



Supporting Information

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Paclitaxel-Initiated, Controlled Polymerization of Lactide for the Formulation of Polymeric Nanoparticulate Delivery Vehicles

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General

D,L-Lactide (LA) was purchased from TCI America (Portland, OR, USA). It was recrystallized three times in toluene and stored at -30°C in a glove box. The BDI ligands and the corresponding metal catalysts (BDI)Mn(TMS)₂ (M = Mg, Zn) were prepared by following the published procedure¹ and stored at -30°C in a glove box. All anhydrous solvents were purified through alumina columns and kept anhydrous by molecular sieves. Paclitaxel (Ptxl) and docetaxel (Dtxl) were purchased from the LC Laboratories (Woburn, MA, USA), and used as received. All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and used as received unless otherwise noted. The molecular weights of PLA or drug-PLA were determined on a gel permeation chromatography (GPC) equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS 18-angle laser light scattering detector (MALLS) and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The wavelength of HELEOS detector was set at 658 nm. Size exclusion columns used for the separation of PLA or drug-PLA conjugates were series connected on the GPC (Phenogel columns 100 Å, 500 Å, 10³ Å and 10⁴ Å, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA). THF (HPLC grade) was used as mobile phase for GPC. The low resolution electrospray ionization mass spectrometry (LR-ESI-MS) experiments were conducted on a Waters Quattro II mass spectrometer. HPLC analysis was performed on a System Gold system (Beckman Coulter, Fullerton, CA, USA) equipped with a 126P solvent module, a System Gold 128 UV detector and an analytical pentafluorophenyl column (Curosil-PFP, 250 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA). The UV wavelengths for Ptxl, Dtxl and CPT were set at 227, 237 and 350 nm, respectively. NMR analyses were conducted on a Varian U500, VXR500 or UI500NB (500 MHz). The sizes and polydispersities of NCs were determined on a ZetaPALS dynamic light-scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). The SEM analysis of nanoparticles were performed on a Hitachi-S4700 high resolution scanning electron microscopy. PC-3 cells (ATCC, Manassas, VA, USA) used for MTT assay were cultured in Ham's F12K medium containing 10% FBS (Fetal Bovine Serum), 1000 units/mL aqueous Penicillin G and 100 μg/mL streptomycin.

General procedure for the preparation of Ptxl-LA₁₀₀ and Ptxl-LA₁₀₀ NCs

Ptxl-mediated ring-opening polymerization of LA in the presence of a metal catalyst. In a glove box, Ptxl (8.5 mg, 0.01 mmol) was dissolved in anhydrous THF (2 mL). (BDI)MgN(TMS)₂¹ (6.2 mg, 0.01 mmol) was added and allowed to react with Ptxl for 15-20 min. LA (144.0 mg, 1.0 mmol) in THF (1 mL) was added dropwise to the mixture of Ptxl and (BDI)MgN(TMS)₂ under vigorous stirring. The polymerization was monitored using FT-IR by following the lactone band at 1772 cm⁻¹ or using ¹H-NMR by

checking the methine (-CH-) peak of LA around 5.2-5.0 ppm. After the polymerization was complete, an aliquot of the polymerization solution was analyzed using HPLC to quantify the unreacted Ptxl in order to determine the incorporation efficiency of Ptxl in Ptxl-PLA. One drop of water was added to the polymerization solution to hydrolyze the Mg-Ptxl oxide. The resulting Ptxl-LA₁₀₀ was precipitated with ethyl ether (10 mL), washed with ether and toluene to remove BDI ligand, dried under vacuum and characterized by GPC. Complete removal of BDI from Ptxl-PLA was verified by TLC.

Nanoprecipitation The Ptxl-LA₁₀₀ conjugate in DMF (100 μ L, 10 mg/mL) (or in other water-miscible solvent such as THF, acetone, etc.) was dropwise added to nanopure water (2 mL). The resulting NCs were collected by ultrafiltration (15 min, 3000 \times g, Ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA, USA) and characterized by DLS or SEM.

Formation and characterization of Ptxl-LA₂₅/PLGA-mPEG core-shell nanostructure

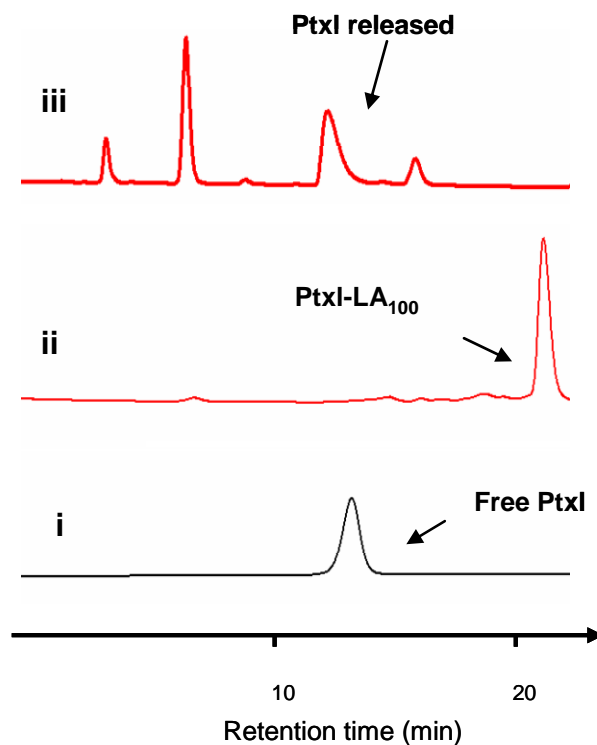
The Ptxl-LA₂₅ conjugate (5 mg/mL in DMF, 100 μ L) was dropwise added to 2 mL nanopure water to give the Ptxl-LA₂₅ NCs. PLGA-mPEG² (MW of the PLGA block = 13kDa, MW of the mPEG block = 5 kDa, 5 mg/mL in DMF, 100 μ L) or mPEG (MW of PEG = 5 kDa, 5 mg/mL in DMF, 100 μ L) was dropwise added to Ptxl-LA₂₅ NC.

The particle sizes were analyzed by DLS. PBS (245 μ L, 10 \times) was added slowly to the mixture. The size of Ptxl-LA₂₅ NC coated with PLGA-mPEG was monitored for 30 mins by DLS or by SEM. The resulting NCs were purified by ultrafiltration; their in vitro toxicities were then evaluated using MTT assay in PC-3 cells (24 hr incubation at 37 $^{\circ}$ C). To determine the release kinetics of Ptxl-PLA, a PBS solution of NCs was equally divided into several portions and then incubated at 37 $^{\circ}$ C. At scheduled time, the release study was terminated. DMF solution was added to dissolve all precipitation. All the samples were then dried, re-dissolved in DCM and reacted with Bu₄NBH₄ for 1.5 hours. A drop of acetic acid was added into the solution. The solution was stirred for 20 min before the solvent was evaporated. The residual solid was reconstituted in acetonitrile for RP HPLC analysis (Curosil, 250 \times 4.6mm, 5 μ ; Phenomenex, Torrance, CA, USA). The release kinetics of Ptxl from Ptxl-LA₂₅ was determined by quantifying PDB (Fig 3a).

Reductive degradation of Ptxl and Ptxl-PLA using Bu₄NBH₄

The reductive degradation of Ptxl at its 13-position was achieved by following the literature reported procedure.³ Ptxl (5 mg, 5.8 mmol) in anhydrous dichloromethane (1 mL) was allowed to react with Bu₄NBH₄ (2.5 mg, 10 mmol) for one and a half hours in a glove box. Acetic acid (\approx 100 μ L) was added to the reaction mixture to terminate the reaction. The reaction solution was stirred for 20 mins followed by complete removal of the solvent. The residual solid was re-dissolved in acetonitrile. An aliquot of such solution was analyzed on a HPLC equipped with a Curosil RP column (250 \times 4.6mm, 5 μ ; Phenomenex, Torrance, CA, USA). The desired fractions were collected and analyzed by for LR-ESI-MS. The Ptxl-PLA conjugate was reductively degraded and analyzed similarly.

Supplementary Figure 1

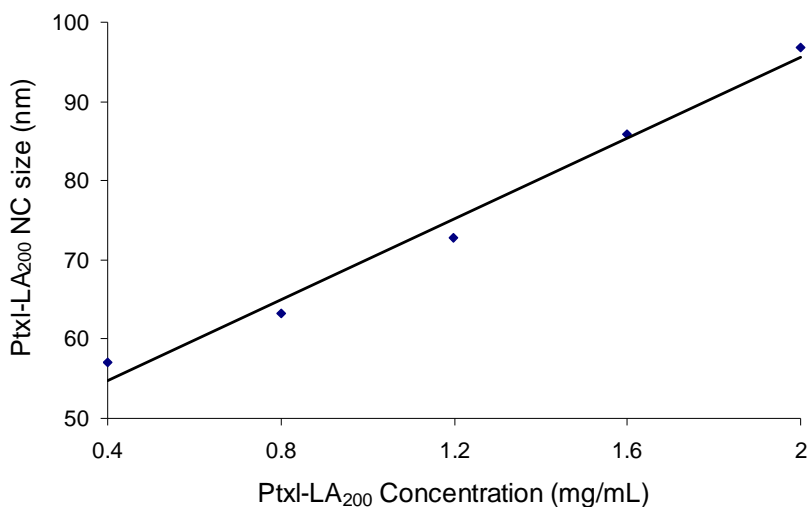


Supplementary Fig. 1. HPLC analysis of (i) Free Ptxl, (ii) Ptxl-LA₁₀₀ and (iii) released Ptxl from Ptxl-LA₁₀₀ after Ptxl-LA₁₀₀ was treated with 1N NaOH for 60 min and extracted with octanol. HPLC condition: RP-HPLC column (Curosil-PFP, 4.6 × 250 mm, 5 μ , Phenomenex, Torrance, CA). Mobile phase was acetonitrile/water with 0.1% TFA (v/v = 50/50). The flow rate was 1.0 mL/min.

Discussion

Ptxl-LA₁₀₀ was completely hydrolyzed after it was treated with NaOH (comparing ii vs iii). The HPLC peaks other than Ptxl in trace iii were the degradation fragments of Ptxl due to the hydrolysis of its intrinsic ester bonds by NaOH.

Supplementary Figure 2



Supplementary Fig. 2 The correlation of Ptxl-LA₂₀₀ nanoconjugate size versus the concentration of Ptxl-LA₂₀₀ during nanoprecipitation.

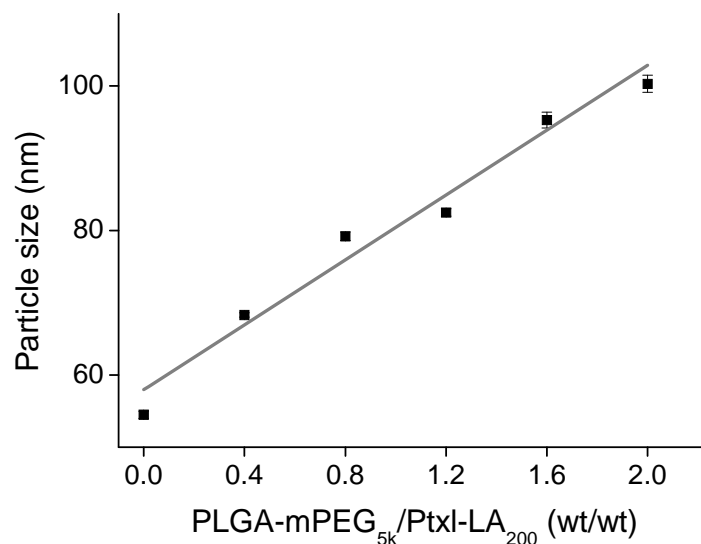
Procedure

Stock solutions of Ptxl-LA₂₀₀ (prepared with (BDI)MgN(TMS)₂) in DMF with concentrations of 0.4, 0.8, 1.2, 1.6 and 2 mg/mL were prepared. These Ptxl-LA₂₀₀ solutions (100 μ L) were separately precipitated in nanopure water (2 mL) under rapid stirring. The sizes of the resulting nanoconjugates were characterized by DLS.

Discussion

When the solvent type and the solvent/water ratio are fixed, the particle size usually has a linear correlation with the polymer concentration because the number of particles remain roughly unchanged.⁴ The linear relationship between particle size and Ptxl-LA₂₀₀ concentration shows that the NC size can be precisely tuned by controlling the concentration of polymer-drug conjugate during nanoprecipitation.

Supplementary Figure 3



Supplementary Fig. 3 Linear correlation of the size of Ptxl-LA₂₀₀/PLGA-mPEG_{5k} PEGylated NC vs. mass ratio of PLGA-mPEG_{5k}/Ptxl-LA₂₀₀

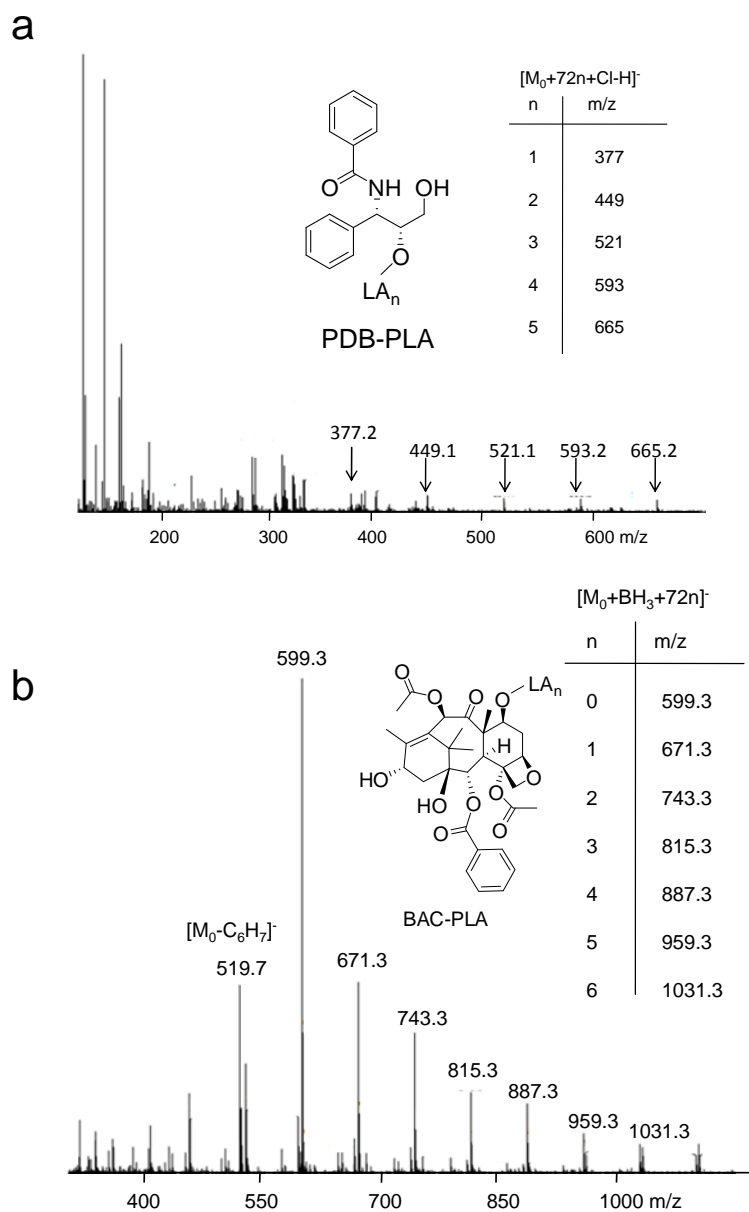
Procedure

Ptxl-LA₂₀₀ conjugate (2 mg/mL in DMF, 100 μ L, prepared with (BDI)ZnN(TMS)₂) was dropwise added to 2 mL nanopure water to give Ptxl-LA₂₀₀ NCs. PLGA-mPEG_{5k} (MW of the PLGA block = 13kDa, MW of the mPEG block = 5 kDa, 2 mg/mL in DMF) was then sequentially added into Ptxl-LA₂₀₀ NC solution under vigorous stirring. The sizes of the resulting Ptxl-LA₂₀₀/ PLGA-mPEG_{5k} NCs were characterized by DLS.

Discussion

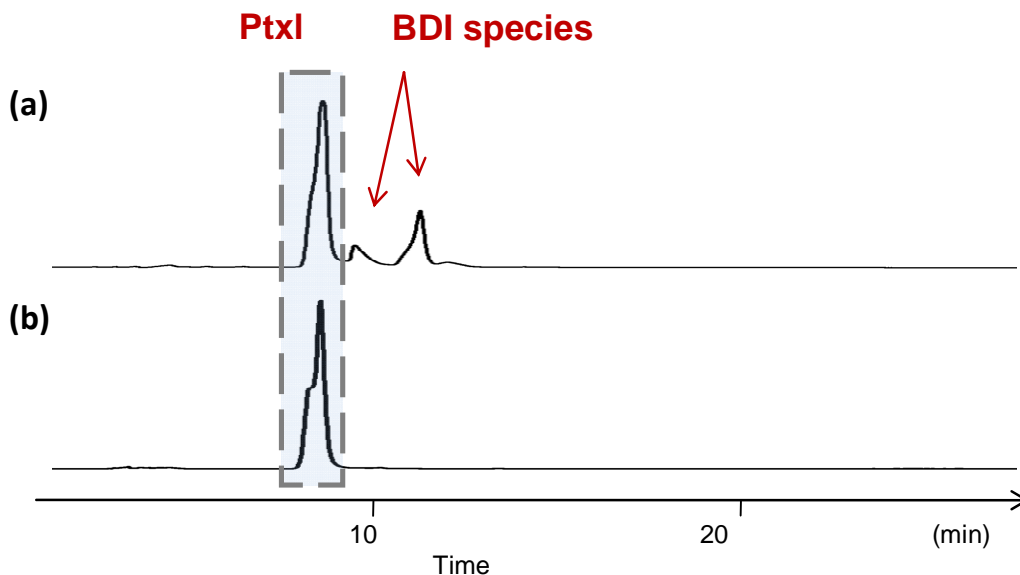
The linear correlation of NC size versus the amount of PLGA-mPEG suggested that PLGA-mPEG_{5k} preferentially precipitated on the surface of NCs instead of self-assembling to form micelles. The hydrophobic interaction between the NC surface and the PLGA block is responsible for the formation of PEG shell on the surface of NCs. The core-shell structure of nanoconjugate/PLGA-mPEG_{5k} has been confirmed by TEM (data not shown)

Supplementary Figure 4



Supplementary Fig. 4 Characterization of PDB-PLA and BAC-PLA using ESI-MS. (a) ESI-MS (negative mode) analysis of Ptxl-PLA prepared by Ptxl/(BDI)ZnN(TMS)₂ (1/1 molar ratio) mediated LA (5 eq.) polymerization followed by treatment with Bu₄NBH₄ (b) ESI-MS (negative mode) analysis of the Ptxl-PLA degradation fragments collected from prep-HPLC with elution time between PDB and BAC (see Fig 3c). Ptxl-PLA was prepared through Mg(N(TMS)₂)₂ mediated LA polymerization (Mg/Ptxl/LA = 1/1/5) and then treated with Bu₄NBH₄ before HPLC separation.

Supplementary Figure 5



Supplementary Fig. 5 HPLC analysis of (a) Ptxl mixing with (BDI)ZnN(TMS)₂ and (b) Ptxl. The samples were analyzed by RP-HPLC equipped with Curosil column (250 × 4.6mm, 5μ, Phenomenex, Torrance, CA, USA) at 227 nm.

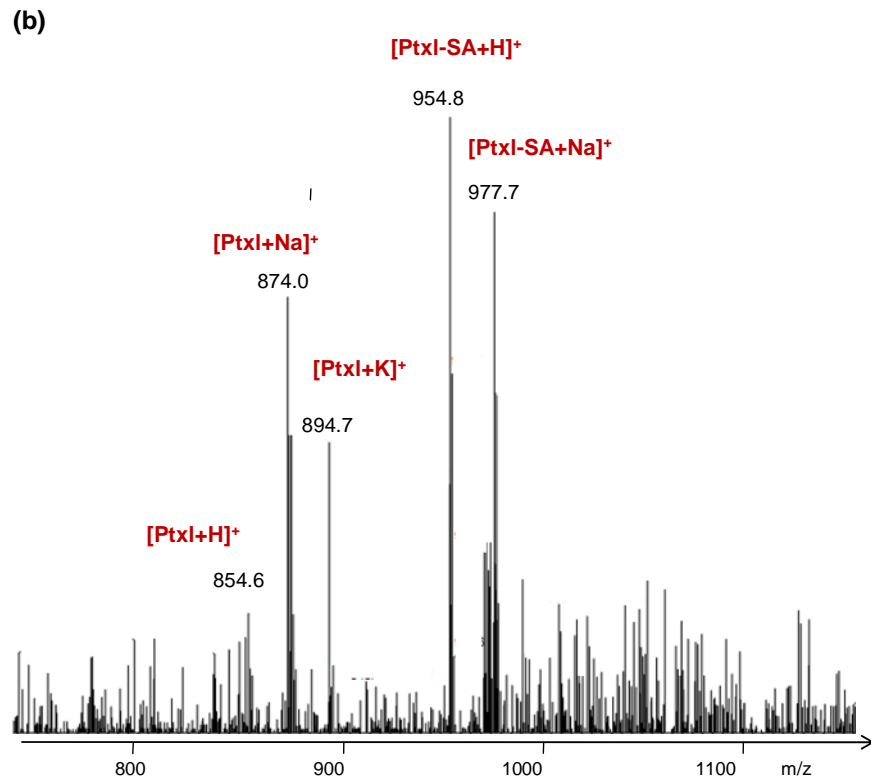
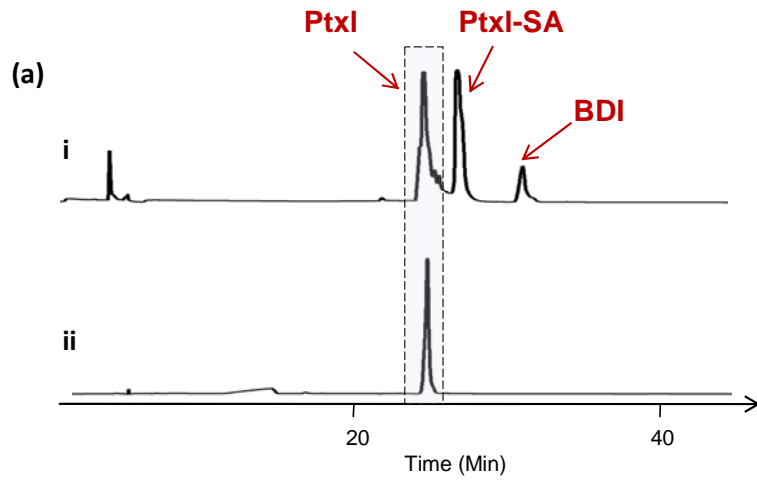
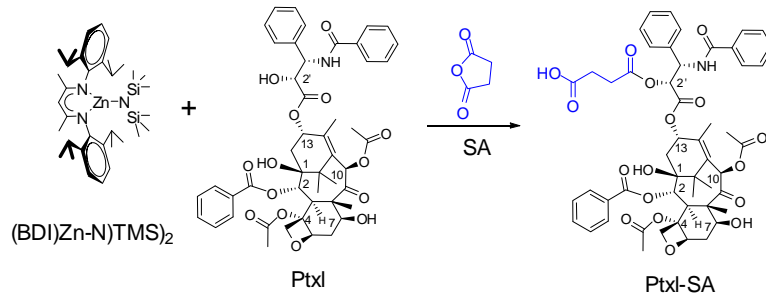
Experimental procedure:

In a glove box, Ptxl (8.5 mg, 0.01 mmol) was mixed with (BDI)ZnN(TMS)₂ (6.4 mg, 0.01 mmol) in 100 μL THF. The mixture was stirred for 15 min at room temperature. To the mixture 100 μL methanol and 20μL acetic acid were added. The solution was stirred at room temperature for an additional 30 min. All solvents were evaporated under vacuum. The sample was then reconstituted using a mixture of acetonitrile and methanol (v/v = 1/1) and then injected to HPLC for analysis (trace a). Free Ptxl was treated similarly prior to the HPLC analysis (trace b).

Discussion:

The formulation process of Ptxl-PLA NC includes initiation, propagation, nanoprecipitation and PEGylation. It is very unlikely that nanoprecipitation and PEGylation processes have deleterious effect on Ptxl. Chain propagation involves the insertion of LA monomer to the metal-alkoxide of the terminal PLA, and Ptxl is not directly involved. Therefore, it is also very unlikely that Ptxl is deleteriously affected by chain propagation. Thus, if the overall process has deleterious impact on Ptxl, such effect must occur during the chain initiation step. To verify whether Ptxl gets degraded by the metal catalyst, we evaluated the coordination of catalyst and drug without addition of LA monomers. The HPLC analysis clearly showed that metal catalyst had no deleterious effect on Ptxl. Ptxl molecule remains intact during initiation and therefore should maintain its original form in Ptxl-PLA NCs, the final product.

Supplementary Figure 6



Supplementary Fig. 6 (a) HPLC analysis of (i) Ptxl mixing with (BDI)ZnN(TMS)₂ followed by addition of succinic anhydride (SA), and (ii) Ptxl. The samples were analyzed by RP-HPLC equipped with Curosil column (250 × 4.6mm, 5μ, Phenomenex, Torrance, CA, USA) at 227 nm. (b) ESI-MS (positive mode) analysis of Ptxl/SA/(BDI)ZnN(TMS)₂ (molar ratio 1/1.1/1.1).

Experimental procedure:

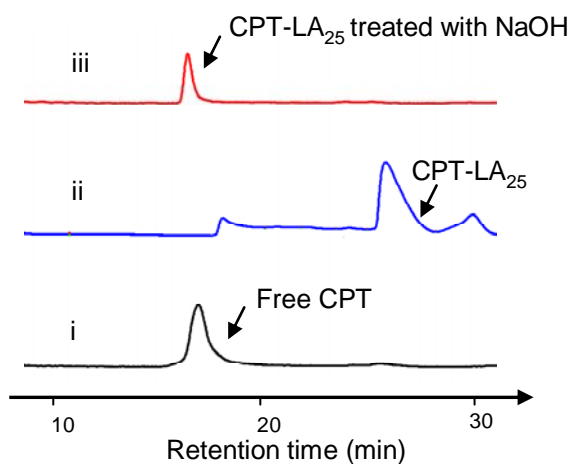
Succinic anhydride was recrystallized in dichloromethane twice prior to be used in this study. In a glove box, Ptxl (8.5 mg, 0.01 mmol) was mixed with (BDI)ZnN(TMS)₂ (7.0 mg, 1.1 equiv) in 100 μL anhydrous THF. The mixture was stirred for 15 min and then 1.1 eq succinic anhydride (1.1 mg) in 100 μL THF was added. The mixture was stirred for 2 hrs at room temperature. To the mixture, 100 μL methanol and 20 μL acetic acid were added. The solution was stirred at room temperature for an additional 30 min. All solvents were evaporated under vacuum. The sample was reconstituted in a mixture of acetonitrile and methanol (v/v = 1/1) and then injected to HPLC for analysis (trace i in (a)). An aliquot of such solution was analyzed by ESI-MS (positive mode) (b). Free Ptxl was analyzed similarly on HPLC (trace ii in (a)).

Discussion:

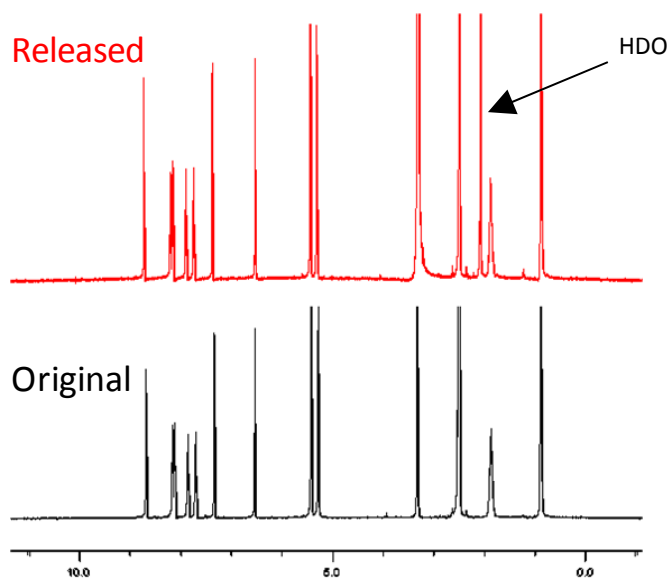
After demonstrating that catalyst has no deleterious effect on Ptxl for the coordination of catalyst and Ptxl (Fig. S5), we next designed a study to mimic the initiation step in order to evaluate whether Ptxl was degraded in this step. We only found two peaks in trace i of (a): the unreacted paclitaxel and the paclitaxel-succinic acid conjugate. No other Ptxl degradation species were observed, indicating that the initiation step should not have deleterious effect on Ptxl in the metal-Ptxl initiated LA polymerizations.

Supplementary Figure 7

a)



b)



Supplementary Fig. 7 (a) HPLC analysis of CPT-initiated polymerization and hydrolysis. (i) authentic CPT (black trace); (ii) the LA polymerization solution (M/I = 100/1) mediated by (BDI)Mg-CPT (red trace); (iii) CPT-PLA treated with 1N NaOH (trace iii, blue). (b) ¹H-NMR (DMSO-d₆) spectra of authentic CPT, and the CPT released and collected (trace iii).

Procedure

We aim to confirm that CPT remains intact during CPT-initiated polymerization, nanoprecipitation and hydrolysis.

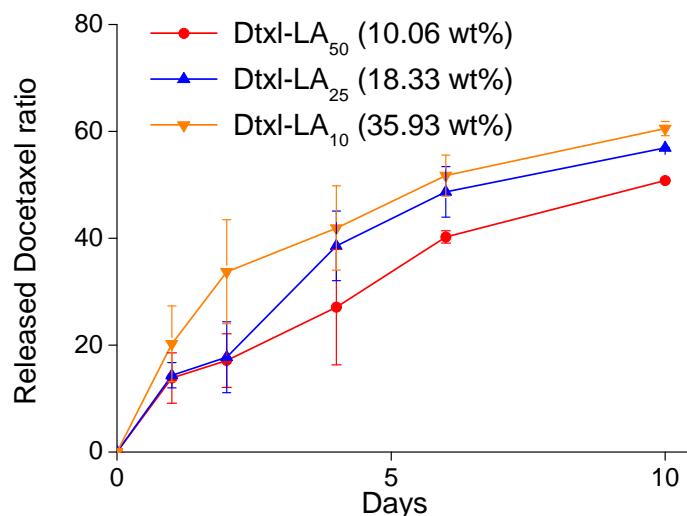
CPT-mediated polymerization of LA in the presence of BDIMg-N(TMS)₂ was set up similarly as Ptxl.

CPT-LA₁₀ NC in water (1 mL, 1 mg/mL) was treated with 1N NaOH (1 mL) for 12 hours. The solution was then tuned to pH 2 using phosphoric acid. The color of the solution turned yellow. The solvent was removed under vacuum. The mixture was then injected to a semi-prep HPLC column (Jupitor Proteo column, 90 Å, 250 × 21.20 mm, 10µ, Phenomenex, Torrance, CA, USA). The fraction that has identical elution time as authentic CPT was collected. Solvent was removed under vacuum. The obtained yellow oily compound was dissolved in phosphoric acid/methanol (v/v = 1/1). The pH of the solution was adjusted to 3 to 4 using 0.1N NaOH. CPT was extracted with chloroform (5 × 100 ml). The organic phase was dried with magnesium sulfate. Magnesium sulfate was removed by filtration. Solvent was removed under vacuum. The slight yellow solid obtained was analyzed by ¹H NMR in DMSO-d₆. Its NMR spectrum was then compared with that of authentic CPT.

Discussion:

The CPT released and collected from the hydrolysis of CPT-PLA NC has identical ¹H NMR spectrum as that of original CPT, which indicates that CPT remain unchanged in its chemical structure throughout the polymerization, nanoprecipitation and hydrolysis processes.

Supplementary Figure 8



Supplementary Fig. 8 Hydrolysis of Dtxl-PLA nanoconjugates with various drug loadings.

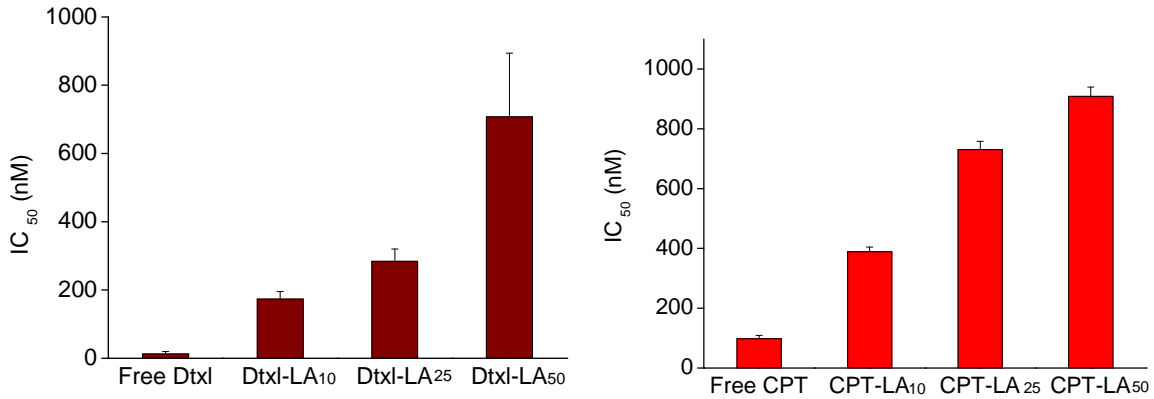
Procedure

Dtxl-LA_n NCs used in this study were prepared using the same polymerization and nanoprecipitation methods as for Ptxl-PLA NCs. NCs were first dispersed in a mixture of 1× PBS/1-octanol (v/v = 1/1) (1mg/mL) in which 1-octanol was used for the partition and extraction of the released Dtxl.⁵ The solution was then divided in equal volume to separate Eppendorf tubes and incubated at 37°C. At schedule time, the hydrolysis in corresponding vials was terminated. The octanol phase was separated from the aqueous phase, and directly injected into HPLC (RP-HPLC column Curosil, 250 × 4.6 mm, 5μ; Phenomenex, Torrance, CA, USA) to quantify the released Dtxl at 265 nm.

Discussion:

Hydrolysis of Dtxl-PLA NCs in PBS showed no burst release effects. The release of Dtxl from Dtxl-LA₅₀ NC was slower than that from Dtxl-LA₂₅ NC and Dtxl-LA₁₀ NC presumably because of the higher MW of Dtxl-LA₅₀ and more compact particle aggregation.

Supplementary Figure 9



Supplementary Fig. 9 Determination of IC₅₀ of Dtxl-LA_n and CPT-LA_n NCs using MTT cytotoxic assays in PC-3 cells (72 hrs incubation at 37°C)

Procedure

PC-3 cells were plated in a 96-well plate for 24 hrs (10,000 cells per well) before the addition of nanoconjugates. Cells were washed with pre-warmed PBS. Freshly prepared NCs (prepared in 1× PBS, 100 μL) were added to cells. The cells were incubated for 72 hrs in the 5% CO₂ incubator at 37°C. After that, the medium was removed. Standard MTT assay protocols were followed thereafter.

Discussion

The toxicities of the Dtxl-LA_n and CPT-LA_n NCs showed similar correlation with drug loadings as that of Ptxl-LA_n NCs. In general, NCs with high drug loadings can release corresponding drug faster than those with low drug loadings, and therefore show high toxicities.

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