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HDAC Inhibitor Conjugated Polymeric Prodrug Micelles for Doxorubicin Delivery

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Abstract

Amphiphilic diblock copolymers bearing histone deacetylase inhibitors (HDACi) (4-phenyl butyric acid and valproic acid) were synthesized by the ring-opening polymerization of γ -4-phenylbutyrate- ϵ -caprolactone (PBACL), γ -valproate- ϵ -caprolactone (VPACL), and ϵ -caprolactone (CL) from a poly(ethylene glycol) macroinitiator (PEG). These amphiphilic diblock copolymers self-assembled into stable pro-drug micelles and demonstrated excellent biocompatibility. High loading of doxorubicin (DOX) up to 5.1 wt% was achieved. Optimized micelles enabled sustained drug release in a concentration-dependent manner over time to expand the therapeutic window of cytotoxic small molecule drugs.

Graphical abstract

Amphiphilic diblock copolymers bearing histone deacetylase inhibitors (HDACi) (4-phenyl butyric acid and valproic acid) are reported for micellar drug delivery.

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Introduction

Polymeric micelles from diblock copolymers that consist of hydrophilic and hydrophobic blocks have gained considerable attention in medical applications such as delivery systems for poorly water soluble drugs.^{1, 2} Amphiphilic block copolymers self-assemble to form micelles with the hydrophobic part of the polymer on the inside (core) and hydrophilic on the outside (shell). The encapsulation of poorly water soluble drugs in the hydrophobic core due to the hydrophobic interactions enables their delivery.² The hydrophilic shell provides the protection in the aqueous environment which allows for a longer blood circulation time. Since most of the anticancer drugs are poorly soluble in water, these can be encapsulated in the micellar core by physical or chemical interactions with the core forming segment to enhance the solubility and hence the bioavailability of the drug. Herein, we report two amphiphilic block copolymers synthesized by the ring-opening polymerization of γ -substituted ϵ -caprolactone monomers bearing two different HDACi pendant groups. These amphiphilic copolymers can self-assemble into core-shell structures to allow loading of hydrophobic drugs such as DOX.

The release rate of the encapsulated drugs depends on the composition of the hydrophobic core, which subsequently affects the antitumor efficacy.³ The ability of the core to encapsulate drug is dependent upon the compatibility between the hydrophobic core and the drug molecule.⁴ Amphiphilic diblock copolymers of PEG and polyphosphoester with different side groups (butyl, hexyl, octyl) have been reported to tune the hydrophobicity of the micellar core.⁵ Widely used core-forming hydrophobic polymers for drug delivery can be classified into the following groups: poly(propylene oxide) (PPO),⁶ poly(esters) such as poly(lactic acid)⁷ and poly(ϵ -caprolactone) (PCL),^{8, 9} poly(L-amino acids) such as poly(L-lysine),¹⁰ and polystyrene.¹¹ The encapsulation of drug within hydrophobic cores constructed from these polymers mainly occurs via hydrophobic interactions that are thermodynamically driven. PEG is the most common shell-forming polymer used in FDA-approved bioconjugates. PEG is non-toxic, hydrophilic, and repels protein adsorption. In an aqueous environment, PEG is highly hydrated and forms a dense shell. The shell limits the micelle-micelle interaction and the micelle-protein interactions.¹

Histone acetylation plays an important role in gene expression. HDAC removes the acetyl group from lysine residues on the tails of histone proteins. This allows the phosphate

backbone of DNA to tightly bind around the histones and leads to condensed and transcriptionally silenced chromatin. HDAC activity plays a key role in cell differentiation,¹² embryogenesis,¹³ cancer,¹⁴ neurodegenerative diseases,¹⁵ immunological responses,¹⁶ metabolic homeostasis¹⁷ and many other biological phenomena. Inhibition of HDAC activity stimulates tumor cells to enter apoptosis; therefore, the utility of HDACi for the treatment of cancer has been investigated and several HDACi have now entered clinical trials. Among various structurally different HDACis, the short chain fatty acids like 4-phenylbutyric acid (PBA) and valproic acid (VPA) have been identified as anti-proliferative agents.^{18, 19} To date, several structurally distinct classes of HDACi have advanced into Phase I and Phase II clinical trials in solid tumors and hematological malignancies, including VPA and PBA.²⁰ The first HDACi (Vorinostat) was approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma. In this study, the HDACi VPA and PBA are attached to functional caprolactone monomers through ester linkages, followed by polymerization. The combination of PEG, HDACi substituted poly(ϵ -caprolactone)s and unsubstituted poly(ϵ -caprolactone)s resulted in amphiphilic block copolymers that self-assemble into pro-drug micelles which can be employed to deliver anticancer drugs.

Results and discussion

Monomer synthesis

VPA and PBA are HDACi that possess anti-cancer properties. The goal of this study was to incorporate VPA and PBA into biodegradable amphiphilic polymers as a novel approach in the delivery of drug combinations. Biocompatible and biodegradable aliphatic polyesters are widely used in drug delivery applications.^{21–25} Among them ϵ -caprolactone has gained the most interest due to its ease of functionalization and hydrolyzability. To date a large number of functionalized poly(ϵ -caprolactone)s have been reported for drug delivery applications.^{26–29}

Steglich esterification was chosen to couple the carboxylic acid of the HDACi and the hydroxyl group of the 1,4 cyclohexane diol, in both the case of **M1** and **M2**.³⁰ The synthesis of monomer **M1** was carried out according to the procedure described in Scheme 1. PBA was reacted with 1,4-cyclohexanediol in the presence of 4-dimethylaminopyridine (DMAP) and N, N'-dicyclohexylcarbodiimide (DCC) to obtain 4-hydroxycyclohexyl-4-phenylbutanoate [**1**]. Dicyclohexylurea, as the by-product, was easily removed by filtration. Oxidation with chromic acid yielded 4-oxocyclohexyl-4-phenylbutanoate [**2**]. Finally, the Baeyer–Villiger oxidation with m-chloroperoxybenzoic acid generated the γ -4-phenylbutyrate- ϵ -caprolactone monomer **M1** in ~40% overall yield. ¹H NMR, ¹³C NMR, and C/H elemental analysis confirmed the formation of the monomer. Monomer **M2** was synthesized according to the previously published method.³¹

Block copolymer synthesis

We hypothesized that hydrophobic pendent groups in the micellar core may result in higher DOX encapsulation due to the hydrophobic interactions. Furthermore the incorporation of PCL may increase the void volume in the micellar core thereby with the overall goal of encapsulating a greater amount of DOX. However, previous studies from our group revealed

that the interaction between the drug and the polymer is more important than the void volume core in determining the drug loading capacity.³² Therefore we have synthesized two different hydrophobic pendent groups featuring amphiphilic diblock copolymers with a substantial amount of PCL.

PEG was chosen to generate the hydrophilic block. Two different block copolymers, **P1** and **P2**, were synthesized using PEG as the macroinitiator. **P1** contains a hydrophobic block consisting of randomly incorporated **M1** and CL, while **P2** contains a hydrophobic block consisting of randomly incorporated **M2** and CL (Scheme 2). The ring-opening polymerization of the caprolactone monomers was carried out using stannous (II) 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$) catalyst at 110 °C. The molar ratio [PEG]: [**M1**]: [CL]: [$\text{Sn}(\text{Oct})_2$] = 1: 100: 125: 1 and ratio [PEG]: [**M2**]: [CL]: [$\text{Sn}(\text{Oct})_2$] = 1: 100: 125: 1 were used for the synthesis of **P1** and **P2**, respectively.

The ^1H NMR spectra for **P1** and **P2** are shown in S9 and S10. The polymer compositions are summarized in Table 1. The composition of the copolymers was determined using ^1H NMR. The molecular weights were determined using SEC and ^1H NMR. The signal at ~ 3.38 ppm was assigned to the methoxy end group of the PEG block. The peaks at ~3.64 ppm are due to the methylene protons of the PEG block. The multiplet at 1.39 ppm includes the methylene protons of the polycaprolactone block. The two singlets at 5.00 and 4.99 are due to the methylene protons of PBA and VPA repeating units respectively. The composition of the copolymers was determined by integrating the methylene protons of the PCL block, methylene protons of PBA, and methyl protons of the VPA vs. the methylene protons of the PEG block. The M_n values obtained from ^1H NMR were higher than the values determined by SEC analysis. These discrepancies can be attributed to the differences in the hydrodynamic volume of the diblock copolymers and poly(styrene) standards used for the calibration.^{21, 25}

The degradability of polymers **P1** and **P2** was studied at three different pHs: pH 7.4 in PBS, pH 6, and pH 5 buffers at 37 °C, over 5 days (Fig. 1). The polymers degraded over time due to the hydrolysis of ester groups in the backbone. The molecular weights decreased by 50% after 5 days for all pHs and the highest degradation rate was observed at pH 5.

Self-assembly

The critical micelle concentration (CMC) of the block copolymers **P1** and **P2** was determined by fluorescence spectroscopy using pyrene as a fluorescent probe.^{24, 25} The intensity ratio ($I^{337.5}/I^{334.5}$) was plotted against the logarithm of the polymer concentration. The CMC value was obtained as the point of intersection of two tangents drawn to the curve at high and low concentrations (Fig. 2). The CMC values of all the polymers are in the range of 10^{-4} g/L indicating high thermodynamic stability of the micelles.

The hydrodynamic diameters (D_h) of the polymeric micelles **P1** and **P2** were 91 and 96 nm respectively (Table 1 and Figure 3). The size of copolymer micelles for **P1** and **P2** are larger than the previously reported values for amphiphilic block copolymers containing hydrophobic γ -substituted poly(ϵ -caprolactone)s despite the comparable molecular weights and ratios between the hydrophilic and hydrophobic blocks.^{32, 33} The larger hydrodynamic

size is most likely due to the bulky substituents which could result in an increase of the volume of the hydrophobic core. The sizes of micelles in TEM (transmission electron microscope) images were smaller than those measured by DLS (Fig. 4). The micelles sizes measured by TEM were 38 and 71 nm for **P1** and **P2** respectively. In TEM, micelles can undergo dehydration during the sample preparation which causes the PEG shell to shrink or collapse.³⁴ Furthermore, TEM reports number-average dimensions, whereas DLS reports an intensity-average dimension. Therefore, TEM can result in smaller sizes relative to DLS.

Doxorubicin encapsulation

DOX loaded micelles were formed by nanoprecipitation followed by dialysis. To determine the amount of encapsulated DOX, a known amount of DOX loaded micelles were mixed with DMSO and sonicated. The absorbance of the solution at 485 nm was fitted to a pre-established standard curve.

DOX loaded micelles were prepared with a polymer:drug feed weight ratio of 10:1 and the encapsulation efficiency (wt % EE) and drug loading capacity (wt % LC) were calculated using the equations below and are shown in Table 2.

$$\text{wt\%EE} = \frac{\text{wt of encapsulated DOX}}{\text{wt of total DOX added}} \times 100$$

$$\text{wt\%LC} = \frac{\text{wt of encapsulated DOX}}{\text{wt of polymer}} \times 100$$

Polymer **P1** showed the highest loading capacity (5.10%) possibly due to the π - π interactions with the DOX and the PBA. Polymer **P2** showed much lower loading capacity (1.62%), which can be attributed to increased steric bulk of the α -propyl pendant groups.

The sizes of the DOX-loaded micelles were determined using DLS (Figure 5) and TEM (Fig. 6). The narrow dispersities shown in DLS indicate the formation of highly uniform micelles. The sizes of the DOX loaded micelles as measured by TEM were 92 and 90 nm for **P1** and **P2** respectively. The larger size of the DOX loaded micelles compared to empty micelles is suggestive of successful DOX loading into the micelle core.

In vitro drug release

The *in vitro* release profile of **P1** was evaluated at 37 °C in buffers at different pHs (pH 5.0, pH 6.0, and pH 7.4) and is shown in Fig. 7. At pH 7.4, only 11% of DOX was released within 24 h. At pH 5.0 and pH 6.0, approximately 30% of DOX was released. However, after 45 hours the DOX release was 66% at pH 5.0. At pH 6 and pH 7.4 the releases were 52% and 13% respectively. In the acidic buffer, micelles can degrade faster due to the hydrolysis of the ester groups in the backbone of the polymer thus releasing the loaded DOX. The micelles are very stable in normal physiological conditions (pH 7.4) but at endocytic pH, the micelle degradation can ensue, enabling DOX release. Additionally,

overall the prepared DOX loaded polymeric micelles fit in the desired range, 10–200 nm, to take advantage of the EPR effect.^{22, 35, 36}

Biological Evaluation of DOX-loaded HDACi substituted poly(ϵ -caprolactone)s amphiphilic diblock copolymers

To investigate the biological utility of HDACi substituted poly(ϵ -caprolactone)s amphiphilic diblock copolymers the cytotoxicity of empty **P1** and **P2** micelles was evaluated. First, empty micelles were incubated with HeLa cervical cancer cells for 60 hours. Cell viability was assessed at different micelle concentrations using the CellTiter-Glo assay (Fig. 8). The results were compared with equivalent doses of free PBA and VPA. **P1** and **P2** micelles did not exhibit appreciable cytotoxicity below 170 ng/ μ L. Above this concentration, some decrease in cell number was measured, which coincided with data for free PBA and VPA. HDACi in the carboxylate class, such as VPA and PBA, typically have lower inhibitory activity when compared with other HDACi.¹⁸ Therefore, it may be necessary to find a way to increase the dose of these HDACi within the polymeric micelles to show higher HDACi activity.

Having verified the ability of the micelles to encapsulate and release DOX in physiologically relevant conditions, we evaluated the cellular uptake behavior of DOX-loaded **P1** and **P2** micelles (Fig. 9). DOX exhibits intrinsic fluorescence which enabled monitoring of DOX-loaded micelles into cells. DAPI was used to stain the nuclei of cells. Free DOX displayed typical cellular uptake, producing a bright, diffuse cytoplasmic signal after 2 hours of incubation with HeLa cells (Fig. 9 bottom). **P1** micelles effectively entrapped DOX, leading to endocytosis into HeLa cells, suggested by the punctate fluorescence pattern inside the cells within the first 4 hours of incubation (Fig. 9, top). Merged bright field and fluorescence images clearly show effective uptake into all cells. In contrast, **P2** micelles, which show low DOX loading capacity (Table 2), are not effectively endocytosed. Using the same imaging settings, **P2** micelles displayed much weaker intracellular DOX fluorescence signal as compared to **P1** micelles (Fig. 9, middle). These results suggest that the phenyl side chains in the **P1** polymer may stabilize the DOX-loaded micelles through enhanced hydrophobic packing (π - π stacking), leading to more stable micelles with enhanced cellular uptake.

The cancer cell killing potential of the DOX-loaded **P1** and **P2** micelles was then examined. Dose response curves illustrate the dose-dependent effects on cell viability after 60 hours of incubation. Combination of free DOX with PBA or VPA displayed the highest decrease in cell viability. As shown in Figure 9 and 10, the free drugs can enter and kill cancer cells through established mechanisms.³⁷ Encapsulation of DOX inside of micelles provides an opportunity to enable controlled release and tumor targeting. Indeed, encapsulation of the same amount of DOX inside of **P1** and **P2** polymeric micelles delayed cell killing, thereby broadening the therapeutic window. Among the tested polymers, **P1** showed the greatest ability to encapsulate DOX into stable micelles. **P1** micelles promoted cellular uptake within the first few hours of incubation (Fig. 9). This likely led to enhanced cell killing versus **P2** micelles.

Next, the effect of the dose on cell viability for the DOX-loaded micelles on cytotoxicity was examined (Figure 10). When evaluating effective cell killing by polymer weight (Fig. 10a), **P1** exhibited improved delivery over polymer **P2**, which was less effective. In all cases, the block copolymers mediated slower DOX release compared to adding free DOX, when compared by weight or DOX concentration. The dose-dependent killing of **P1** micelles provides a longer treatment window, and potentially permits co-delivery of HDACi and DOX, which could synergize to increase efficacy. All polymers show better release toxicity profiles than free drugs alone, suggesting the ability of these degradable copolymers to slowly release DOX and increase the therapeutic window.

Finally, we performed a time course evaluation of the effect of the DOX-loaded micelles on cell viability (Fig. 11). All polymers showed improved cell killing with increased incubation time. **P1** likely mediated fast uptake, resulting in 40% toxicity at 30 minutes. Subsequently, this level of toxicity was sustained until 16 h. This suggests a possible burst release initially, followed by sustained controlled release as the copolymer degrades. Alternatively, some micelle instability in serum-containing media could prematurely release DOX, resulting in an early onset of toxicity, but this is less likely given the measured stability over several days at pH 7.4 (Fig. 7). All three copolymers mediated slower cell killing versus free DOX, suggesting a slow release mechanism and an expanded therapeutic window. Between **P1** and **P2**, both side chains are hydrophobic, but the **P1** phenyl group may provide better DOX encapsulation and slow release due to π - π stacking interactions. Cumulatively, these results indicate that substituted poly(ϵ -caprolactone)s amphiphilic diblock copolymers are applicable drug carriers that can enable sustained drug release in a concentration-dependent manner over time to expand the therapeutic window of cytotoxic small molecule drugs.

Conclusions

Novel 4-phenyl butyric acid and valproic acid, conjugated amphiphilic diblock copolymers were synthesized for the delivery of the anticancer drug DOX. The polymers self-assembled into prodrug micelles and the micellar core was used to load the doxorubicin. The introduction of the 4-phenylbutyric acid into the polymers resulted the loading up to 5.1 wt % with DOX. The DOX loaded histone deacetylase inhibitor conjugated prodrug micelles fit into the targeted nanoscale to exploit EPR effect and enabled sustained drug release in a concentration-dependent manner over time to expand the therapeutic window of cytotoxic small molecule drugs. Future efforts will be in increasing the amount of HDACi incorporated into the polymer in order to increase the therapeutic benefit of the HDACi.

Experimental

Materials

Doxorubicin hydrochloride (DOX·HCl, 99%), methoxy poly(ethylene glycol) (mPEG, M_n = 2000 g/mol) and all other chemicals were purchased from Sigma-Aldrich Co. and were used without further purification unless otherwise noted. Stannous(II) 2-ethylhexanoate and benzyl alcohol were purified by vacuum distillation prior to use. All polymerization reactions were conducted under purified nitrogen. The polymerization glassware and

syringes were dried at 120 °C for at least 24 hours and then were cooled under nitrogen before use.

Analysis

¹H NMR spectra of the synthesized monomers and polymers were recorded on a Bruker AVANCE III 500 MHz NMR instrument at 25 °C in CDCl₃. ¹H NMR data are reported in parts per million as chemical shift relative to tetramethylsilane (TMS) as the internal standard. Polydispersity indices of the synthesized polymers were measured by size exclusion chromatography (SEC) analysis on a Viscotek VE 3580 system equipped with ViscoGEL columns (GMHHR-M), connected to a refractive index (RI) detector. GPC (SEC) solvent/sample module (GPCmax) was used with HPLC grade THF as the eluent, and calibration was based on polystyrene standards. An Agilent UV/Vis spectrophotometer was employed for DOX loading determination (absorbance). TEM imaging of the DOX-loaded micelles was performed on a Tecnai G2 Spirit Biotwin microscope by FEI and images were analyzed using ImageJ software. Samples were prepared by treating copper mesh grid with 1 mg mL⁻¹ aqueous micelle solution for 2 minutes, followed by staining with 2% phosphotungstic acid for 30 seconds.

Synthetic procedures

Monomer γ -2-propylpentanoate- ϵ -caprolactone (**M2**) was synthesized according to previously described procedures.³¹

Synthesis of 4-hydroxycyclohexyl-4-phenylbutanoate (1)—To a stirred solution of 4-phenylbutyric acid (5.00 g, 0.030 mol) in 30 mL DMF was added DMAP (0.37 g, 0.003 mol) and 1,4-cyclohexanediol (7.06 g, 0.0608 mol). DCC (6.899 g, 0.033) was added to the reaction mixture at 0 °C, which was then stirred for 10 min at 0 °C and 4h at RT. Precipitated urea was then filtered off and residue was taken up in CH₂Cl₂ and organic layer washed with water (3× 50 mL). The CH₂Cl₂ solution was washed with 0.5N HCl (2× 50mL) and with saturated NaHCO₃ solution. Organic layer was dried over anhydrous MgSO₄ and concentrated in vacuo. The product was isolated by flash chromatography (R_f = 0.2 in 70:30, Hexane: ethyl acetate) to yield 3.7 g of pale yellow oil (0.014 mol, 50%). ¹H NMR (500 MHz, CDCl₃): δ 1.45 (m, 2H), 1.62 (m, 2H), 1.87 (m, 2H), 1.99 (m, 4H), 2.32 (m, 2H), 2.66 (m, 2H), 3.80 (m, 1H), 4.78 (m, 2H), 4.89 (m, 1H), 7.19 (m, 3H), 7.30 (m, 2H). ¹³C NMR (500 MHz, CDCl₃): δ 173.0, 141.8, 128.0, 125.9, 71.6, 68.9, 35.1, 33.9, 32.2, 27.4, 26.6.

Synthesis of 4-oxocyclohexyl-4-phenylbutanoate (2)—Sulfuric acid 2M (10 mL) was added slowly to a solution of potassium dichromate (4.48 g, 0.015 mol) in water. The chromic acid was added slowly to a 4-hydroxycyclohexyl-4-phenylbutanoate (2.66 g, 0.010 mol) solution in 5 mL of DMF. After 3 h, the solution was extracted with diethyl ether (3×50 mL) and the ether layers were washed with water (3×50 mL). The ether layer was dried over magnesium sulfate and concentrated in vacuo. The product was isolated by flash chromatography (R_f = 0.4 in 70:30, hexane: ethyl acetate) to yield 2.17 g of pale yellow oil (0.008 mol, 85%).

^1H NMR (500 MHz, CDCl_3): δ 2.02 (m, 2H), 2.09 (m, 4H), 2.40 (m, 4H), 2.54 (m, 2H), 2.70 (m, 2H), 5.20 (m, 1H), 7.21 (m, 3H), 7.32 (m, 2H). ^{13}C NMR (500 MHz, CDCl_3): δ 209.8, 172.8, 129.0, 141.2, 126.1, 68.5, 37.3, 35.1, 33.8, 30.5, 26.5.

Synthesis of γ -4-Phenylbutyrate- ϵ -caprolactone (M1)—4-oxocyclohexyl 4-phenylbutanoate (2.17 g, 0.008 mol) was added to a solution of 77% m-chloroperoxybenzoic acid (2.86 g, 0.016 mol) in 20 mL of chloroform under stirring. The reaction was left overnight at RT. Potassium carbonate (4 g) and 10 mL of water were added to solution and stirred vigorously for 2 h. The organic layer was dried over magnesium sulfate and concentrated in vacuo. The product was isolated by flash chromatography (R_f = 0.3 in ethyl acetate) to yield 2.1 g (0.008 mol, 90%) of pale yellow oil.

^1H NMR (500 MHz, CDCl_3): δ 2.00 (m, 3H), 2.20 (m, 1H), 2.40 (m, 2H), 2.55 (m, 1H), 2.68 (m, 2H), 2.91 (m, 2H), 4.16 (m, 1H), 4.44 (m, 1H), 5.14 (m, 1H), 7.21 (m, 3H), 7.32 (m, 2H). ^{13}C NMR (500 MHz, CDCl_3): δ 174.9, 172.4, 141.0, 128.5, 126.1, 69.8, 63.5, 35.0, 33.6, 33.7, 28.5, 27.6, 26.4. Anal. Calculated for $\text{C}_{14}\text{H}_{26}\text{O}_3$: C, 69.54%; H, 7.3%; O, 23.16%. Found: C, 68.65%; H, 7.52%; O, 23.82%.

Synthesis of terpolymer P1—To an oven-dried 10 mL Schlenk flask, PEG (0.072 g, 0.036 mmol) and monomer **M1** (1.00 g, 3.6 mmol) with CL (0.52 g, 4.5 mmol) were introduced. The flask was dried under vacuum for an hour and the vacuum in the Schlenk flask was cancelled with nitrogen. The reaction was introduced to a thermostat-controlled oil bath heated to 110 °C. A solution of $\text{Sn}(\text{Oct})_2$ containing (0.015 g, 0.036 mmol) in 0.2 mL dry toluene was prepared in glove box and added to the flask under nitrogen. After the polymerization was carried out overnight, the reaction mixture was removed from heat and dissolved in 2 mL THF, and then was poured into a beaker containing hexane, where it crashed out. The polymer suspension was refrigerated overnight and then decanted and dried under vacuum. The polymer was analyzed by ^1H NMR and SEC. $M_n^{\text{NMR}} = 27000 \text{ g mol}^{-1}$, $M_n^{\text{SEC}} = 8900 \text{ g mol}^{-1}$, $\text{PDI}^{\text{SEC}} = 1.73$. ^1H NMR (500 MHz, CDCl_3): δ 1.37 (m, 2H), 1.62 (m, 6H), 1.90 (m, 4H), 2.29 (m, 6H), 2.63 (m, 2H), 3.64 (m, 4H), 4.20 (m, 4H), 5.0 (m, 1H), 7.21 (m, 5H).

Synthesis of terpolymer P2—To an oven-dried 10 mL Schlenk flask, PEG (0.069 g, 0.035 mmol) and **M2** (1.00 g, 3.9 mmol) and CL (0.56 g, 4.9 mmol) were introduced. The flask was dried under vacuum for an hour and the vacuum in the Schlenk flask was cancelled with nitrogen. A thermostat-controlled oil bath was heated to 110 °C. A solution of $\text{Sn}(\text{Oct})_2$, containing (0.016 g, 0.039 mmol) in 0.2 mL dry toluene was prepared in glove box and added to the flask under nitrogen, which was immediately lowered into the oil bath. After the polymerization carried out overnight the reaction mixture was removed from heat and dissolved in 2 mL THF, and then was poured into a beaker containing hexane, where it crashed out. The polymer suspension was refrigerated overnight and then decanted and dried under vacuum. The polymer was analyzed by ^1H NMR and SEC. $M_n^{\text{NMR}} = 21000 \text{ g mol}^{-1}$, $M_n^{\text{SEC}} = 44800 \text{ g mol}^{-1}$, $\text{PDI}^{\text{SEC}} = 1.76$. ^1H NMR (500 MHz, CDCl_3): δ 0.88 (m, 6H), 1.27 (m, 4H), 1.39 (m, 4H), 1.56 (m, 2H), 1.63 (m, 4H), 1.88 (m, 4H), 2.29 (m, 5H), 3.63 (m, 4H), 4.02 (m, 4H), 5.00 (m, 1H).

Characterization

Preparation of micelles—Polymeric micelles of the terpolymers were formed by nanoprecipitation and dialysis. In general, the terpolymer (10 mg) was dissolved in THF (0.5 mL) and added dropwise into 10 mL of deionized water while sonicating. The suspension was transferred to SnakeSkin® dialysis tubing (MWCO 3500 Da) and dialyzed against a minimum of 3000 mL deionized water over a 24 hour period. Finally, the dialysis tube contents were filtered through a Nylon syringe filter (0.45 µm pore size), and a 1 mg mL⁻¹ solution of polymeric micelles was obtained.

Preparation of DOX-loaded micelles—DOX-loaded micelles were prepared using a previously published method. First, the DOX·HCl was neutralized with 3 equivalents of triethylamine in THF:DMSO 5:1. An aliquot (containing 0.5 mg DOX) of the neutralized DOX solution was added to the terpolymer solution (5 mg in 0.2 mL THF). The DOX–polymer solution was then added dropwise into 5 mL of deionized water while sonicating. The suspension was transferred to dialysis tubing (MWCO 3500 Da) and dialyzed against a minimum of 1500 mL deionized water over a 12 hour period. The water was exchanged for phosphate buffered saline (1500 mL, pH 7.4 PBS 1×) and dialysis continued for another 12 hours. Finally, the dialysis tube contents were filtered through a Nylon syringe filter (0.45 µm pore size), and a 1 mg mL⁻¹ solution of DOX-loaded micelles was obtained. To determine drug loading capacity (LC) and drug loading efficiency (LE), 1.5 mL of the micelle suspension was added to 1.5 mL dimethylsulfoxide (DMSO) and subjected to bath sonication (~20 min) to release the DOX from the micelles. After sonication, the absorbance of the solution at 485 nm was fitted to a pre-established standard curve of DOX in PBS/DMSO to determine the concentration of DOX.^{21, 33}

Analysis of micelles by dynamic light scattering (DLS)—Aqueous suspensions of polymeric micelles were prepared as described above. The micelle suspensions were filtered through a 0.45 µm Nylon syringe filter. The micelles were analyzed using dynamic light scattering with a Malvern Zetasizer Nano ZS instrument equipped with a He–Ne laser (633 nm) and 173° backscatter detector to determine their hydrodynamic diameters.

Determination of CMC—The critical micelle concentration was determined using fluorescent spectroscopy. Pyrene was used as the fluorescent probe and a constant pyrene concentration of 6.67×10^{-5} g L⁻¹ was used. Samples of polymer of varying concentrations were combined with a small amount of pyrene in small amount of THF. These solutions were added dropwise into 10 mL of deionized water. The solutions were stirred for a minimum of 6 hours to allow the micelles to assemble as the THF evaporated. Fluorescence spectra of the polymer/pyrene solutions were collected with a Perkin-Elmer LS 50 BL luminescence spectrometer at 25 °C with emission wavelength set at 390 nm. The ratio of the intensities of the pyrene excitation peaks at 337.5 nm and 334.5 nm were recorded and plotted against the log of the polymer concentration.

Demonstration of polymer degradability—The biodegradability of polymers **P1** and **P2** was measured. Polymers (20 mg) were dissolved in 2.0 mL of pH 7.4 PBS, pH 6 PBS and pH 5 acetate buffer solutions separately in closed containers and placed in a

thermostatted bath at 37 °C for 5 days. Periodically, samples were extracted from the solution and analyzed by SEC to monitor the change in M_n from $t = 0$ days to $t = 5$ days. The data is plotted as % of initial M_n versus days spent in the PBS solution at 37 °C.

In vitro DOX release—A 1 mg/mL solution of DOX-loaded **P1** micelle solution was prepared. The 4 mL solution was transferred to dialysis membrane tubes with a molecular weight cut-off of 3500. The tubes were then immersed in 3 separate beakers, each containing 30 mL pH 7.4 PBS, 30 mL pH 6 PBS, and 30 mL pH 5 acetate buffer solutions. The solutions were stirred at a constant speed. The release of DOX from the micelles was measured at 37 °C. At specific time intervals, 3 mL of solutions were withdrawn from the each release medium and replaced with fresh solutions. The DOX content in the samples was analyzed using the UV-vis spectrophotometer. Absorbance measurements in the release media at 485 nm were used to calculate the cumulative DOX release.

Biological Studies

Unless otherwise indicated, all tissue culture was performed in high glucose Dulbecco's Modified Eagle Medium (Hyclone) supplemented with 5% fetal bovine serum (Sigma-Aldrich). Cells were grown in a humidified environment at 37 °C, 5% CO₂. Cell viability studies were performed using the CellTiter-Glo assay (Promega) according to the manufacturer's recommended protocol.

Empty micelles cytotoxicity studies—HeLa cells were seeded in white opaque flat-bottom 96-well plates at a cell density of 2,500 cells per well in 100 μ L growth medium. After 24 h to allow cell adhesion, the medium was exchanged for 180 μ L fresh growth medium. Empty micelles (1 mg/mL in PBS) were serially diluted 2-fold down to 0.016 mg/mL in PBS. Micelles dilutions (100 μ L) were added to the cells via multichannel pipette for final micelle concentrations of 357 ng/ μ L, 179 ng/ μ L, 89 ng/ μ L, 45 ng/ μ L, 22 ng/ μ L, 11 ng/ μ L, or 5.5 ng/ μ L respectively. Free HDAC inhibitor dosing of PBA and VPA was performed in parallel and was calculated based on the HDACi-CL monomer incorporation in the polymer. The micelles were incubated for 24 hours with the cells. After this time, the micelles and medium were removed and replaced with 200 μ L fresh growth medium. The cells were allowed to grow for an additional 36 hours (60 h total incubation time). Cell viability was evaluated by the CellTiter-Glo assay and normalized to the PBS control, N = 4 or 5.

DOX-loaded micelles cytotoxicity studies—HeLa cells were seeded in white opaque flat-bottom 96-well plates at a cell density of 2,500 cells per well in 100 μ L growth medium. After 24 h to allow cell adhesion, the medium was exchanged for 180 μ L fresh growth medium. DOX-loaded micelles (1 mg/mL in PBS) were diluted in PBS to 0.5 mg/mL the serially 4-fold to a final concentration of 1.22×10^{-4} mg/mL. Micelles dilutions (100 μ L) were added to the cells via multichannel pipette for final micelle concentrations of 357 ng/ μ L, 179 ng/ μ L, 45 ng/ μ L, 11 ng/ μ L, 2.8 ng/ μ L, 0.70 ng/ μ L, 0.17 ng/ μ L, 0.04 ng/ μ L respectively. Free DOX dosing was given assuming a 5% micelle loading by weight, while free HDAC inhibitor dosing of PBA and VPA was calculated based on the HDACi-CL monomer incorporation in the polymer. The micelles were incubated for 24 hours with the

cells. After this time, the micelles and medium were removed and replaced with 200 μ L fresh growth medium. The cells were allowed to grow for an additional 36 hours (60 h total incubation time). Cell viability was evaluated by the CellTiter-Glo assay and normalized to the PBS control, N = 4 or 5.

Time course cytotoxicity studies with DOX-loaded micelles—HeLa cells were seeded in white opaque flat-bottom 96-well plates at a cell density of 2,500 cells per well in 100 μ L growth medium. After 24 h to allow cell adhesion, the medium was exchanged for 200 μ L fresh growth medium. 25 μ L of DOX-loaded micelles (1 mg/mL in PBS) were added to the respective wells via multichannel pipette for a final micelle dose of 11.11 ng/ μ L micelle. Free DOX was added assuming a 5% micelle loading by weight, while free HDAC inhibitor dosing of PBA and VPA was calculated based on the HDACi-CL monomer incorporation in the polymer. The micelles were incubated for 0.5, 1, 2, 4, 16, or 24 hours with the cells. After each time point, the cells were washed with 100 μ L PBS and 200 μ L fresh DMEM complete medium was added. The cells were incubated until a final time point of 61h. Cell viability was evaluated by the CellTiter-Glo assay and normalized to the PBS control, N= 4.

Imaging studies—HeLa cells were seeded in 8-well coverslip bottom chamber slides (Lab-Tek) at a density of 30,000 cells per well and allowed to adhere overnight. After the initial adhesion period the medium was exchanged for 300 μ L fresh growth medium. DOX loaded micelles in PBS (1 mg/mL) were added for a concentration of 0.25 μ g/mL micelle. Free DOX in PBS was added to the cells to mimic a dose of 5% loading. The micelles were incubated for 4 hours while free DOX was incubated for 2 hours. After the uptake time period, the cells were washed 3 times with 100 μ L PBS, fixed with 4% paraformaldehyde (room temperature 10 minutes), washed 3 times with 100 μ L PBS, and the nuclei were counterstained with DAPI. Confocal laser scanning microscopy was performed on a Zeiss LSM700, and the images were processed using ImageJ (NIH).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Degradability of polymer **P1**: decrease in M_n as a function of time, performed in pH 7.4 PBS, pH 6 PBS, and pH 5 acetate buffer solutions at 37 °C over time.

Fig. 2.

Dependence of ratio $I^{337.5}/I^{334.5}$ from pyrene excitation spectra as a function of polymer concentration: **P1** and **P2**. [Py] 4.0×10^{-7} M, $\lambda_{\text{em}} = 390$ nm

Fig. 3.
DLS measurements: hydrodynamic diameters (D_h) of micelles of **P1** and **P2**.

Fig. 4.
TEM images of empty micelles **P1** and **P2**. Scale bars =100 nm

Fig. 5.
DLS measurements: hydrodynamic diameters (D_h) of DOX loaded micelles of **P1** and **P2**.

Fig. 6.
TEM images of DOX loaded micelles of **P1** and **P2**. Scale bars are set at 100 nm.

Fig. 7. Release profiles of DOX from DOX-loaded micelles of P1 incubated at 37°C under different pH conditions.

Fig. 8. Cell viability in HeLa cells after 60h total incubation time (24h exposure to polymer, 36h continued growth after washing). Data is normalized to PBS treated control cells and plotted as the mean $\pm$ standard deviation, N = 4 or 5.

Fig. 9.

Uptake of DOX-loaded micelles in HeLa cells. **P1** and **P2** micelles were incubated at a concentration of 250 ng/ μ L for 4 h. Free DOX was incubated for 2 hours at 12.5 ng/ μ L which would correspond to 5% DOX loading.

Fig. 10.

The effect of micelle concentration on cell viability. Panel A shows the data plotted as a function of polymer mass (μg). Panel B shows the data plotted as a function of DOX concentration (mol/L). Data is normalized to PBS treated control cells and plotted as the mean $\pm$ standard deviation, N = 4 or 5.

Fig. 11.

Time course study of DOX-loaded micelles. Micelle concentration was 111 ng/ μ L, while free drugs were dosed assuming 5% DOX loading and using the calculated HDACi monomer incorporation. Data is normalized to PBS treated control cells and plotted as the mean $\pm$ standard deviation, N =4.

Scheme 1. Synthesis of γ -4-phenylbuterate- ϵ -caprolactone (**M1**)

Scheme 2.

Synthesis of poly(ethylene glycol)-*b*-poly(γ -2-propylpentanoate- ϵ -caprolactone-*ran*- ϵ -caprolactone) and poly(ethylene glycol)-*b*-poly(γ -4-phenylbutyrate- ϵ -caprolactone-*ran*- ϵ -caprolactone) diblock copolymers (**P1** and **P2**)

Table 1Summary of amphiphilic diblock terpolymers **P1** and **P2**

Polymer	M ^{SEC} _n (g mol ⁻¹)	M ^{NMR} _n (g mol ⁻¹)	PDI ^a	PEG ^b (mol %)	HDACI ^b (mol %)	Cl ^b (mol %)	CMC ^c (g L ⁻¹)	D _n ^d (nm)
P1	8900	27000	1.73	25	30	45	3.16×10 ⁻⁴	91.2 ± 4.5
P2	21000	44800	1.76	13	25	62	1.00×10 ⁻⁴	95.5 ± 2.0

^aEstimated by size exclusion chromatography.^bDetermined by ¹H NMR.^cDetermined by fluorescence spectroscopy using pyrene as the fluorescence probe.^dHydrodynamic diameter was measured by DLS.

Table 2

DOX-loaded terpolymer micelles

Polymer	D _h (nm)	DOX ^a	Micelle Dispersity	EE % (wt %)	LC (wt %)
P1 ^{DOX}	150.4 ± 0.2		0.208	52.41	5.10
P2 ^{DOX}	134.4 ± 3.0		0.139	17.35	1.62

^aHydrodynamic diameter and dispersity were measured by DLS, N=3