Nanoparticles for Drug Delivery Prepared from Amphiphilic PLGA Zwitterionic Block Copolymers with Sharp Contrast in Polarity between Two Blocks**

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Supplementary Information

Materials. 2-bromoisobutyryl bromide, t-Boc-aminoethyl alcohol, 2-(Dimethylamino)ethyl methacrylate, tert-butyl bromoacetate, Cu(I)Br, 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA), N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), 2,2,2-Trifluoroethanol (TFE), and 4-Aminophenyl β-D-galactopyranoside (NH2-galactose) were purchased from Sigma-Aldrich, St. Louis, MO. Trifluoroacetic acid (TFA) and N-Hydroxysuccinimide (NHS) were purchased from Acros Organics USA, Morris Plains, NJ. Poly(D,L-lactide-co-glycolide)s (PLGA) with a 50:50 monomer ratio were purchased from Durect Corporation, Pelham, AL. Docetaxel (Dtxl) was purchased from LC Laboratories, Woburn, MA. 5-(aminomethyl)fluorescein hydrochloride, and 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD) were purchased from Invitrogen, Carlsbad, CA.

2-aminoethyl 2-bromoisobutyrate. The ATRP initiators with NH₂ functional groups were synthesized via a modified method based on established procedures reported previously. [1] Briefly, 3.57 g 2-bromoisobutyryl bromide was added to a solution of 2.5 g t-Boc-aminoethyl alcohol and 1.73 g triethylamine in 8 ml methylene chloride in an ice bath. After 4 h reaction, the salts were filtered off and the filtrate was extracted with saturated sodium bicarbonate solution. Methylene chloride phase was dried over magnesium sulfate and evaporated. The resulting t-Boc-aminoethyl 2-bromoisobutyrate was treated by 15 ml trifluoroacetic acid (TFA) for 2 h and crystallized upon addition of ethyl ether (yield 95%). ¹H NMR (DMSO-d₆) δ (ppm): 1.93 (s, 6H, -C(CH₃)₂Br), 3.16 (s, 2H, TFA·NH₃⁺CH₂CH₂OCO), 4.31 (t, J=5Hz, 2H, TFA·NH₃⁺CH₂CH₂OCO), 8.22 (s, 3H, TFA·NH₃⁺CH₂CH₂OCO).
2-tert-butoxy-N-(2-(methacryloyloxy)ethyl)-N,N-dimethyl-2-oxoethanaminium (CB-tBu monomer). 5 g 2-(Dimethylamino)ethyl methacrylate and 8.68 g tert-butyl bromoacetate were reacted in 20 ml acetonitrile for 24 h at 50 °C under N₂ protection. Upon addition of 250 ml ethyl ether to the reaction mixture, the formed white crystalline was isolated and dried. The resulting CB-tBu monomers were immediately stored in a desiccator at -20 °C (yield 96%).

¹H NMR (D₂O) δ (ppm): 1.44 (s, 9H, -OC(CH₃)₃), 1.87 (s, 3H, CH₂=C(CH₃)COO⁻), 3.31 (s, 6H, -CH₂N(CH₃)₂CH₂COO⁻), 3.98 (t, J=3Hz, 2H, CH₂=C(CH₃)COOCH₂CH₂N(CH₃)₂CH₂⁻), 4.28 (s, 2H, -CH₂N(CH₃)₂CH₂COO⁻), 4.60 (t, J=3Hz, 2H, CH₂=C(CH₃)COOCH₂CH₂N(CH₃)₂CH₂⁻), 5.73 and 6.10 (s, 2H, CH₂=C(CH₃)COO⁻).

Synthesis of PCB-tBu polymers. The ATRP of CB-tBu monomers was carried out as follows: 74 mg Cu(I)Br and 148.6 mg HMTETA were placed into a Schlenk tube and underwent three vacuum-nitrogen cycles. Then, 7 ml degassed DMF was added to make solution A. Similarly, 1.8 g CB-tBu monomers and 80 mg 2-aminoethyl 2-bromoisobutyrate were placed into another Schlenk tube with oxygen fully excluded, followed by addition of 8 ml degassed DMF to make solution B. Polymerization was started by transferring solution B into solution A under N₂ protection. After reaction at 60 °C for 24 h, the polymers were first precipitated in ethyl ether, then were dissolved in a small amount of ethanol and precipitated in acetone repeatedly to remove residual monomers, initiators and catalysts. The resulting PCB-tBu was dried under vacuum before further use.

PLGA-b-PCB-tBu Conjugation. The conjugation process was via NHS/EDC chemistry. Briefly, 3.2 g COOH terminated PLGA (0.20 dl/g), 86.4 mg NHS and 147.2 mg EDC were reacted in 6 ml methylene chloride for 4 h at room temperature. Then, 5 ml ethyl ether was added to obtain white precipitates. The resulting PLGA-NHS was washed with cold ethyl ether/methanol mixture (2/1, v/v) to remove any NHS and EDC residuals, then vacuum-dried.
before use. TFA\(^{-}\)NH\(_3^+\) terminated pCBMA-tBu was treated with an excess of triethylamine to remove TFA protection. NH\(_2\) terminated pCBMA-tBu was purified via filtration, precipitated into ethyl ether, and vacuum-dried. 878 mg NH\(_2\) terminated PCB-tBu and 1.68 g PLGA-NHS were conjugated in the presence of 50 \(\mu\)l triethylamine in 7 ml acetonitrile at 60 °C for 20 h. The resulting PLGA-PCB-tBu was precipitated in cold methanol. PCBMA-tBu contaminant was removed by repeating the washing cycle. The formation of PLGA-PCBMA-tBu conjugation was confirmed and the weight ratio (PLGA/PCB) was determined to be 6/1 (mole ratio 12.4/1) by \(^1\)H NMR (Acetonitrile-d\(_3\)) \(\delta\) (ppm): 1.55 (m, 3H, -COCH(CH\(_3\))O-, in PLGA, and 9H, -OC(CH\(_3\))\(_3\) in PCB-tBu), 3.65 (br, 6H, -CH\(_2\)N(CH\(_3\))\(_2\)CH\(_2\)COOC(CH\(_3\))\(_3\), in PCB-tBu), 4.85 (m, 2H, -COCH\(_2\)O-, in PLGA), 5.22 (m, 1H, -COCH(CH\(_3\))O-, in PLGA). All the polymers were dried in vacuum before use.

**Hydrolysis of tBu ester groups.** In our control experiments, PLGA treated with TFA for up to 6 h did not show significant molecular weight changes, and PCB-tBu after 1 h TFA treatment showed no signal at 1.44ppm in \(^1\)H NMR (D\(_2\)O), indicating that tBu ester groups are fully removed. To confirm that ester bonds at methacrylates were not destroyed following the TFA treatment, we did the hydrolysis of CB-tBu monomers in TFA for 1 h and identified the hydrolyzed products to be CB zwitterionic monomers by \(^1\)H NMR (D\(_2\)O) \(\delta\) (ppm): 1.89 (s, 3H, CH\(_2\)=C(CH\(_3\))COO-), 3.31 (s, 6H, -CH\(_2\)N(CH\(_3\))\(_2\)CH\(_2\)COO-), 3.98 (t, J=3Hz, 2H, CH\(_2\)=C(CH\(_3\))COOCH\(_2\)CH\(_2\)N(CH\(_3\))\(_2\)CH\(_2\)COO-), 4.48 (s, 2H, -CH\(_2\)N(CH\(_3\))\(_2\)CH\(_2\)COO-), 4.54 (s, 2H, CH\(_2\)=C(CH\(_3\))COOCH\(_2\)CH\(_2\)N(CH\(_3\))\(_2\)CH\(_2\)COO-), 5.75 and 6.06 (s, 2H, CH\(_2\)=C(CH\(_3\))COO-). To obtain PLGA-co-PCB, PLGA-co-PCB-tBu was treated with TFA for 1 h to remove tBu ester groups. The resulting PLGA-co-PCB was precipitated into ethyl ether, and re-dissolved in a small amount of TFE and precipitated in ethyl ether repeatedly. After vacuum dry, the copolymers are ready for NP formulation. Weight ratio (PLGA/PCB) was determined to be 10/1 by \(^1\)H NMR (Trifluoroacetic Acid-d) \(\delta\) (ppm): 5.50 (m, 1H, -COCH(CH\(_3\))O-, in PLGA),
5.10 (m, 2H, -COCH$_2$O-, in PLGA), 1.77 (d, 3H, -COCH(CH$_3$)$_2$O-, in PLGA), 3.64 (br, 6H, -CH$_2$N(CH$_3$)$_2$CH$_2$COO-, in PCB).

The molecular weight and distribution of PCB homopolymers (derived by hydrolysis of PCB-tBu polymers) are determined by a Waters Alliance 2695 Separations Module equipped with a Waters Ultradehydrogel 1000 column and a Waters 2414 reflex detector. The mobile phase was 100mM NaCl aqueous solution at a flow rate of 0.7 ml/min at 35 °C. Poly(ethylene oxide) from Polymer Laboratories were used as standards. Gel permeation chromatography shows a molecular weight (Mn) of 13640 with polydispersity of 1.12.

**Formulation of PLGA-PCB NPs.** Solvent displacement (nanoprecipitation) method was used to formulate NPs. PLGA-PCB copolymers were dissolved in TFE/MeOH 1/1 v/v cosolvent at the concentration of 0.5 mg/ml. Water (water: organic solvent volume ration, 4:1) was transferred to copolymer solution in a dropwise manner under 1000 rpm stir. After 2 h, solvents were exchanged to PBS and resulting PLGA-PCB NPs were concentrated to the desired concentration by an Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA, US) with 100,000 Da MW cutoff. PLGA NPs were prepared via established method [2]. Docetaxel was loaded in the NPs by mixing with polymers in the organic solvent and followed the above-mentioned procedures to formulate PLGA-PCB/Dtxl NPs. The mean diameter, polydispersity index (PDI) and zeta-potential of NPs were determined by Zetasier Nano-ZS (Malvern Instruments Ltd, Malvern, WR, UK) in triplicates. PDI ranging from 0 to 1.00 was used to characterize the NP size distribution. NPs are considered as monodisperse when PDI < 0.10.

**Drug Loading and Releasing Kinetics.** After nanoprecipitation of polymers (either PLGA or PLGA-PCB) with docetaxel, preparation solution containing drug-loaded NPs and free drugs went through a Microcon centrifugal filter (Millipore, Billerica, MA) with 100,000 Da MW
cutoff. The drug contents in the filtrates (containing free drugs) along with the preparation solution were compared to determine the drug loading (drug/polymers, w/w). In drug releasing studies, Dtxl-loaded NP solutions were placed into Slide-A-Lyzer MINI dialysis microtubes (3500Da MW cutoff, Pierce, Rockford, IL) at 100 μl (0.33 mg/ml) per tube. Those tubes were dialyzed against 1 L PBS at 37 °C with gentle stirring. PBS was refreshed every 24 h. At varied time points, three microtubes were taken to determine drug content retained by the NPs. All aqueous samples were mixed with equal-volume acetonitrile overnight to fully release the drug before running HPLC. Docetaxel content was quantified in triplicates by a Waters Alliance 2695 Separations Module equipped with a reverse-phase C18 column (Econosil, 250 x 4.6 mm, 5 μm, Alltech, Deerfield, IL, USA) and a UV detector (wavelength of 227 nm). The mobile phase was water and acetonitrile (v/v 50/50) at a flow rate of 0.5 ml/min at room temperature. The retention time for free Doc was 13.75 min.

**PLGA-PCB NP functionalization with dye molecules and targeting ligands.** Molecules of interest can be immobilized onto PLGA-PCB NPs via EDC/NHS chemistry. To conjugate dye molecules, NPs were incubated with 400 mM EDC and 200 mM NHS in water for 20 min, and washed with pure water to remove unreacted EDC and NHS. 0.5 mg NHS-activated NPs in 100 μl water were reacted with 25 μl of 5-(aminomethyl)fluorescein hydrochloride (Invitrogen, Carlsbad, CA, US) at the concentration of 10 mg/ml in 10 mM sodium borate buffer, pH = 9 at dark place for 2.5 h. The resulting dye-NP conjugates were washed with pH 9 buffer and water, resuspended in water, and analyzed with the FACScan flow cytometer (Becton Dickinson, San Jose, CA) at the concentration of 0.5 mg/ml. For the immobilization of targeting ligands, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD) was formulated into PLGA-PCB NPs with initial drug loading of 0.25wt %. The resulted PLGA-PCB/NBD NPs were activated by 400 mM EDC and 200 mM NHS in water for 20 min. 0.5 mg NHS-activated NPs in 100 μl water were reacted with 10 μl of NH₂-
galactose at the concentration of 25 mg/ml in 10 mM sodium borate buffer, pH = 9 for 1 h. The resulting galactose-NP conjugates were washed with pH 9 buffer and PBS, and resuspended in PBS before cell incubation. HepG2 cells were grown in 24-well plates in full MEM medium (Hyclone, Logan, UT) supplemented with non-essential amino acid, sodium pyruvate and 10% fetal bovine serum (FBS) under 5% CO₂ at 37 °C to allow 50% confluence. Cells were then washed with pre-warmed PBS and incubated with 400 μl/well NP-containing MEM medium without FBS supplement (PLGA-PCB/NBD-Galactose NP concentration: 1.25 mg/ml). After 2 h, cells were washed with PBS and supplemented with 400 μl/well FBS-containing medium. After 20 h incubation at 37 °C, cells were visualized using a Nikon TE2000U microscope. For control experiments, EDC was absence while all other conditions and procedures were exactly the same.

**Cytotoxicity assays for PLGA-PCB NPs**

We evaluated the cytotoxicity of the NPs using a Vybrant® MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly, HepG2 cells were grown in 96-well plates in full MEM medium supplemented with non-essential amino acid, sodium pyruvate and 10% FBS under 5% CO₂ at 37 °C to allow 80-90% confluence. For each well, cells were washed with PBS and incubated with 200 μl full medium containing varied concentration of either PLGA-PCB or PLGA NPs for 24 h. Cells were washed with PBS to remove NPs and incubated with 100 μl full medium plus 50 μl of 12 mM MTT stock solution for another 4 h. Then, MTT-containing medium was replaced with 150 μl DMSO to dissolve the formed crystal at 37 °C for 10 min. Absorbance (Abs) was measured at 570 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) with pure DMSO as the blank reading. Cells with no NP incubation were used as the controls and cell viability upon NPs treatment was estimated in triplicate: cell viability (%) = Abs_{sample} / Abs_{control} x 100.
Figure S1. Docetaxel release profiles from PLGA-PCB NPs and PLGA NPs. Drug loading for both NPs was 1wt %. PCB modification did not change drug releasing behavior much over unmodified PLGA.

Figure S2. NP stability in a) 10 wt % BSA solution in PBS, and b) 100% FBS solution at 37°C. NP size (Mean ± SD, N = 3) was plotted as a function of time.
Figure S3. Cytotoxicity of PLGA-PCB NPs and PLGA NPs on HepG2 cells. NPs are incubated with the cells at indicated concentrations for 24h, and immediately assayed for cell viability in triplicate. Similar to PLGA NPs, PLGA-PCB NPs exhibit no cytotoxicity at concentrations up to 10 mg/ml.
