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Degradable Brush Polymer-Drug Conjugate for pH-Responsive Release of Doxorubicin

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To achieve high precision and efficacy in disease treatment, biodegradability and environmental responsivity are highly desired for drug delivery systems. Having a polylactide (PLA)-based biodegradable scaffold conjugated with doxorubicin (DOX) moieties via pH-responsive linkages, a brush polymer-drug conjugate (BPDC) was synthesized and studied. The biodegradable scaffold, PLA-*graft*-aldehyde/polyethylene glycol (PLA-*g*-ALD/PEG), was prepared via copper-catalyzed alkyne-azide click reaction. Subsequently, the BPDC was obtained by conjugating doxorubicin with the scaffold through an acid-sensitive Schiff base linkage. Well-controlled structures of the resulting BPDC and its precursors were verified by proton nuclear magnetic resonance and gel permeation chromatography characterizations. As revealed by dynamic light scattering and transmission electron microscopy, the BPDC had well-defined nanostructure with the size of 10-30 nm. Drug release study of the BPDC demonstrated much faster release of DOX at the pH of 5.5 than at the pH of 7.4. Both cell internalization and cytotoxicity studies of the BPDC in MCF-7 breast cancer cells indicated its significant potential for application as a novel anticancer nanomedicine.

Introduction

As cancer is becoming one of the major causes of mortality in the world, the development of anticancer therapy has gained unprecedented attention over the recent decades.[1-5] DOX, also known as hydroxydaunorubicin, is an antracycline antibiotic that has been commonly used in the chemotherapy of a variety of cancers, such as acute leukaemia, Hodgkin's disease, non-Hodgkin's lymphomas, bone and soft tissue sarcoma, and cancers of the bladder, breast, stomach, lung, ovaries and thyroid.[6] It destroys tumor cells by intercalating into DNA and inhibiting the progression of the DNA polymerase and topoisomerase II. Despite its anticancer efficacy, the dosage of DOX is limited by its severe side effects such as cardiotoxicity which can cause life-threatening heart damage.[7] Furthermore, similar to that of many other small molecule drugs, intravenous administration of DOX (as the hydrochloride salt to promote the water solubility) lacks tumor selectivity and results in fast blood and renal clearance. Therefore, DOX prodrugs have been extensively developed to address these problems.[8]

Early development of DOX prodrugs lays in the conjugation of small moieties through the reactive aliphatic carbonyl group or primary amine of DOX.[8] With the discovery of enhanced permeability and retention (EPR) effect,[9] macromolecules have been extensively used to construct macromolecular prodrugs which are so-called polymer-drug conjugates (PDCs).[1] Because a tumor tissue is more acidic than a normal tissue, pH-responsive drug release is highly preferred in targeted drug release for solid tumor. Therefore, a variety of acid-sensitive cleavable linkages have been developed for polymer-DOX conjugates.[10-11] Hydrazone linkage is acid labile and can hydrolyze under acidic aqueous environment to yield the corresponding carbonyl and hydrazine compounds within half-life of several hours.[12-14] Carbamate linkage was designed for acid labile hydrolysis, as well as selective hydrolysis by human carboxylesterases, to release active drugs.[15] Belonging to Schiff base linkages, benzyl imine formed by the reaction of benzyl aldehyde of scaffolds with primary amine of DOX can also be cleaved readily under acidic conditions.[16-17] Amide linkage is susceptible to lysosomal acid hydrolysis but has considerably longer half-life for DOX release than other linkages mentioned above.[18]

Besides the cleavage linkages, the selection of an appropriate polymeric scaffold is also critical for the design of PDCs.[19-21] Vinyl polymers with pendent functionalities, such as *N*-(2-hydroxypropyl) methacrylamide (HPMA)-based copolymers, have been extensively studied for developing PDCs since 1970s.[22-24] However, when these vinyl polymers have the molecular sizes suitable for inducing EPR effects, their non-degradability may also raise concern regarding long-term side effects. Being approved by the FDA for human applications, polyethylene glycol (PEG) has been regarded as an excellent polymer for drug delivery because of its non-toxicity, non-immunogenicity, biocompatibility and low non-specific binding.[11,25-27] In the early development of PEG-drug conjugates, PEG with molecular weight (MW) up to ~40 kDa was directly used to conjugate with drug.[28-32] Recently, PEG with MW of several kDa has been widely used as one of the components to

construct nanoscopic polymer scaffolds via degradable linkages, because PEG is non-degradable and requires relatively low MW to facilitate eventual renal clearance. Meanwhile, biodegradable polymers, especially aliphatic polyesters, become increasingly appealing for drug delivery.[33-35] Intrinsically, conventional aliphatic polyesters such as polylactide (PLA), poly(lactide-*co*-glycolide) (PLGA) and poly(ε-caprolactone) (PCL) are hydrophobic and only possess functionalities at chain ends. Therefore, typically polyester-based amphiphilic copolymers were utilized for the preparation of PDCs through terminal functionality of polymers.[36-39] Accordingly, only one or two chain end groups can be provided by each polyester chain for drug conjugation, thereby the drug loading capacity of these polyesters has been significantly restricted. Thus, with an increasing demand on polyesters with multiple reactive sites per chain, functional polyesters have been actively pursued.[40-49] A variety of functionalities, such as hydroxyl, amine, carboxyl acid, allyl and alkyne groups, have been successfully introduced to the repeat units of polyesters. The resulting functional polyesters have demonstrated great potential as polymer scaffolds for developing novel biomaterials for therapeutic delivery.[40,50-58] For instance, Emrick and co-workers prepared alkyne-functionalized polyesters and further used the "clickable" polyesters to prepare PDCs with high drug loadings.[50-51] Farokhzad, Langer and co-workers also prepared hydroxyl-functionalized PLA-based copolymers by a polycondensation approach, and conjugated a platinum (IV) prodrug with their hydroxyl groups for a combination drug therapy of prostate cancer.[52]

Although linear or lightly branched polymers are typically utilized as base polymers in PDCs, recently brush polymer-drug conjugates (BPDCs) have been established as a new class of PDCs with nanoscopic structural features.[59-61] Because the first examples of BPDCs are non-degradable, different strategies have been developed to incorporate degradability with the brush polymer-based drug delivery systems. As demonstrated by Johnson and co-worker, drug-conjugated brush polymer-based nanoparticles can be prepared by "brush-first" cross-linking polymerization approach using degradable cross-linkers, and the degradation of these nanoparticles can yield brush polymers with hydrodynamic sizes (~5 nm) small enough for renal clearance.[62-63] With a long term interest in developing novel therapeutic delivery systems, we prefer to introduce degradability to BPDCs through degradable backbones. We have successfully synthesized alkyne-functionalized PLAs and utilized them to construct two types of BPDCs with degradable PLA-based backbones carrying PEG-based grafts and paclitaxel (PTXL) drug moieties, through highly efficient azide-alkyne click reaction.[64-65] These PEGylated brush polymer scaffolds not only possess nanoscopic size and significant water solubility, but also exhibit remarkable biodegradability and non-cytotoxicity. Therefore, our BPDC platform can also be appealing for the delivery of other drugs by conjugating them with cleavable linkages.

Recently, we investigated degradable PDCs for sustained delivery of DOX. In our previous study, PLA-*g*-DOX, an acid-sensitive linear polymer-DOX conjugate with a high drug loading, was prepared. Due to its lack of water-solubility, it was further converted into pegylated PLA-*g*-DOX-based nanoparticles by nanoprecipitation with a PEG-containing surfactant. These nanoparticles exhibited acid-triggered DOX release behaviour and significant therapeutic efficacy towards cancer cells. Herein, we report a novel BPDC, PLA-*g*-DOX/PEG, for DOX delivery through a pH-labile Schiff base linkage between DOX and a brush polymer scaffold. Besides synthesis and characterization, the drug release profile and *in vitro* biomedical assessment of the BPDC are also addressed in this paper. With brush-like architecture, the BPDC is structurally different with PLA-*g*-DOX and its derived nanoparticles. Because the BPDC is water-soluble and can directly serve as pegylated nanotherapeutics for sustained DOX delivery without the assistance of external reagents, it potentially may be more relevant to clinical applications in cancer treatment.

Experimental

Measurement

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Varian INOVA-500 spectrometer at room temperature, and the samples were dissolved in $CDCl_3$ containing 1.0 vol% tetramethylsilane (TMS) as an internal reference. FT-IR spectra were obtained on a Bruker Tensor 27 system using attenuated total reflectance (ATR) sampling accessories. The concentration of DOX was determined using a Shimadzu 3101PC UV-Vis-NIR scanning spectrophotometer, based on the characteristic UV-Vis absorption of DOX at 490 nm.

Gel permeation chromatography (GPC) data were obtained from a Viscotek GPC system equipped with a VE-3580 refractive index (RI) detector and a VE-3210 UV-Vis detector. DMF (HPLC) with 0.1 M LiBr was used as the solvent for polymers and the eluent for GPC with a flow rate of 0.5 mL/min at 55 ºC. The GPC instrument was calibrated with narrowly dispersed linear polystyrene standards purchased from Varian.

Dynamic light scattering (DLS) measurements were performed using a Nano ZS90 Zetasizer (Malvern Instruments). A 4 mW 633 nm HeNe laser was used as the light source and all experiments were performed at a temperature of 25.0 ºC at a measuring angle of 90º to the incident laser beam. An aqueous solution of BPDC (1 mg/mL) prepared by simply mixing BPDC with water was used for the DLS measurement.

Transmission electron microscopy (TEM) images were obtained using a JEOL 2010 microscope. Each TEM sample was prepared by dropwise addition of 10 μL of aqueous solution of BPDC (1.3 mg/mL) on a carbon-coated copper grid, followed by negative staining using 10 μL of 0.5% uranyl acetate solution.

Materials

6-Bromo-1-hexanol (97 %), 4-dimethylaminopyridine (DMAP; 99+%) and N,N'-dicyclohexylcarbodiimide (DCC; 99%), methoxypolyethylene glycol azide (azide-PEG_{2000}, M_n = 2,000 Da) were purchased from Sigma-Aldrich. Sodium azide (99%), triethylamine (TEA; 99+%), copper(I) bromide (98+%), *N,N,N',N',N''*-pentamethyldiethylenetriamine (PMDETA, 99%), dichloromethane (DCM; HPLC), tetrahydrofuran (THF; HPLC), ethyl acetate (HPLC), hexane (HPLC), N,N'-dimethylformamide (DMF; HPLC) and methyl sulfoxide (DMSO; HPLC) were purchased from Fisher Scientific. DOX hydrochloride salt (DOX·HCl; 99+%) was purchased from LC laboratories (Woburn, MA). Alkyne-functionalized PLA (**1**) and 6-azidohexyl

4-formylbenzoate (**2**) were prepared following the synthetic approaches we reported previously.[16,65]

Synthesis of PLA-*g*-ALD/PEG (3)

To a 5-mL ampoule, alkyne-functionalized PLA **1** (30 mg, 0.178 mmol of alkyne groups), 6-azidohexyl 4-formylbenzoate **2** (24.5 mg, 0.0891 mmol), azide-PEG_{2000} (178 mg, 0.0891 mmol) and CuBr (25.5 mg, 0.178 mmol) and DMF (2 mL) were added. Then the reaction mixture was degassed by three freeze-pump-thaw cycles followed by adding PMDETA (30.8 mg, 0.178 mmol). After stirring at room termperture for 24 h, the reaction mixture was purified by passing through a short alumina oxide column and precipitation in cold ethyl ether. Finally, **3** was obtained as a colorless polymer with an isolated yield of 71%. ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 10.09 (s, 1 H from **2**, C_6H_4CHO), 8.17 and 7.95 (m, 4 H from **2**, C_6H_4CHO), **7**.62 (s, 1 H from triazole formed by **1** and azide-PEG_{2000}, $C{=}CHN_3$), 7.50 (s, 1 H from triazole fromed by **1** and **2**, $C{=}CHN_3$), 5.41 and 5.14 (m, 2 H from monomer unit of **1**, $OCHCOO$), 4.50 (s, 2 H from azide-PEG_{2000}, CH_2CH_2N), 4.32 (s, 4 H from **2**, CH_2CH_2OCO and CH_2CH_2N), 3.85-3.40 (br m, 4 H from azide-PEG_{2000}, CH_2CH_2O), 3.38 (s, 3 H from azide-PEG_{2000}, CH_3O), 3.38-3.18 (br m, 2H from monomer unit of **1**, $CHCH_2C$), 1.92-1.38 (br m, 3 H from monomer unit of **1**, CH_3; 10 H from **2**, $N(CH_2)_5CH_2$).

Synthesis of PLA-*g*-DOX/PEG (4)

In a 8-mL vial, DOX·HCl (9.86 mg, 0.017 mmol) was dissolved in DMSO (4.0 mL) and then TEA (3.44 mg, 0.034 mmol) was added to neutralize HCl. The mixture was stirred in the dark for 0.5 h and then was dropwise added to a solution of **3** (50 mg, with 0.017 mmol of aldehyde groups) in DMSO. After stirring for 24 h, the reaction mixture was subjected to dialysis against THF for 2 days to give 52 mg of **4** as a red polymer with an isolated yield of 94%. ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 10.09 (s, 1 H from **2**, C_6H_4CHO), 8.40 (s, 1H from benzyl imine, N=CH), 8.20-7.34 (m, all Ar-H from **2** and DOX, 1 H from triazole formed by **1** and **2** or azide-PEG_{2000}, $C{=}CHN_3$), 5.00−5.60 (br m, 2H from monomer unit of **1**, $OCHCOO$; 2H from DOX, CH and OH), 4.70-4.95 (br m, 3H from DOX, CH_2OH and OH), 4.50 (s, 2 H from azide-PEG_{2000}, CH_2CH_2N), 4.32 (s, 4 H from **2**, CH_2CH_2OCO and CH_2CH_2N), 4.20−2.70 (br m, 2H from monomer unit of **1**, $CHCH_2C$; all CH_2O and OCH_3 protons from azide-PEG_{2000}; 7 H from DOX, NCH and OCH_3, $CHOH$, and $ArCH_2C$), 2.60-1.20 (br m, all CH_3 from **1**; 8 H from **2**, $NCH_2(CH_2)_4CH_2$; 7H from DOX, 2 × $CHCH_2CH$ and CH_3).

DOX Release Study

Eight mg of BPDC **4** was dissolved in 2 mL of phosphate buffer pH 7.4 and pH 5.5 (10 mM), respectively, and then transferred to a dialysis bag (MW cutoff: 3500 Da) against 50 mL of corresponding buffer medium. At each time interval, 5 mL of exterior buffur medium was withdrawn for UV measurement. The concentration of DOX was determined based on a calibration curve which was acquired from a series of DOX solutions with predetermined concentrations.

Cytotoxicity Assay

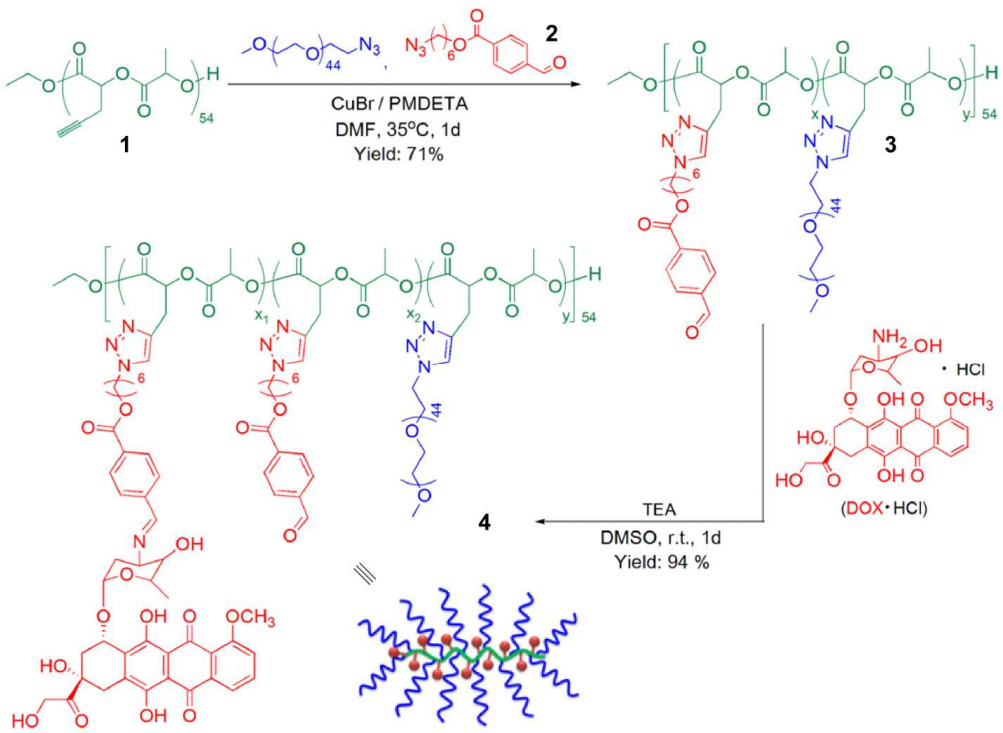
The cytotoxicity of BPDC **4** against MCF-7 human breast cancer cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madision, WI). The cells were seeded onto a 96-well plate at a density of 5×10^3 to 1×10^4 cells per well in 180 μL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and then 20 μL of samples with different concentrations of **4** in water were added. The cells were incubated for 48 h at 37 °C in 5% CO_2 atmosphere followed by adding 110 μL of MTS solution. After incubating the cells for another 2 h, the resulting solutions were measured for absorbance at 490 nm by a multiwell plate reader (Opsys MR, Dynex). The cell viability was calculated as a percentage of absorbance of the sample well compared to that of the control well with untreated cells. The cytotoxicity of free DOX and PLA-*g*-ALD/PEG **3** as controls against MCF-7 was evaluated with a similar procedure.

Confocal Imaging

Prior to the experiments, studied cells were seeded on a square glass coverslip in 35 mm cell culture dishes at 40-50% confluence. After the culture dishes were treated with an aqueous solution of DOX·HCl or BPDC **4** with the final DOX concentration of 5 μg/mL, the samples were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2 for 4 and/or 24 h. Dishes were rinsed with DMEM for 3 times to wash out any free particles before imaging. Cell imaging was obtained using a Leica TCS-SP2/AOBS confocal microscope, equipped with excitation laser lines of 405, 442, 458, 476, 488, 496, 543, 633 nm and capable of spectral detection in the range of 400-720 nm. All of the confocal images were taken under the same conditions (the parameters of photodetector gain, pinhole size and exposure time were kept constant).

Results and Discussion

To create the brush polymer scaffold which can directly react with DOX and form Schiff base linkage, highly efficient azide-alkyne click reaction of alkyne-functionalized PLA **1** with 6-azidohexyl 4-formylbenzoate **2** and azide-PEG_{2000} was designed (Scheme 1). As we reported previously,[65] **1** was synthesized by ring-opening polymerization of alkyne-functionalized lactide initiated by ethanol. ^{1}H NMR results indicated that the number-average degree of polymerization (DP_n) of **1** was 54 and thus the M_n of **1** was 9.1 kDa. GPC analysis showed that **1** had a narrow PDI of 1.15 relative to linear polystyrenes. Both ^{1}H NMR and GPC data confirmed the well-defined structure of **1**. While **2** was utilized to convert alkyne side group to the amine-reactive benzyl aldehyde group, azide-PEG_{2000} was grafted onto the PLA-based backbone along with **2** to create stealth shielding and promote water solubility of the entire structure. In the presence of copper(I) bromide and PMDETA ([alkyne of **1**]$_0$:[**2**]$_0$:[azide-PEG_{2000}]$_0$:[CuBr]$_0$:[PMDETA]$_0$ = 1.0:0.5:0.5:1.0:1.0), the click reaction of **2** and azide-PEG_{2000} with **1** was conducted in DMF at r.t for 24 h.[65] The high convertion of the click reaction was indicated by an essential disappearance of the characteristic peak of azide-PEG_{2000} in the GPC curve of reaction mixture. The resulting PLA-*g*-ALD/PEG **3** was obtained in 71% isolated yield after passing the final reaction solution through a short alumina column to remove catalysts (eluent: CH_2Cl_2), followed by precipitation in diethyl ether.



Scheme 1 Synthesis of PLA-*g*-DOX/PEG, BPDC **4**

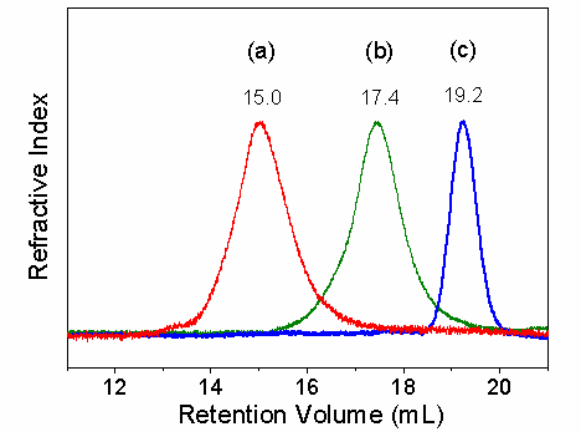


Fig. 1 GPC curves of PLA-*g*-ALD/PEG **3** (a), alkyne-functionalized PLA **1** (b), and azide-PEG_{2000} (c) using DMF with 0.1 M LiBr as the eluent.

PLA-*g*-ALD/PEG **3** was characterized by GPC and ^{1}H NMR analysis. The GPC curves of **3** compared with reactants (**1** and azide-PEG_{2000}) are shown in Figure 1. M_n and PDI of **3** estimated from GPC measurement were 55.4 kDa and 1.33 relative to linear polystyrene. Because of the compact macromolecular structure of **3**, the M_n of **3** might be considerably underestimated through GPC measurement. ^{1}H NMR analysis verified its chemical structure. From its ^{1}H NMR spectrum (Figure 2, top), the molar fraction of **2** grafted to **1** (relative to PLA-based backbone unit) was determined to be 0.46 by comparing the resonance intensities of aldehyde protons from **2** at 10.09 ppm with those of CH protons from monomer units of the backbone at 5.41 and 5.14 ppm. The molar fraction of azide-PEG_{2000} grafted to **3** (relative to PLA-based backbone unit) was determined to be 0.54 by comparing the resonance intensities of CH_2 protons from azide-PEG_{2000} at 3.40-3.85 ppm with those of CH protons from repeating units of **1** at 5.40 and 5.14 ppm. Thus M_n estimated from ^{1}H NMR data was 69.6 kDa. The slightly higher-than-expected experimental grafting density of PEG_{2000} chains may be ascribed to the workup process in which PEG-enriched speicies could be more readily recovered.

The conjugation reaction between **3** and DOX·HCl was conducted in DMSO at room temperature for 24 h, in the presence of TEA which was added to neutralize HCl and activate the amine group of DOX ([aldehyde group from **3**]$_0$: [DOX]$_0$: [TEA]$_0$ = 1 : 1 : 2). The successful conjugation of DOX with **3** was verified by GPC measurement (Figure 3).[16] While precursor **3** showed signals using RI detector and could not be detected by UV-Vis detector at 490 nm, DOX·HCl was sensitive to the UV-Vis detector but did not give appreciable signal with the RI detector. After the conjugation reaction, the resulting BPDC **4** showed monomodal GPC peaks by both RI and UV-Vis detectors, indicating that DOX was successfully conjugated onto **3**. Interestingly, BPDC **4** and the precursor **3** showed very similar RI curves with the same peak positions at 15.0 mL, suggesting that the hydrodynamic volumes of **3** and **4** were identical. Relative to PEG side chains, DOX side moieties were short and sterically confined through the polymer backbone, and therefore, the entire hydrodynamic volume may not be affected considerably no matter whether DOX is conjugated or not. On the other hand, UV-Vis detector showed an elution volume of 15.5 mL for the peak position of **4**. Because the two detectors were linked in series with the RI detector at first, the 0.5 mL difference in elution volumes measured by the two detectors was ascribed to the volume of mobile phase required to elute between the two detectors. The UV-Vis peak of **4** can be exactly overlaid with the RI peak by a shift of 0.5 mL and an adjustment of height ratio, indicating that there is no appreciable concern regarding compositional heterogeneity in DOX conjugation.

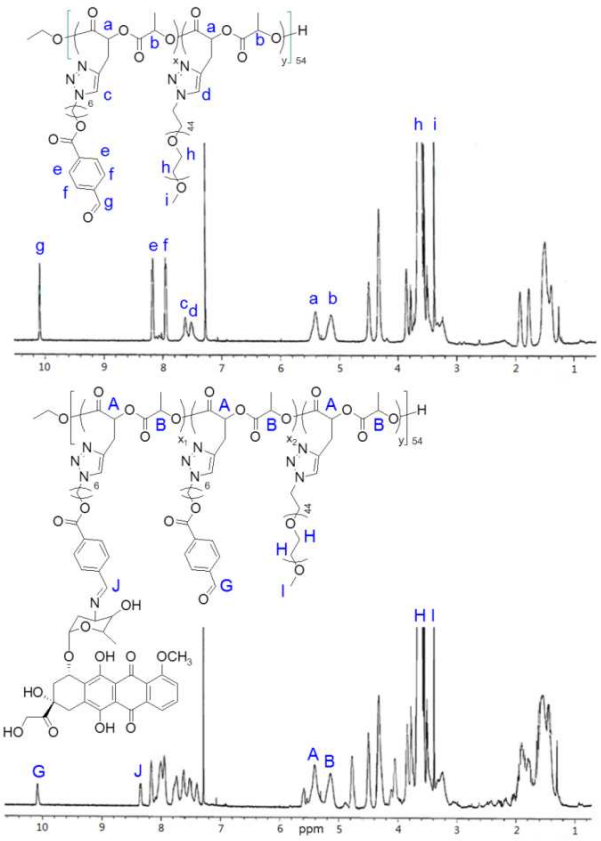


Fig. 2 [1]H NMR spectra of PLA-*g*-ALD/PEG **3** (top) and PLA-*g*-DOX/PEG, BPDC **4** (bottom) in $CDCl_3$.

[1]H NMR analysis verified the chemical structure of **4**. From Figure 2 (bottom), the molar fraction of conjugated DOX (relative to PLA-based backbone unit) was determined to be 0.25 by comparing the resonance intensities of imine protons of the Schiff base conjugation linkage at 8.40 ppm with those protons from unreacted aldehyde groups at 10.09 ppm.[16] Thus M_n of **4** estimated from [1]H NMR spectrum was 76.7 kDa. According to the [1]H NMR results, the DOX loading effeciency was 55% and the DOX loading amount was 9.2 wt%. The DOX loading amount determined by UV-vis measurement was 9.3 wt%, which is very close to the experimental value by [1]H NMR spectroscopy. The formation of benzyl imime linkage between DOX and the polymer scaffold can be deduced from the reduction of the resonance intensities of aldehyde proton at 10.09 ppm after conjugation, coupled with the appearance of a variety of resonances from DOX protons (Figure 2).

Due to its hydrophilic PEG side chain, **4** can quickly be dissolved in water without the need of any external assistance. The size and size distribution of **4** in aqueous solution were measured by DLS analysis (Figure 4). It showed that **4** had a hydrodynamic size around 13.1 ± 3.2 nm. TEM image also indicated that **4** had a uniform spherical morphology with diameter around 10-30 nm, which agrees well with the DLS result. Because kidney and reticuloendothelial clearance can effectively eliminate nanostructures with smaller sizes (<10 nm) and larger sizes (~100-200 nm) respectively,[66-67] the nanoscopic size of **4** in aqueous solution is in the preferred range (10-100 nm) to promote EPR effect and to achieve high bioavailability.[1]

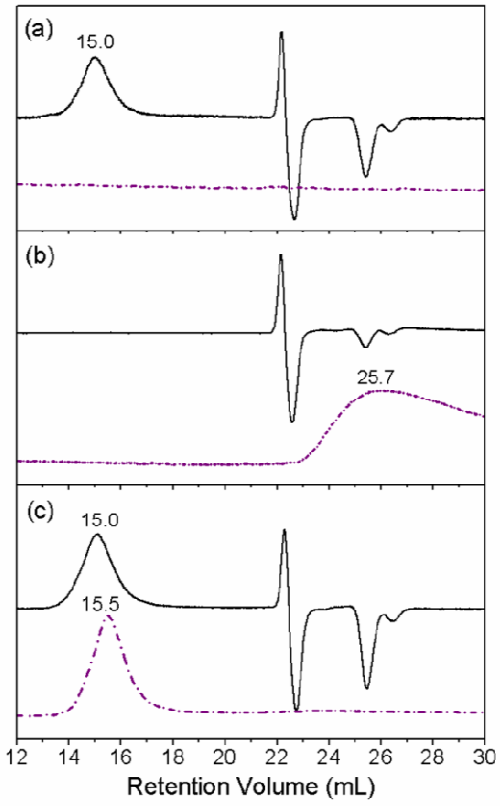


Fig. 3 GPC curves of PLA-*g*-ALD/PEG **3** (a), DOX·HCl (b) and PLA-*g*-DOX/PEG, BPDC **4** (c), based on RI signal (black solid lines) and UV-Vis signal at 490 nm (purple dash dots) using DMF with 0.1 M LiBr as the eluent. The delay volume between the RI and the UV-Vis detectors was not substracted in the GPC curves.

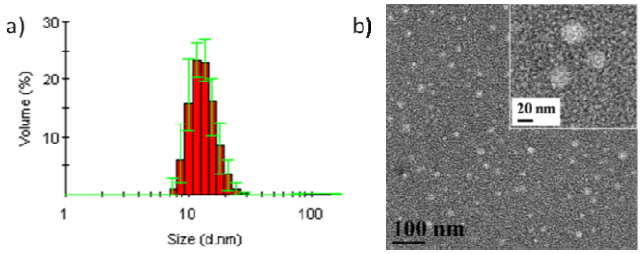


Fig. 4 DLS profile (a) and TEM image (b) of PLA-*g*-DOX/PEG **4**.

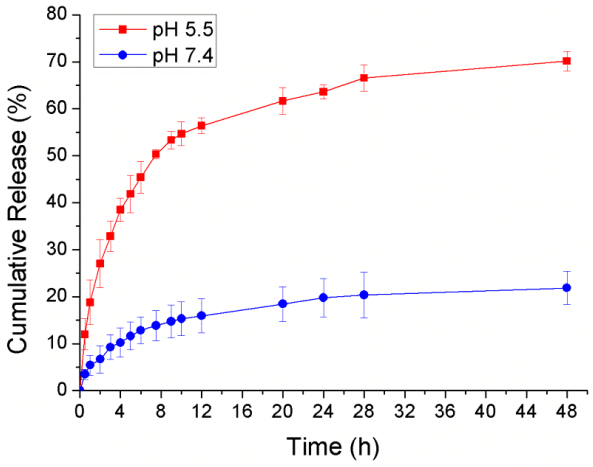


Fig. 5 Drug release profile of DOX from PLA-*g*-DOX/PEG **4** at pH 5.5 and pH 7.4.

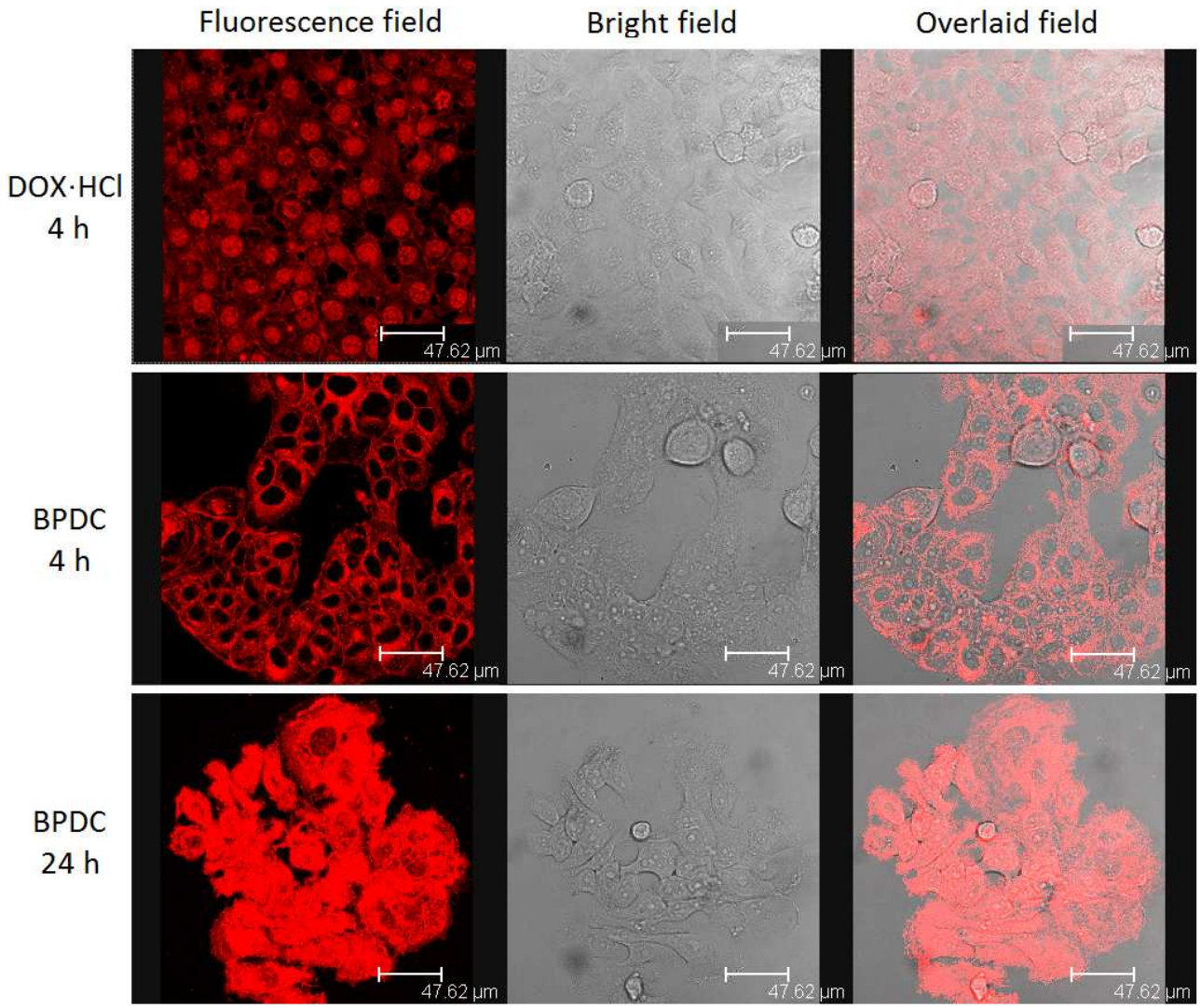


Fig. 6 Confocal images of MCF-7 cells after 4 h incubation of DOX·HCl, and 4 and 24 h incubation of BPDC **4** (scale bar: 47.62 μm).

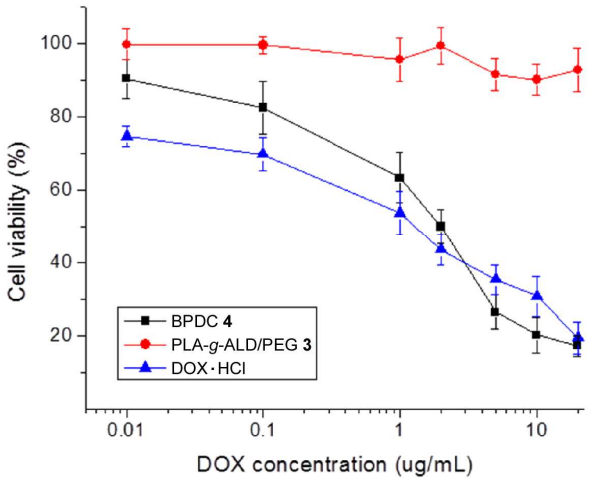


Fig. 7 Cytotoxicity of BPDC **4** against MCF-7 breast cancer cells after 48 h of incubation (PLA-*g*-ALD/PEG **3** and DOX·HCl as controls; **3** was in the same molar concentrations as BPDC **4**).

Using a dialysis approach, *in vitro* drug release behavior of PLA-*g*-DOX/PEG **4** was investigated at physiological pH (7.4) and under a slightly acidic condition (pH 5.5) at 37 °C, in order to demonstrate pH-responisve DOX realese from **4**. As illustrated in Figure 5, the drug release at pH 5.5 was much faster than that at pH 7.4. It was observed that 50% of the drug was released from **4** after 7 h at pH 5.5, while only 20% of DOX was released after 48 h in pH 7.4 buffer. The pH-responsive drug release was attributed to the acid labile benzyl imine Schiff base linkage.

Uptake of BPDC **4** by MCF-7 cells was studied. After the incubation of MCF-7 cells with BPDC **4** for 4 h, fluorescence (red) signals from DOX were observed mainly in cytoplasm (Figure 6). In constrast, cell incubation with DOX·HCl for 4 h resulted in strong fluorescence in cell nuclei but relatively weak fluorescence in cytoplasm. Because BPDC **4** can effectively transport DOX moieties into cytoplasm while free DOX can rapidly enter into nuleis, their internalization mechanisms should be different.[68] Our observation also suggests that most of the conjugated DOX moieties on **4** had not been released within 4 h. On the other hand, when the incubation time of **4** increased to 24 h, the DOX fluorescence signals became much more intensive indicating more BPDC macromolecules were taken up by the cells. Moreover, fluorescence was also observed in the nuleis of

many cells, indicating the release of significant amount of DOX from BPDC. Because DOX suppresses tumor proliferation by intercalating DNA,[69] the successful delivery of DOX via BPDC **4** into nuclei of MCF-7 cells suggests the significant potential applications of **4** in cancer treatment.

In vitro cytotoxicity of BPDC **4** relative to free DOX and the brush polymer scaffold was evaluated by the MTS assay against MCF-7 breast cancer cells (Figure 7). It was found that the base polymer, **3**, showed no considerable cytotoxicity at all experimental concentrations after 48 h of incubation. Both BPDC **4** (IC_{50} ~ 2.0 μg/mL) and free DOX (IC_{50} ~ 1.5 μg/mL) exhibited significant cytotoxicity, with different concentration-dependence. BPDC **4** resulted in higher cell viability than DOX·HCl at the concentration of 1 μg/mL or less, and this may be ascribed to the very low intracellur DOX concentrations as a result of time-consuming DOX release from **4** under the incubation concentration range. However, **4** exhibited higher therapeutic efficacy in killing MCF-7 cells than DOX·HCl at the concentrations of 5 and 10 μg/mL, presumably because the efficient cell uptake and sustained DOX release of **4** at such incubation concentrations can help to maintain relatively long time period of therapeutic effective DOX concentrations within MCF-7 cells.

Conclusion

A novel degradable BPDC was successfully designed and prepared for pH-responsive DOX release. Alkyne-functionalized PLA was used as a biodegradable backbone and was further grafted with PEG side chains and aldehyde side groups by its efficient and quantitative click reaction with azide-functionalized PEG and 6-azidohexyl 4-formylbenzoate. The resulting PLA-*g*-ALD/PEG polymer scaffold was then conjugated with DOX, with the formation of Schiff base linkage between the polymer and DOX. The water-soluble BPDC exhibited nanoscopic size of 10-30 nm, when dispersed in water. With Schiff base linkages, BPDC showed remarkable a pH-responsive drug release behavior with the half-life of 7 h under pH 5.5. The BPDC can enter the cytoplasm of MCF-7 cells within 4 h, and deliver DOX moieties into the nuclei within 24 h. Moreover, the cytotoxicity assessment revealed that the BPDC was more therapeutically effective towards MCF-7 cells than free DOX when the concentrations are 5 and 10 μg/mL. The BPDC has a significant DOX loading (9.2 wt%), which potentially may be further improved by using the DOX-enriched core domain for the encapsulation of DOX. Based on the promising *in vitro* results, *in vivo* studies are required to further verify the applicability of the BPDC for effective anti-cancer therapy.

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Notes and references

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TOC:

We report the synthesis, characterization and *in vitro* assessment of a degradable brush polymer-drug conjugate which can enable acid-triggered release of doxorubicin (DOX).