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Biodegradable zwitterionic sulfobetaine polymer and its conjugate with paclitaxel for sustained drug delivery

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Abstract

A fully biodegradable zwitterionic polymer and the corresponding conjugate with paclitaxel (PTX) were synthesized as promising biomaterials. Allyl-functionalized polylactide (PLA) was employed as the precursor of polymer backbones. UV-induced thiol-ene reaction was conducted to conjugate thiol-functionalized sulfobetaine (SB) with the PLA-based backbone. The resulting zwitterionic polymer did not exhibit considerable cytotoxicity. A polymer-drug conjugate was also obtained by thiol-ene reaction of both thiol-functionalized SB and PTX with allyl-functionalized PLA. The conjugate could readily form narrowly-dispersed nanoparticles in aqueous solutions with a volume-average hydrodynamic diameter ($D_{h,v}$) of 19.3 ± 0.2 nm. Such a polymer-drug conjugate-based drug delivery system showed full degradability, well-suppressed non-specific interaction with biomolecules, and sustained drug release. *In vitro* assessments also confirmed the significant anti-cancer efficacy of the conjugate. After 72 h incubation with PLA-SB/PTX containing 10 $\mu\text{g/mL}$ of PTX, the cell viabilities of A549, MCF7, and PaCa-2 cells were as low as 20.0 ± 2.5 %, 1.7 ± 1.7 %, and 14.8 ± 0.9 %, respectively. Both flow cytometry and confocal microscopy suggested that the conjugates could be easily uptaken by A549 cells before the major release of PTX moieties. Overall, this work elucidates promising potentials of biodegradable zwitterionic polymer-based materials in biomedical applications.

Graphical abstract

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Keywords

zwitterionic polymer; biodegradable polymer; drug delivery; paclitaxel; polymer-drug conjugate

1. Introduction

The development of novel and transformative materials for biomedical applications is a central aspect of biomaterial research. With minimal long-term side effects and systemic toxicity, biodegradable polymers have been broadly used in biomedical fields [1–7]. For instance, aliphatic polyesters, such as poly(lactic acid) (PLA) and polycaprolactone (PCL), are important biodegradable polymers that have been approved by FDA for specific *in vivo* clinical applications [8–10]. However, typical biodegradable polymers are hydrophobic and lack functionalities, and there is a significant need to develop well-defined hydrophilic biodegradable polymers carrying special functionalities to enhance their biomedical applicability [11–20].

Poly(ethylene glycol) (PEG) is an important type of polymer with significant biomedical applications [21]. There is a broad variety of PEGylated therapeutics currently on the market for the treatment of numerous kinds of diseases [22–24]. PEGylation not only confers the attached therapeutics a protective hydration layer to increase their circulation time and bioavailability by mitigating clearance mechanisms, but also can improve the stability and enhance aqueous solubility of therapeutics [21]. However, reduced bioactivity and immunogenicity of PEGylated therapeutics severely restrict their biomedical efficacy [25]. It is urgently required to develop new polymers as improved alternatives of PEG.

Recently zwitterionic polymers have attracted significant interests due to their special properties and potential biomedical applications [26]. Zwitterionic polymers generally are more hydrophilic than PEG and may exert improved protection and solubility enhancement to therapeutics. Zwitterionic polymer-modified nanoparticles exhibited excellent long-circulation abilities [27–29]. Zwitterionic polymer-specific antibodies are also absent [30]. A variety of conjugates of zwitterionic polymers with therapeutic agents (i.e. drugs and proteins) have been reported [31–34], and it has been illustrated that conjugation with zwitterionic polymers would not compromise bioactivity or binding affinity of proteins. However, the zwitterionic polymers with polymethacrylate or polyacrylamide backbones that have been studied intensively are non-biodegradable, and side effects and systemic toxicity resulted from polymer accumulation may present remarkable health risks for clinical applications of such zwitterionic polymer-modified therapeutics. Such concerns may be somewhat reduced but would not be fully dissipated by preparing block copolymers,

composite materials, or surface-modified polymer nanoparticles to integrate non-degradable zwitterionic polymers with degradable polymeric structures [28, 35–37]. Therefore, synthesis and studies of biodegradable zwitterionic polymers have received considerable attention in the past several years [38]. Biodegradable zwitterionic polymers based on poly(amino acid)s, polysaccharides (such as chitosan and starch), and polycarbonates have been prepared [39–42]. These biodegradable zwitterionic polymers may possess either mixed oppositely charged moieties [39, 40], or integrated zwitterions [42, 43]. Noticeable efforts in incorporating zwitterions with aliphatic polyesters have also been made [44–47], but zwitterionic polymers with well-defined aliphatic polymer-based backbones carrying pendant zwitterions have not been obtained. Moreover, to the best of our knowledge, the conjugates of biodegradable zwitterionic polymers with therapeutic agents have not been reported.

Herein we report a fully biodegradable zwitterionic polymer derived from well-defined aliphatic polyester, as well as the corresponding polymer-drug conjugate. PLA-based main-chain was designed for the zwitterionic polymer and the conjugate because of its biodegradability. Integrated zwitterion moieties, instead of mixed oppositely charged moieties, were incorporated with the biodegradable main-chains to guarantee electrically-neutral zwitterionic properties of the resulting materials without requiring additional synthetic control. With an anionic sulfonate group and a cationic trimethyl ammonium group, sulfobetaine (SB) was further selected as the zwitterion because of its superhydrophilicity [48–50]. Paclitaxel (PTX), a potent clinic anticancer drug, was chosen as a representative drug in our design of the polymer-drug conjugate [51]. Relative to drug encapsulation systems, drug delivery systems based on polymer-drug conjugates can eliminate unfavorable burst release that reduces drug bioavailability and induces severe systemic toxicity [52, 53]. The objective of this study is to establish the synthetic approaches for aliphatic polyester-based zwitterionic sulfobetaine polymers and the corresponding PTX-containing polymer-drug conjugates, and demonstrate their merits as novel biomaterials through comprehensive characterization and in vitro studies. This work serves as a valid basis for future investigations on broad varieties of biodegradable zwitterionic polymers and polymer-drug conjugates.

2. Materials and methods

2.1. Materials

(3*S*)-*cis*-3,6-Dimethyl-1,4-dioxane-2,5-dione (L-LA, 98%), 4-(dimethylamino)pyridine (DMAP, 99%, prilled), fibrinogen from human plasma, and poly(D,L-lactide) were purchased from Sigma-Aldrich. 3-Dimethylamino-1-propyl chloride hydrochloride (98%) and 1,3-propanesultone (99%) were purchased from Alfa Aesar. Thioacetic acid (>95.0%, GC) were purchased from TCI America. Triethylamine (NEt₃, 99%) were purchased from Fisher Scientific. 2,2-Dimethoxy-2-phenylacetophenone (DMPA, 99%), Nile red (99%), and sodium periodate (99%, for analysis) were purchased from Acros Organics. Benzyl alcohol (BnOH) was purchased from J. T. Baker. Paclitaxel (PTX, 99%) was purchased from AvaChem Scientific. Proteinase K (*Tritirachium album*/Molecular Biology) was purchased from Fisher BioReagents. Ruthenium dioxide (99.9%) was purchased from Pfaltz & Bauer.

Dichloromethane (DCM, HPLC), chloroform (CHCl₃, HPLC), hexanes (HPLC), ethyl acetate (HPLC), diethyl ether (HPLC), acetone (Certified ACS), methanol (MeOH, HPLC), acetonitrile (HPLC), *N,N*-dimethylformamide (DMF; HPLC) were purchased from Fisher Scientific. DCM and DMF were dried by distillation over CaH₂. Compounds **1–5**, 3-(dimethylamino)propyl thioacetate and SB thioacetate were synthesized (see Supplementary Data for details).

2.2. Measurements

¹H NMR spectra were recorded at 500 MHz on a Varian INOVA-500 Spectrometer at 25 °C. CDCl₃ (with tetramethylsilane as an internal standard), D₂O, CD₃OD, methyl sulfoxide-*d*₆ (DMSO-*d*₆) were used as solvents for NMR measurements. Number-average molecular weight (*M*_n) and molecular weight dispersity () of allyl-functionalized polylactide **1** were determined by gel permeation chromatography (GPC). A Viscotek GPC system was used with a VE-1122 pump, two mixed-bed organic columns (PAS-103M-UL and PAS-105M-M), and a VE-3580 refractive index (RI) detector. DMF with 0.01 M LiBr was used as eluent for GPC measurement (flow rate: 0.5 mL/min, 55 °C). The polymer was dissolved in DMF at a concentration of 3 mg/mL, and the injection volume was 0.1 mL. The system was calibrated with polystyrene standards (< 1.1) obtained from Varian Inc.

Transmission electron microscopy (TEM) images were obtained with a JEOL 2010 microscope. TEM samples were prepared using 400 mesh carbon-coated copper grids. Dilute solutions of PLA-SB **4** and PLA-SB/PTX **5** in water were dip coated onto the TEM grids. When the water was completely dried under vacuum, the samples were stained by freshly prepared 0.5 % solution of ruthenium tetroxide. The staining agent was prepared by reaction between sodium periodate and ruthenium dioxide in water.

Dynamic light scattering (DLS) was used to determine hydrodynamic diameters (*D*_h) and size distributions of PLA-SB **4** and PLA-SB/PTX **5**. The measurements were performed on Zetasizer Nano ZS90 (Malvern Instruments Ltd.) with a 4 mM 633 nm HeNe laser as the light source. The temperature was maintained at 25°C, and the measuring angle was 90°C. Both PLA-SB **4** and PLA-SB/PTX **5** were simply dispersed in water (1 mg/mL).

Nonspecific interactions between PLA-SB/PTX **5** and fibrinogen was monitored by DLS [54]. PLA-SB/PTX **5** and fibrinogen were dispersed in water at the concentration of 1 mg/mL, respectively. Then the two solutions, as well as the mixed solution, were measured by DLS.

The enzymatic degradation of PLA-SB/PTX **5** was also monitored by DLS [55]. PLA-SB/PTX **5** was dispersed in water and proteinase K solution (0.2 mg/mL 0.1 M Tris-HCl buffer, pH = 8.5), respectively. The concentration of **5** was both 1 mg/mL in each case. The solutions were incubated in a shaking bed at 37 °C. At different time intervals, 1 mL of both solutions were withdrawn for DLS measurements.

The drug release profiles of PLA-SB/PTX **5** were studied using high-performance liquid chromatography (HPLC) with a diode array detector (DAD) (model: Agilent 1260 Infinity). The sample was dissolved in 1X PBS (pH = 7.4) and 1X PBS (pH = 5.5) respectively, with

the concentration of 0.15 mg/mL. The solutions were incubated in a shaking bed at 37 °C. At different time intervals, 3 mL of both solutions were withdrawn and extracted by 3 mL DCM for three times. The DCM phases were concentrated and redissolved in acetonitrile for HPLC measurements. A reversed phase C18-column (ZORBAX Elipse XDB-C18, 4.6 × 150 mm, 5 μ) was used for chromatographic separations. The mobile phase consisted of Milli-Q water and HPLC grade acetonitrile, with linear gradients of water/acetonitrile (9:1~6:4 v/v 0~3 min, 6:4~3:7 v/v 3~10 min, 3:7~6:4 v/v 10~18 min, 6:4~9:1 18~20 min). The eluent flow rate was 1.0 mL/min and the column compartment temperature was set at 30 °C. For each sample, the injection volume was 20 μ L. PTX moieties were detected by ultraviolet (UV) absorbance at 227 nm. A calibration curve was obtained in advance, which was used to calculate the PTX concentration according to UV absorbance.

2.3. Cell culture

A549, MCF7, and PaCa-2 cells were obtained from the American Type Culture Collection (Manassas, VA). The A549 and MCF7 cells were cultured in RPMI 1640 medium (Life Technologies; Grand Island, NY; 11875-093) supplemented with 10% fetal bovine serum (FBS; Life Technologies; 26140-079) and 1% penicillin streptomycin (PS; Life Technologies; 15140-122). PaCa-2 cells were cultured in DMEM medium (Life Technologies; Grand Island, NY; 11965-092) supplemented with 10% FBS and 1% penicillin streptomycin. The cells were seeded in the P100 petri dish (Greiner Bio-one Monroe NC) at 2×10^5 cells/mL and incubated in the CO₂ incubator at 37 °C. The cells were subcultured every 2 days.

2.4. Cell viability

A549, MCF7, PaCa-2 cells were seeded in 96 wells plate (Greiner Bio-one; 655180) at the density of 1×10^4 per well. The cells were then incubated in the CO₂ incubator at 37 °C overnight. PTX, PLA-SB **4**, PLA-SB/PTX **5**, and four unconjugated control groups (SB, PTX+SB, PLA+PTX, PLA+PTX+SB) were dissolved or dispersed in PBS buffer; then PTX, **5**, PTX+SB, PLA+PTX, PLA+PTX+SB were added to the cells at the PTX concentrations of 0 (PBS control), 0.001, 0.01, 0.1, 1 and 10 μ g/mL, and **4** was added to the cells in concentrations equal to the polymer concentrations of **5**. At 24 and 72 hours post treatment, the cell viability was measured using the alamarBlue assay (Invitrogen; DAL1025) following the manufacturer's protocol. Briefly, the medium was removed; then 110 μ L phenol red free RPMI (Invitrogen; 11835-030) containing 10% alamarBlue reagent (Invitrogen; DAL1025) and 10% FBS was added to each well. The cells were incubated in the CO₂ incubator at 37 °C for 3 hours, protected from light. Then, 95 μ L medium was transferred to another 96 wells plate. The fluorescence intensity was measured by TECAN microplate reader (San Jose, CA) with the excitation and emission wavelengths at 560 nm and 590 nm, respectively. The cell viability was calculated and normalized to PBS controls.

2.5. Cellular uptake analysis

The hydrophobic Nile red was used as the dye to study the cellular uptake of PLA-SB/PTX **5**. The Nile red was encapsulated in PLA-SB/PTX **5** nanoparticles by a reported method [56–58]. In a 2 mL vial, PLA-SB/PTX **5** and Nile red were completely dissolved in small amount

of mixed solvent (CHCl_3 and MeOH). Then a large amount of deionized water was added. The mixture was stirred overnight under flowing nitrogen. Then the mixture was dried under vacuum to remove all the organic solvent, followed by filtration to remove large particles. Extraction was further conducted for three times with DCM to remove trace amount of free Nile red. No red color could be observed in DCM phase during the second and third times of extraction, while the water phase was always red, suggesting that all Nile red in the water phase was encapsulated within PLA-SB/PTX **5**. A549 cells were seeded in the 6 well plates (Greiner Bio-one; 657160) at 2×10^5 cells/well. The cells were then incubated in the CO_2 incubator at 37°C overnight. Free Nile red and Nile red-loaded PLA-SB/PTX **5** were added into cells with the same Nile red concentration. After 4 hours of incubation at 37°C , the cells were harvested by trypsin treatment and fixed in 4% paraformaldehyde (Acros, 41678-5000) for cellular uptake analysis by flow cytometry and confocal microscopy. For flow cytometry analysis, the fluorescence intensity of Nile red was measured by BD Fortessa flow cytometer (BD bioscience, San Jose, CA) in the PE-Texas Red channel. Total 10,000 events were collected for each sample, and the mean fluorescence intensity $\pm$ standard deviation of Nile red was reported. For the confocal microscopy imaging, the cell nuclei were stained with $1\text{ }\mu\text{g/mL}$ DAPI (Sigma Aldrich; D8417) at room temperature for 10 min. The cells were then mounted on glass slides. The fluorescence of DAPI and Nile red was observed using LSM 710 confocal microscope (ZEISS, Dublin, CA) through the DAPI filter (440 nm) and Nile red filter (560 nm) respectively.

2.6. Statistical analysis

All the results were reported as mean $\pm$ standard deviation (SD) of three independent experiments. One-way ANOVA was employed to assess the statistical significance of the experimental data. $*p < 0.05$ was considered as statistical significant.

3. Results and discussion

3.1. Synthesis

The synthesis of the biodegradable zwitterionic polymer and polymer-drug conjugate is illustrated in Scheme 1. UV-induced thiol-ene functionalization strategy was employed because of the high reaction efficiency and functional group tolerance of thiol-ene reactions under mild conditions [59, 60]. Following the method we reported previously [55, 61], allyl-functionalized PLA (**1**) was synthesized with 75% yield *via* ring-opening polymerization (ROP) of allyl-functionalized lactide (ALA) with lactide (LA) using benzyl alcohol (BnOH) as the initiator and 4-dimethylaminopyridine (DMAP) as the organocatalyst. Its narrow molecular weight dispersity ($M_w/M_n = 1.16$) was revealed by characterization using gel permeation chromatography (GPC; Fig. S1). Its composition (53 mol% of LA; 47 mol% of ALA) and degree of polymerization ($\text{DP} = 145$) were determined by ^1H NMR analysis (Fig. S2). Thiol-functionalized SB (SB-SH, **2**) was prepared with an overall yield of 55% through the nucleophilic substitution reaction of 3-(dimethylamino)propyl chloride hydrochloride with thioacetic acid [62], followed by the reaction of the resulting 3-(dimethylamino)propyl thioacetate with 1,3-propanesultone [63], and the NaOH-catalyzed hydrolysis of the thioacetate groups of the intermediate product (Fig. S3) [64]. Thiol-functionalized PTX (PTX-SH, **3**) was obtained following a literature method (Fig. S4) [65]. Biodegradable

zwitterionic polymer as SB-functionalized PLA (PLA-SB, **4**) and the polymer-drug conjugate (PLA-SB/PTX, **5**) were obtained by thiol-ene reactions of the thiol functionalization agent(s) with the allyl-containing backbone precursor **1** using 2,2-dimethoxy-2-phenylacetophenone (DMPA) as the photoinitiator under UV irradiation ($\lambda_{\max}$ = 365 nm) for 1 h ([allyl]₀: [SB-SH]₀: [DMPA]₀ = 1.0:0.9:0.4 for **4**; [allyl]₀: [SB-SH]₀: [PTX-SH]₀: [DMPA]₀ = 1.0:0.9:0.2:0.5 for **5**), in a mixed solvent of CHCl₃ and methanol (v/v = 5:1). The yields of PLA-SB **4** and PLA-SB/PTX **5** were 83% and 82%, respectively. Noteworthy, thiol-ene reaction of **1** with PTX-SH can also readily yield the corresponding PLA-PTX conjugate; however, it is not dispersible in water, and therefore, not further studied in this work.

3.2. Characterization

PLA-SB **4** and PLA-SB/PTX **5** were characterized systematically to reveal their structural features. According to ¹H NMR analysis (Fig. 1), both of them had 41 mol% of SB-functionalized backbone repeat units, and PLA-SB/PTX **5** also possessed 6 mol% of PTX-functionalized backbone repeat units, corresponding to 17 wt% of PTX. The quantification was made by comparing intensities of characteristic resonances of -CH₂SO₃ protons from SB moieties at 2.87 ppm (peak *o*) and -OCHCHNH- protons from PTX moieties at 5.49 and 5.88 ppm (peaks *c2'* and *c3'*) with these of -CHOCO- protons from PLA-based backbone at 5.30 ppm (peak *b*). Because =CH₂ protons from pendent allyl groups of the backbone polymer also showed resonances at ~5.30 ppm, the net resonance intensities of backbone -CHOCO-protons were obtained by deducting the resonance intensities of =CH₂ protons (equal to two times of the resonance intensities of -CH= protons, peak *d* at 5.50 ppm) from the overall resonance intensities at ~5.30 ppm. No occurrence of side reactions is illustrated by the ¹H NMR spectra of **4** and **5**, because the decreased intensity of -CH= protons at 5.50 ppm quantitatively agreed with the increased intensities of characteristic protons from the grafted zwitterion/PTX moieties. According to the composition of **4** and **5**, it can be deduced that ~97% conversion of SB-SH and ~64% conversion of PTX-SH in the corresponding thiol-ene reactions. The nearly quantitative conversion of SB-SH indicates that mol% of SB moieties in the zwitterionic materials can be readily controlled. Reactivity of PTX-SH is somewhat less than that of SB-SH, because the PTX moiety in PTX-SH is much bulkier and presents more steric hindrance to the thiol reactive site than the SB moiety in SB-SH. Although GPC analysis of **4** and **5** could not be done because each of the common GPC solvents tested did not dissolve them unimolecularly [66, 67], our previous studies on thiol-ene functionalization systems indicated that the PLA-based main-chain was essentially unaffected under the reaction conditions [61].

With both superhydrophilic SB zwitterions and hydrophobic components, **4** and **5** assembled in aqueous solutions, and the corresponding nanostructures were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS analysis showed that PLA-SB **4** had a volume-average hydrodynamic diameter ($D_{h,v}$) of 14.4 ± 0.1 nm with a polydispersity of 0.123, while PLA-SB/PTX **5** had a $D_{h,v}$ of 19.3 ± 0.2 nm with a polydispersity of 0.236 (Fig. 2a and 2b). TEM imaging exhibited that both **4** and **5** had spherical morphologies on TEM grids, with number-average diameter (D_{av}) of 10.1 ± 1.0 nm and 14.1 ± 1.2 nm, respectively (Fig. 2c and 2d; Fig. S5). For each of **4** and **5**, the D_{av}

value was considerably smaller than $D_{h,v}$ value, because D_{av} corresponds to dry state while $D_{h,v}$ corresponds to solvated state in solution of the substance.

3.3. Biodegradability study

Aliphatic polyester-based zwitterionic materials are expected to possess biodegradability resulted from their ester-containing polymer backbones. Using PLA-SB/PTX **5** as the representative sample, biodegradability study was performed. Biodegradability of the PLA-based backbone was verified through the enzymatic degradation experiment monitored by light scattering, using the ratio of remaining intensity of scattered light of the incubated buffer solutions of **5** to the original intensity (I/I_0) as the assessment parameter [55]. As shown in Fig. 3, I/I_0 values of **5** decreased quickly to only ~0.02 within 24 hours in the presence of proteinase K enzyme. On the other hand, the I/I_0 values essentially remained unchanged within 120 hours without the presence of the enzyme, suggesting no remarkable degradation. Such an enzyme-trigger degradation behavior may minimize unfavorable polymer accumulation when the biodegradable zwitterionic polymers are used as scaffolds in biological environment.

3.4. Assessment of non-specific interactions with biomolecule

These biodegradable zwitterionic materials are also expected to have low non-specific interactions with biomolecules owing to the hydration effects of their zwitterionic moieties. Using PLA-SB/PTX **5** as the representative sample, their non-specific interactions with biomolecule were probed. Non-specific interaction of PLA-SB/PTX **5** with biomolecules was studied by choosing fibrinogen (from human plasma), a glycoprotein essential for the formation of blood clots, as the model biomolecule [54]. The mixing process of an aqueous solution of **5** with that of fibrinogen was monitored by DLS (Fig. 4). Bimodal DLS intensity profile of the resulting mixed solution with two peaks corresponding to **5** and fibrinogen respectively was observed. The result indicates a well-suppressed non-specific interaction between **5** and biomolecules, which may help to stabilize **5** in plasma and promote its circulation time.

3.5. Drug release study

Because drug release behavior is critically important for drug delivery systems, the PTX release profiles of **5** was probed by analyzing the PBS buffer solutions of **5** incubated at 37 °C using high-performance liquid chromatography (HPLC). As shown in Fig. 5, sustained release of PTX from **5** was observed at both pH of 5.5 and physiological pH of 7.4. PTX release at pH of 5.5 was faster than that at pH of 7.4, due to the acid-labile linkage between PTX moieties and PLA-based main-chain (Fig. S6). The acid-sensitive drug release behavior was quite significant in the early stage (in the first 4 hours, 11.4 ± 0.5 % of PTX release at pH = 5.5 and only 1.6 ± 0.5 % of PTX release at pH = 7.4), but became less noticeably at a larger time scale (after 120 hours, 83.5 ± 4.9 % of PTX release at pH = 5.5 and 72.7 ± 4.1 % of PTX release at pH = 7.4). With the sustained drug release behavior of **5**, burst release of PTX that can reduce drug bioavailability and induce harmful systemic side effects was not observed at both pH conditions. The acid-sensitive drug release is also

preferred for anticancer drug delivery because tumor tissue is more acidic than healthy tissue [68].

3.6. In vitro cytotoxicity study

In vitro cytotoxicity assessment was performed to reveal biocompatibility of PLA-SB **4** and the therapeutic effectiveness of PLA-SB/PTX **5** towards cancer cells. Using free PTX as a control, the cytotoxicity of PLA-SB **4** and PLA-SB/PTX **5** was evaluated by the alamarBlue assay at 24 and 72 h against A549 lung cancer cells (Fig. 6a and 6b), MCF7 breast cancer cells (Fig. 6c and 6d), and PaCa-2 pancreatic cancer cells (Fig. 6e and 6f), respectively [69]. PLA-SB **4** did not exhibit considerable cytotoxicity to any cell lines under the experimental conditions. Even with the concentration up to 1,000 $\mu\text{g/mL}$ (data not shown), each cell line exhibited over 90% viability, suggesting that the biodegradable zwitterionic polymer is a promising biomaterial with high biocompatibility. Although the release of PTX would not complete at 24 or 72 h, PLA-SB/PTX **5** showed significant anti-cancer efficacy similar to that of free PTX. It was remarkable that when the PTX concentrations were higher than 1 $\mu\text{g/mL}$, PLA-SB/PTX had higher efficacy in killing A549 cells and MCF7 cells as compared to free PTX. After 72 h incubation with PLA-SB/PTX containing 10 $\mu\text{g/mL}$ of PTX, the cell viabilities of A549, MCF7, and PaCa-2 cells were as low as $20.0 \pm 2.5\%$, $1.7 \pm 1.7\%$, and $14.8 \pm 0.9\%$, respectively. To strengthen the cell viability data, four more unconjugated control groups (SB, PTX+SB, PLA+PTX, PLA+PTX+SB) have also been studied. Because the synthesized allyl-functionalized PLA **1** was obtained as white solid and cannot be dispersed in PBS even after sonification, a poly(D,L-lactide) from Sigma-Aldrich, which can be dispersed in PBS with the assistance of sonification and is similar to **1** in molecular weight range (Fig. S7), was used as a substitute to **1** in the preparation of PLA+PTX and PLA+PTX+SB. Because of the high biocompatibility of SB and PLA, the cell viabilities treated with SB were similar to the ones treated with PLA-SB. The other three control groups with PTX showed similar anticancer efficacy to free PTX. Table S1 listed the IC_{50} values of different PTX formulations in A549, MCF7, PaCa-2 cells incubated for 72 h. For all the three cell lines, PLA-SB/PTX showed the lowest IC_{50} values, indicating a higher anticancer efficacy. The comparable or even more significant cytotoxicity of PLA-SB/PTX relative to free PTX suggested that PLA-SB/PTX might be able to enter cells more efficiently than free PTX, and this needs to be verified through cellular uptake study.

3.7. In vitro cellular uptake study

Cellular uptake of PLA-SB/PTX **5** was studied using both flow cytometry and confocal microscopy. A549 cells were chosen as the model cancer cells. Nile red, a fluorescent hydrophobic (lipophilic) probe, was employed to visualize cellular uptake, and it was encapsulated by **5** using a reported method (Fig. S8) [56]. A typical time period of 4 h was used as the incubation time. As shown in Fig. 7a, the fluorescence signal of cells treated with nile red-loaded **5** was much stronger than that of cells treated with free nile red. The mean fluorescence intensity of cells treated by nile red-loaded **5** was ~ 27 folds higher than that of free nile red treated cells, and ~ 373 folds higher than that of untreated cells (Fig. 7b). Confocal microscopy images also confirmed that nile red-loaded **5** can be easily uptaken by A549 cells before the major release of PTX moieties (Fig. 7c). Meanwhile, for the cells

treated with free Nile red, no red fluorescence could be observed in the confocal microscopy images, showing much lower cell uptake efficiency of the free Nile red. The above results suggest that cellular uptake of the cargo-loaded delivery scaffolds by endocytosis is more favorable than cellular uptake of hydrophobic cargo via diffusion-based transport process [70, 71], and the presence of zwitterionic moieties in the delivery scaffolds would not invalidate the uptake pathway. Such facilitated cellular uptake of zwitterionic scaffolds relative to hydrophobic molecules is highly preferred for delivery systems of hydrophobic drugs, and may lead to remarkable intracellular drug concentration and result in low cell viability which was observed in the cytotoxicity study. The similar or even higher cytotoxicity of PLA-SB/PTX **5** relative to free PTX can be attributed to the facilitated cellular uptake of **5**, which sufficiently compensates the incomplete availability of PTX moieties of **5** for therapeutic function due to its sustained PTX release process. In addition, the fact that PLA-SB/PTX **5** can effectively encapsulate a hydrophobic substance for cellular uptake also suggests that it may potentially be used for the delivery of multiple drugs based on both conjugation and encapsulation.

4. Conclusions

In summary, novel examples of aliphatic polyester-based zwitterionic polymer and polymer-drug conjugate were reported. They were synthesized by thiol-ene functionalization approaches, and their chemical structures were verified by characterization. Using the PLA-SB/PTX as a representative zwitterionic polymer-drug conjugate, enzymatic degradability, well-suppressed nonspecific interaction with biomolecules, and acid-sensitive sustained drug release were demonstrated. Cytotoxicity and cellular uptake studies further illustrated the biocompatibility of the zwitterionic polymer, the anticancer effectiveness of the polymer-drug conjugate, as well as its ready cellular internalization. Overall, this work indicates the promising application potentials of biodegradable zwitterionic polymer-based biomaterials, and our data encourage broader biomedical studies of the corresponding polymer-drug conjugate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in its online version.

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Statement of Significance

The applicability of FDA-approved biodegradable aliphatic polyesters has been significantly restricted because they are hydrophobic and lack functionalities. Recently zwitterionic polymers have emerged as promising hydrophilic biomaterials, but most of the reported zwitterionic polymers are non-biodegradable. This study reports a novel aliphatic polyester-based zwitterionic polymer and the corresponding polymer-drug conjugate. Their aliphatic polyester and zwitterionic components provide them with high enzymatic degradability and low nonspecific interactions with biomolecules, respectively. While the zwitterionic polymer did not show noticeable cytotoxicity, the corresponding polymer-anticancer drug conjugate exhibited acid-sensitive sustained drug release, remarkable effectiveness in killing cancer cells, as well as the ready cellular internalization. This work lays a foundation for the further development of synthetic biodegradable zwitterionic polymer-based materials which potentially may have broad and significant biomedical applications.

Fig. 1.
 ^1H NMR spectra of a) PLA-SB **4**, and b) PTX-SB/PTX **5** in $\text{CDCl}_3/\text{CD}_3\text{OD}$ (v/v, 1:1).

Fig. 2.

a) and b): Histograms of $D_{h,V}$ of PLA-SB **4** and PLA-SB/PTX **5** in aqueous solutions measured by DLS. For each data point, error bar represents standard deviation resulting from three independent experiments; c) and d): TEM images of **4** and **5** from aqueous solutions (0.1 mg/mL). TEM samples were stained by RuO_4 .

Fig. 3. Enzymatic degradation profiles of PLA-SB/PTX **5** in water (pH = 7.4) and 0.2 mg/mL of proteinase K in 0.1 M Tris-HCl buffer (pH = 8.5) at 37 °C.

Fig. 4. Histograms of intensity-based hydrodynamic diameter of a) of PLA-SB/PTX **5**, b) fibrinogen, and c) the mixture of PLA-SB/PTX **5** and fibrinogen, as obtained by DLS analysis of the corresponding aqueous solutions. For each data point, error bar represents standard deviation resulting from three independent experiments.

Fig. 5.

The hydrolytic PTX release profiles of PLA-SB/PTX 5 in PBS buffer solutions at pH of 7.4 and 5.5 at 37 °C. For each data point, error bar represents standard deviation resulting from three independent experiments. * $p < 0.05$ when the cumulative release was compared at 4 h and 120 h.

Fig. 6.

Cytotoxicity of PTX, PLA-SB, PLA-SB/PTX, SB, PTX+SB, PLA+PTX, and PLA+PTX+SB: a) A549 cells after 24 h treatment; b) A549 cells after 72 h treatment; c) MCF7 cells after 24 h treatment; d) MCF7 cells after 72 h treatment; e) PaCa-2 cells after 24 h treatment; f) PaCa-2 cells after 72 h treatment (PLA-SB had the same polymer concentrations as PLA-SB/PTX, each component in the unconjugated mixtures had the same weight concentration as the same component in PLA-SB/PTX). For each data point, error bar represents standard deviation resulting from three independent experiments. * $p < 0.05$ when the cell viability of A549 and MCF7 was compared after 72 h treatment with 10

µg/mL PTX and PLA-SB/PTX. In PLA+PTX and PLA+PTX+SB, PLA referred to poly(D,L-lactide).

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Fig. 7.

a) Typical flow cytometry data set showing cellular uptake of free Nile red and Nile red-loaded PLA-SB/PTX **5** after 4 h incubation; b) mean fluorescence intensity of Nile red obtained from flow cytometry. For each data point, error bar represents standard deviation resulting from three independent experiments. $*p < 0.05$ when the mean fluorescence of free Nile red treated A549 cells and Nile red-loaded PLA-SB/PTX treated A549 cells was compared; c) confocal microscopy images of A549 cells after 4 h incubation with Nile red and Nile red-loaded PLA-SB/PTX **5**. Cell nuclei were counterstained with DAPI.

Scheme 1.

Synthesis of the biodegradable zwitterionic sulfobetaine polymer and polymer-drug conjugate.