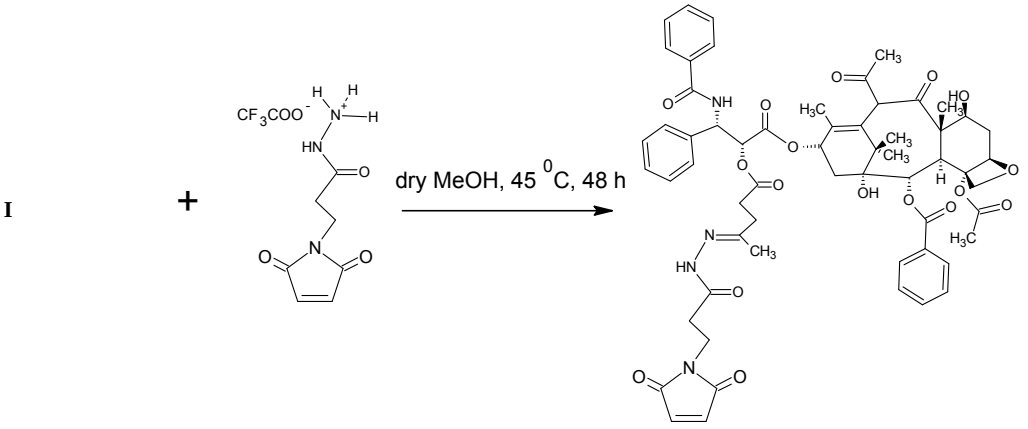
The conjugation ratio of paclitaxel to CP was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the CP-Paclitaxel conjugates and free CP using a Voyager DE-Pro Maldi-MS (Applied Biosystems) instrument equipped with a nitrogen laser (337 nm). The MALDI-TOF-MS samples were prepared in an aqueous 50% acetonitrile solution containing 0.1% TFA, using a sinapinic acid matrix. The conjugation ratio was determined by examining the increase in mass of the conjugate relative to free CP.

Scheme II. Synthesis of CP-Paclitaxel conjugate

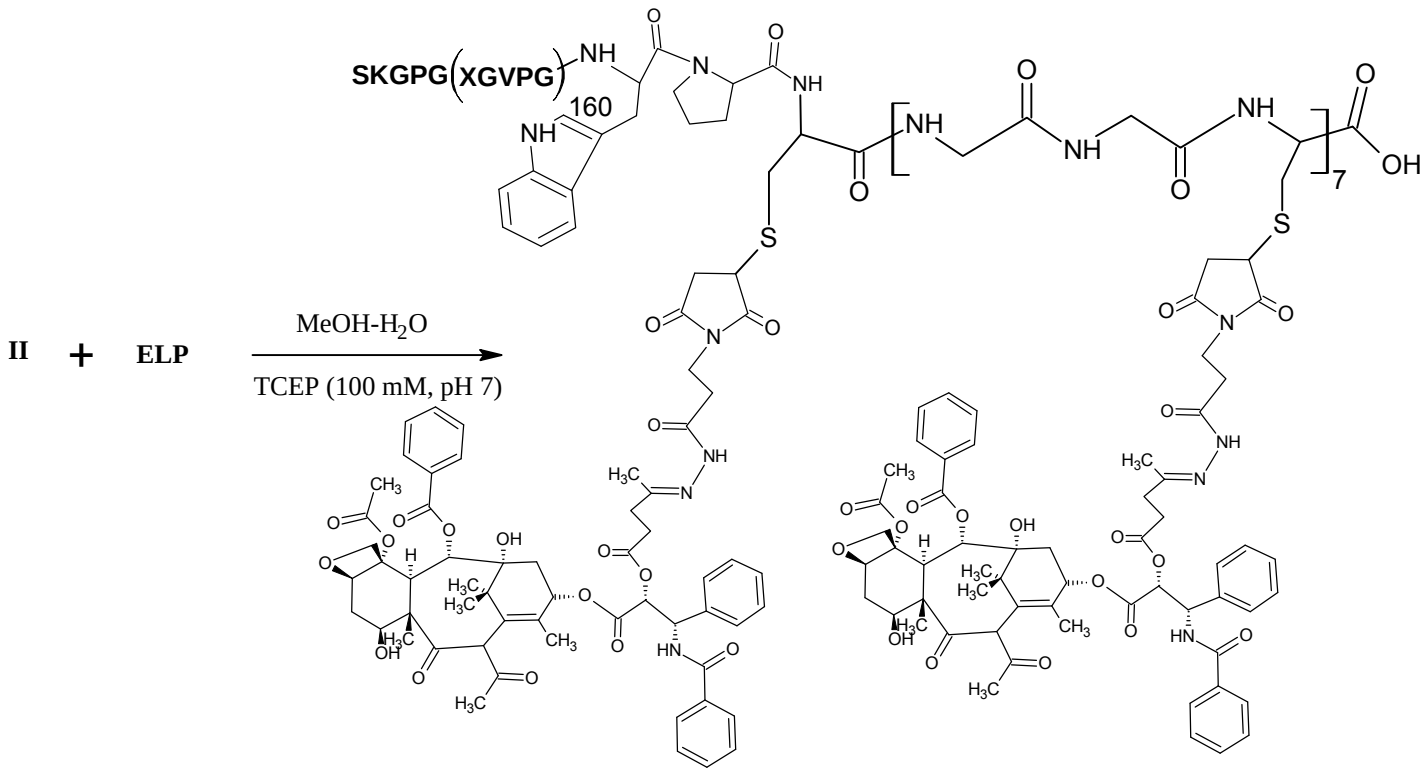


Paclitaxel **Levulinic acid** **I**

I

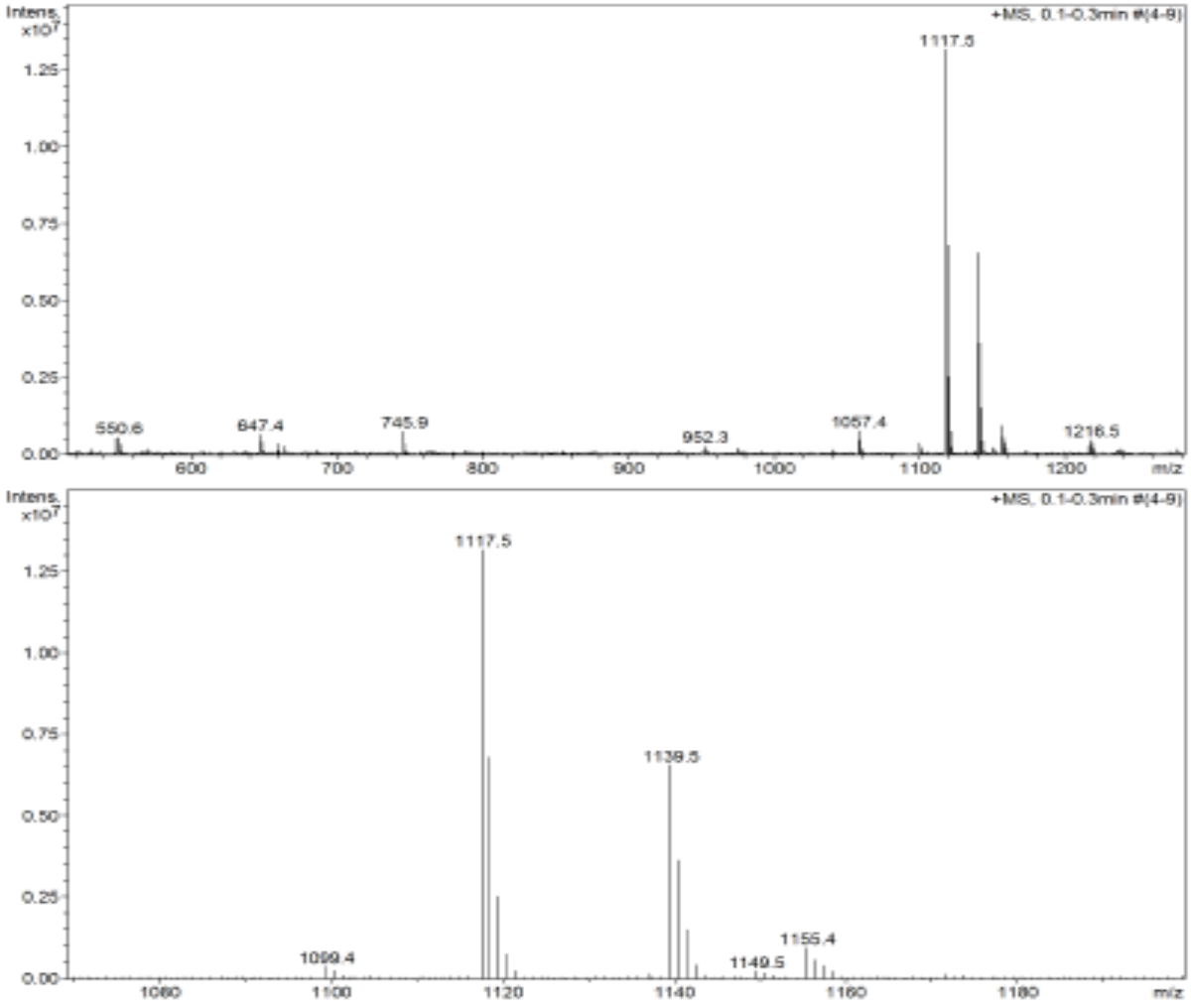


BMPH **II**

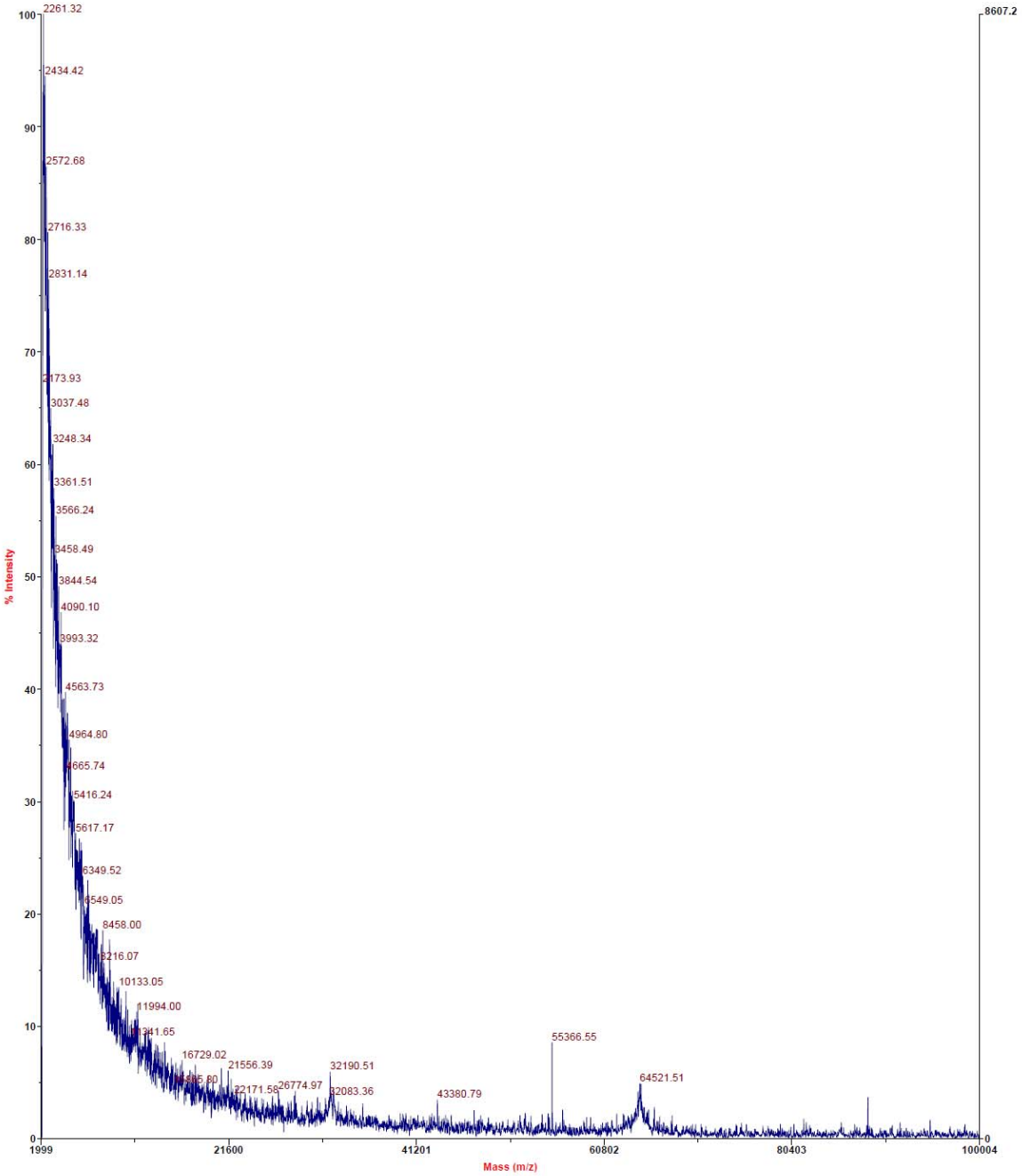


ELP-Paclitaxel Conjugate

ESI-MS of Activated Paclitaxel (II):



MALDI-MS of CP-Paclitaxel Conjugate:



Synthesis of ELP-Oxycodone Conjugate:

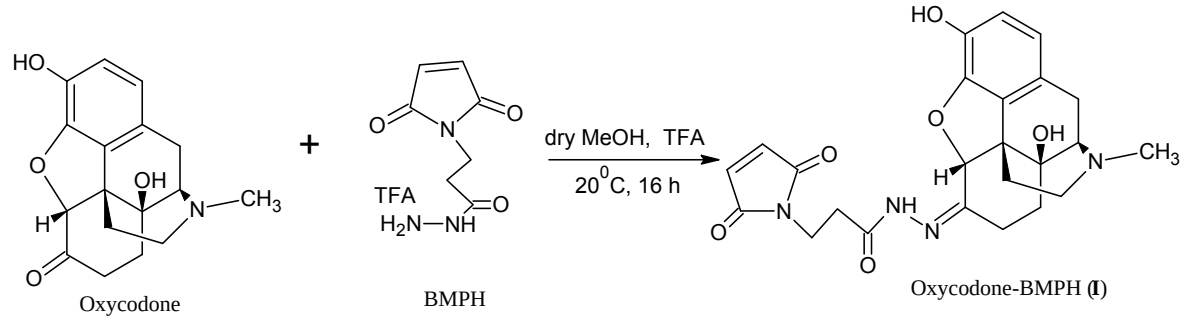
Synthesis of Oxycodone-BMPH (I) conjugate: Oxycodone-BMPH was prepared as described previously [2]. Briefly, oxycodone (25 mg, 79.2 µmoles) and BMPH (28 mg, 95 µmoles) were co-dissolved in 1 mL anhydrous methanol with 2 µL of tri-fluoroacetic acid. The reactants were stirred for 16 hs at 20 °C in the dark. The white precipitate was isolated by centrifugation and washed with MeOH. Drying under vacuum produced the white purified product (**I**). Activated BMPH was immediately used in a second reaction with the ELP. ESI-MS: 481 [M+H], 503 [M+Na].

Conjugation of oxycodone with ELP: Prior to conjugation with activated oxycodone, purified ELP was suspended in reaction buffer (0.1 M NaPO4, 1 mM EDTA, pH 7.0) and reduced with 1 mL of TCEP at neutral pH (100 mM, pH 7.0) at ~5x excess to thiol. Excess TCEP was removed from the solution by initiating the phase transition with sodium chloride (2.5 M) and centrifugation at 4,000 rpm at 25 °C for 10 minutes. The ELP pellet obtained by centrifugation was re-suspended in ~1 mL of reaction buffer. Compound **I** was re-suspended in ~1 mL of methanol-H_2O (1:1) and slowly transferred to the stirring ELP solution. 1 mL of pH neutral TCEP (100 mM) was added and the reactants were stirred for 16 hrs at 20 °C in the dark. After reaction, the methanol was evaporated under a nitrogen stream to a volume ~1 mL. Unreacted oxycodone precipitates were removed via centrifugation at 13,000 rpm at 10 °C for 10 minutes. The supernatant was further purified by initiating the ELP phase transition with sodium chloride (2.5 M) to aggregate the ELP-oxycodone conjugate followed by centrifugation at 4,000 rpm at 25 °C for 10 minutes. The ELP-oxycodone pellet obtained by centrifugation was re-suspended in phosphate buffered saline (PBS) (pH 7.4) at a concentration of ~10 mg mL^{-1} and dialyzed using Spectra/Por 2 membrane (Spectrum labs, MWCO 12-14 kD) in 20 % acetonitrile-water, followed by 100% DI water.

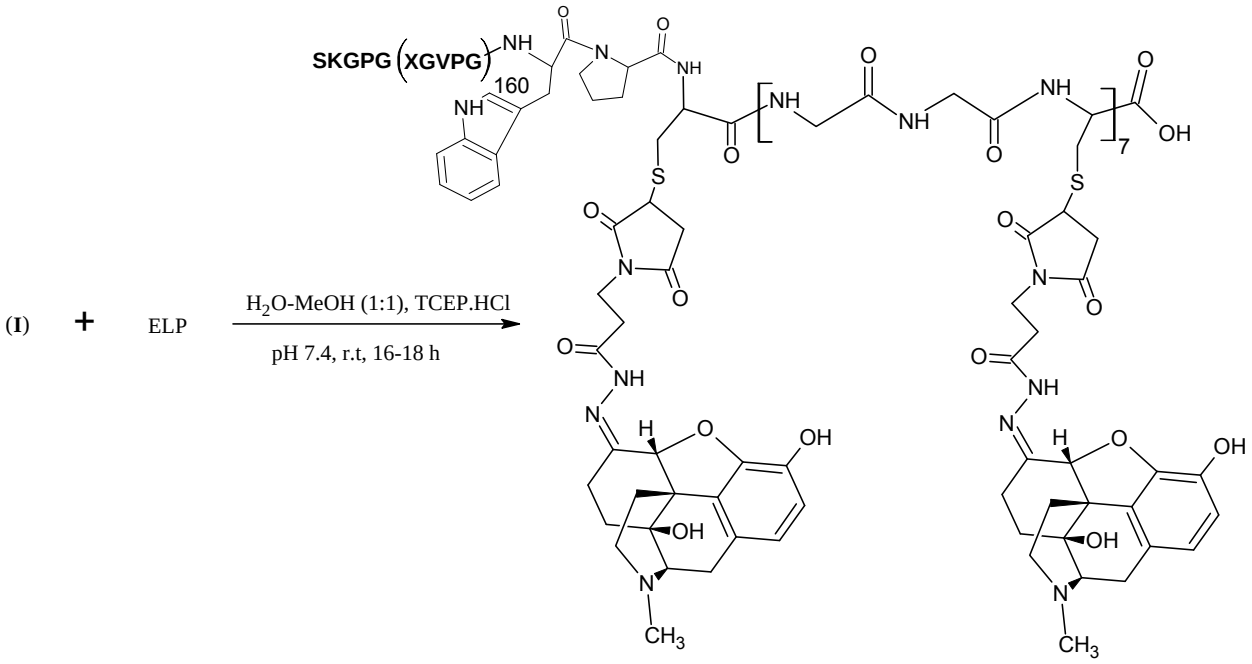
Determination of oxycodone conjugation ratio

The conjugation ratio of paclitaxel to ELP was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the ELP-oxycodone conjugates and free ELP using a Voyager DE-Pro Maldi-MS (Applied Biosystems) instrument equipped with a nitrogen laser (337 nm). The MALDI-TOF-MS samples were prepared in an aqueous 50% acetonitrile solution containing 0.1% TFA, using a sinapinic acid matrix. The conjugation ratio was determined by examining the increase in mass of the conjugate relative to free ELP.

Scheme III. Synthesis of ELP-Oxycodone Conjugate

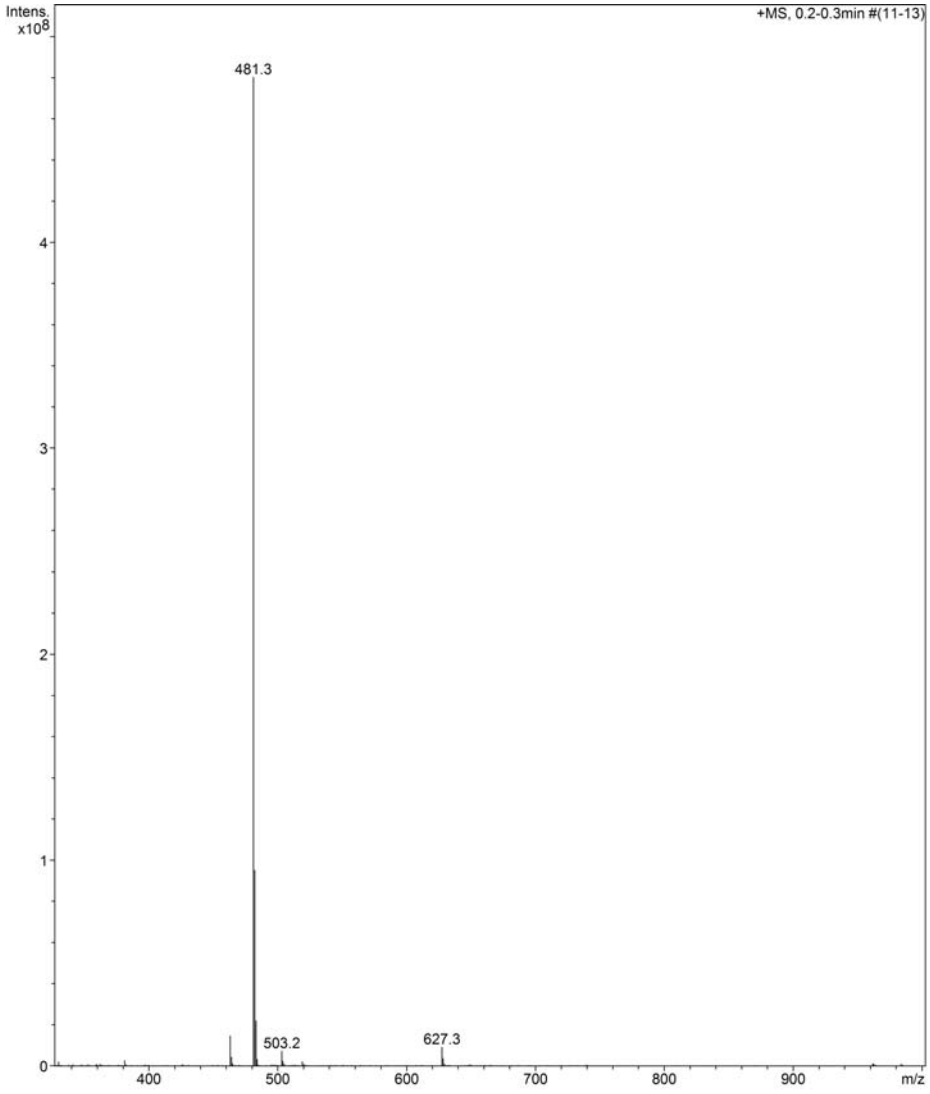


Oxycodone BMPH Oxycodone-BMPH (**I**)

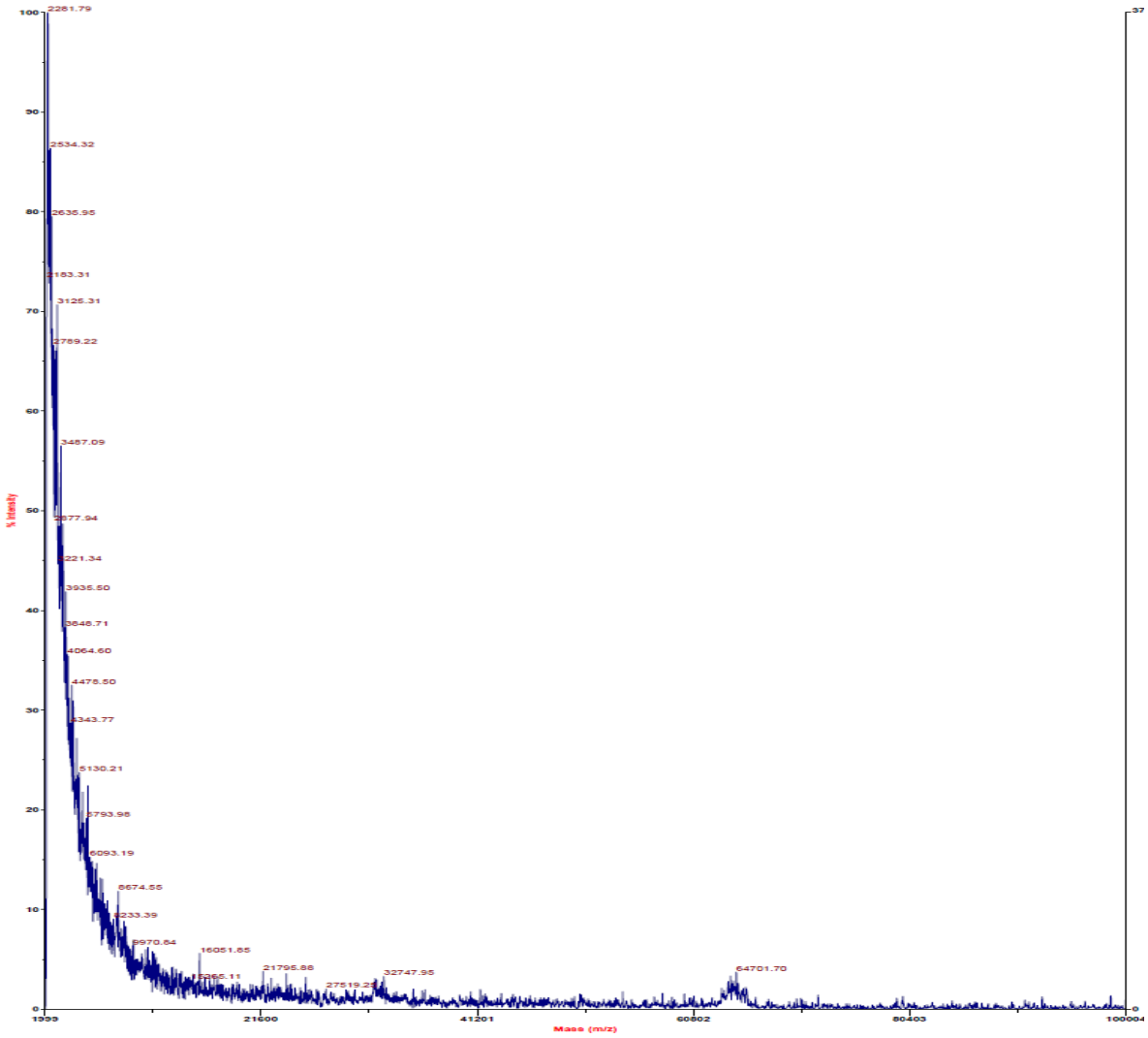


ELP-oxycodone

ESI-MS of Oxycodone-BMPH (I):



MALDI-MS of ELP-oxycodone Conjugate:



	Free Drug-Linker	Conjugate	Delta	Drugs/CP
GEM	615.2	65498.0	2818.6	4.6
PTX	1117.5	64521.5	1842.1	1.6
OXY	481.3	64701.7	2022.3	4.2

SI Table I. MALDI-MS of CP-Drug conjugates. The number of drugs conjugated to each CP was calculated by subtracting the molecular weight of the unconjugated CP (62,679.2 $gmol^{-1}$) from the molecular weight of each construct measured via MALDI-MS.

SI Table II: Characterization of CP conjugates

	Attached Molecule	Log(D)	Molecules/ELP	R_h (nm)	R_g (nm)	ρ	CN	T_t slope	CAC (uM)	Assembly	Type
	Unconjugated Control	--	0	5.7				-5.78	N/A	No	Monomer
1	N-Methoxycarbonylmaleimide	-0.87	6.4	6.3				-5.84	N/A	No	Monomer
2	N-Ethylmaleimide	0.68	6.4	5.7				-5.79	N/A	No	Monomer
3	N-Phenylmaleimide	1.09	3.6	5.5				-6.43	N/A	No	Monomer
4	N-Propylmaleimide	1.22	3.3	5.8				-4.96	N/A	No	Monomer
5	N-tert-Butylmaleimide	1.38	5.9	6.1				-4.82	N/A	No	Monomer
6	N-(Para-tolyl)-maleimide	1.55	3.8	33.5	34.0	1.01	9.1	-1.00	31±5	Yes	Nanoparticle
7	N-Benzylmaleimide	1.78	3.9	40.2	30.6	0.76	18.3	-0.71	1.4±0.2	Yes	Nanoparticle
8	N-(4-ethylphenyl)-maleimide	2.08	5.3	33.4	23.0	0.69	27.1	-0.68	1.2±0.1	Yes	Nanoparticle
9	N-[4-(2-Benzimidazolyl)phenyl]maleimide	2.12	5.5	30.5	26.4	0.87	15.8	-0.74	0.78±0.04	Yes	Nanoparticle
10	N-(9-Acridinyl)maleimide	2.27	5.0	27.3	28.3	1.04	11.7	-0.85	29±2	Yes	Nanoparticle
11	N-(1-naphthyl)-maleimide	2.32	5.6	58.3	47.3	0.81	22.5	--	n.d.	Yes	Nanoparticle
12	2-maleimidofluorene	3.04	5.3	47.5	45.2	0.95	63.4	--	n.d.	Yes	Nanoparticle
13	N-(3-Fluoranthyl)maleimide	4.04	4.1	55.7	47.1	0.85	43.8	--	n.d.	Yes	Nanoparticle
14	N-(1-Pyrenyl)maleimide	4.04	4.3	39.1	33.6	0.86	40.5	-0.79	n.d.	Yes	Nanoparticle
Gem	Gemcitabine	-2.2	5	5.7	37.8	6.59	0.93		N/A	No	Monomer
Oxy	Oxycodone	1.2	4	9.7	17.6	1.81	1.3		N/A	No	Monomer
PTX	Paclitaxel	4.0	2	53.3	57.7	1.08	72.0		6.1±0.6	Yes	Nanoparticle

SI Table II: Code represents the corresponding molecule number in the manuscript (Figure 1). Log(D) represents the hydrophobicity as measured by the logarithm of the distribution coefficient at pH 7.4. Molecules/CP is the number of attached molecules per CP chain, as measured by the Ellman's Assay described above. R_h and R_g are the hydrodynamic radius (nm) and radius of gyration (nm), respectively, determined on the ALV/CGS-3 Goniometer system. The ALV system was also used to calculate the ρ value (form factor, R_g / R_h), and the absolute molecular weight (g/mol). Z (coordination number) describes the number of CPs per nanoparticle, which was determined by dividing the absolute molecular weight of the nanoparticle by the mass of the individual CP chain (62 kDa). Values of 1 for the molecular weight represent an estimation based on DLS data and the transition behavior. T_t slope describes the slope of the T_t as a function of CP concentration graph (manuscript Figure 2A). CAC represents the critical aggregation concentration (μM), as measured by the pyrene assay (manuscript, Figure 3). Assembly indicates whether nanoparticles were formed upon attachment of the corresponding molecule.

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