Preparation and biological evaluation of a novel pH-sensitive poly (β-malic acid) conjugate for antitumor drug delivery

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Abstract. Poly (β-malic acid), referred to as PMLA, has been synthesized and introduced as a polymeric drug carrier due to its desirable biological properties. In the present study, a novel pH-sensitive polymer-drug conjugate based on PMLA, PMLA-Hz-doxorubicin (DOX), was prepared, and another conjugate, PMLA-ami-DOX, was synthesized as a comparison. The structures, conjugation efficiency, and drug release properties of the prodrugs were determined. The cytotoxicity and cell uptake were assessed using the HT1080 human fibrosarcoma cell line as an *in vitro* cell model. The release of DOX in the two conjugates were pH-dependent in PBS buffer at a pH of 5.6, 6.0, 6.8 and 7.4. The quantity of drug released increased with the decrease in pH, and PMLA-ami-DOX released twice as much as PMLA-Hz-DOX (12 h). The cytotoxicity of PMLA-Hz-DOX at pH 7.4 was lower than that of free DOX and increased with the decrease in pH, indicating that the cytotoxicity of PMLA-Hz-DOX was pH-sensitive. Flow cytometry and confocal experiments confirmed the efficiency of the PMLA-Hz-DOX conjugate. Therefore, bonding DOX to PMLA via an acid-sensitive hydrazone bond may be used to reduce its toxic side effects on normal tissues while responding to tumor pH and releasing the drug.

Introduction

Polymeric conjugates of conventional drugs, a conjugation of a drug with a polymer, have several advantages over their low molecular weight precursors. Polymer-drug conjugates can increase the solubility of low solubility or insoluble drugs,

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enhancing drug bioavailability (1-3). In addition, polymeric conjugates can provide passive and/or active targeting of the drug specifically to the site of its action leading to a reduction in antigenic activity of the drug (4-7). Polymer-drug conjugates have introduced a new era of polymeric drug delivery systems with these advantages over the free form of a drug.

Several polymers with desirable biological properties have been employed as a drug delivery platform. Poly (β-malic acid), referred to as PMLA, which is an aliphatic polyester based on malic acid, has a preferable biodegradability and lack of toxicity. PMLA can be degraded into malic acid, an intermediate of tricarboxylic acid cycle, and subsequently degraded into water and CO_2 (8,9). PMLA has free pendant carboxyl groups on each monomer, which can be covalently bound to small-molecule drugs and other functional moieties (10-12). Multi-modification makes PMLA an efficient carrier, making it well-suited as a scaffold for tailored nanoconjugate chemistry.

Polymeric-drug conjugates based on PMLA have been examined over the course of the last two decades (13). Ljubimova *et al* (14) synthesized a targeted polymeric delivery system based on PMLA, named Polycefin, which is constructed of antisense oligonucleotides targeting Laminin-8 and monoclonal anti-transferrin receptor antibody, to target brain tumors and breast cancer. Polycefin was found to accumulate in U87MG brain tumor tissue and had no toxic effects on normal tissue (14-16). Controlled molecular weight PMLA was synthesized with a high yield in our previous study, and the antitumor agent 10-hydroxycamptothecin was attached to PMLA in order to enhance its water solubility and bioavailability (17,18).

To further improve delivery efficiency and cancer-targeting specificity, environment-responsive nanoconjugates have been developed by employing external stimuli, including pH-responsiveness. The application of pH-sensitivity is based on the fact that increased aerobic glycolysis in cancer cells leads to a lower extracellular pH of cancer cells (pH 6.5-7.2) than that in normal tissues (19,20). In addition, once taken up by cells, drug carriers experience a gradient pH moving from endosomes (pH 5.0-6.0) to lysosomes (pH 4.0-4.5) (21-23). One approach is to introduce cleavable bonds, which can be broken to release the drugs conjugated to or encapsulated in the carrier (24-26). In the case of polymer-drug conjugates, pH-sensitive linkages, including hydrazone, hydrazide and

acetal linkages, have been used to directly attach drug molecules to polymers (19,27-29).

In the case of polymer-drug conjugates, pH-sensitive linkages, including hydrazone, hydrazide, and acetal linkages, have been used to directly attach drug molecules to polymers (24). Based on our previous study, a pH-triggered drug release profile based on PMLA, PMLA-Hz-doxorubicin (DOX), was examined in the present study. The anticancer drug DOX was chemically attached to the polymer backbone via a pH-responsive hydrazone bond. By contrast, another conjugate linking DOX via an amide bond was also prepared. The polymeric conjugation protected DOX from being released during systemic circulation, however, once the polymeric drug was internalized by the cancer cells and taken up by the endo/lysosomes, the hydrazone linkage was ruptured due to the acidic microenvironment and DOX was released from the PMLA backbone (Fig. 1). It is anticipated that the pH-triggered release of the nanoconjugate may enhance the drug delivery efficiency and offer a promising therapeutic outcome.

Materials and methods

Materials. L-aspartic acid and trifluoroacetic anhydride were obtained from Aladdin Chemical Co., Ltd. (Shanghai, China) Doxorubicin hydrochloride (DOX·HCl) was purchased from HVSF United (Beijing, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) and N-hydroxysuccinimide (NHS) were purchased from TCI (Shanghai, China). HPLC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Minimum Eagle's medium (MEM) and trypsin were purchased from HyClone; GE Healthcare Life Sciences (Logan, UT, USA) and Gibco; EMD Millipore (Billerica, MA, USA), respectively. The Cell Counting Kit-8, fetal bovine serum (FBS) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from ZETA life (Shanghai, China). All other chemicals used in the present study were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and all reagents were of analytical grade without further purification.

Synthesis of PMLA. PMLA was synthesized as described previously by ring-opening polymerization, starting from L-aspartic acid (17,18). PMLA with a molecular weight of 10 kDa was used in the present study, which is a suitable molecular weight for drug conjugates. The yield of PMLA was 5.6%. The synthetic route for PMLA is illustrated in Fig. 2.

Preparation of PMLA-Hz-DOX and PMLA-ami-DOX. PMLA (116 mg, 1 mmol with regard to repeat units), NHS (1 mmol) and EDC·HCl (1.5 smmol) were dissolved in 15 ml of DMSO and stirred at room temperature for 6 h to activate the carboxyl group. Subsequently, 2 mmol $N_2H_4\cdot H_2O$ was added. After 6 h, 0.2 mmol DOX was added to the solution and stirred at room temperature for another 6 h in the dark. The solution was dialyzed (MWCO: 5 kDa) against DMSO for 24 h to remove unreacted and produced small molecules and then dialyzed (MWCO: 5 kDa) against deionized water at 4°C in the dark for 24 h. Finally, the solution was lyophilized to yield PMLA-Hz-DOX as red powder, the yield was 64.5%.

The carboxyl group was activated, as above, and then 0.2 mmol DOX was added and stirred at room temperature overnight in the dark. The solution was dialyzed (MWCO: 5 kDa) against DMSO for 24 h to remove unreacted regent and produced small molecules and then dialyzed (MWCO: 5 kDa) against deionized water at 4°C in the dark for 24 h. Finally, the solution was lyophilized to yield PMLA-ami-DOX as red powder with a yield of 76.6%. The synthetic routes for these two conjugates are illustrated in Fig. 3.

UV-vis spectroscopy (Shimadzu Corporation, Tokyo, Japan) was used to quantify the quantity of DOX conjugated on the polymers. Briefly, the DOX-conjugated polymers were dissolved in deionized water and the absorbance of the solutions at 254 nm was measured. Using a calibration curve obtained by measuring the absorbance of different concentrations of free DOX in deionized water at 254 nm, the DOX content in the polymer was calculated. The DOX loading rate was calculated follows: DOX loading rate (%)=(weight of DOX in conjugate/weight of conjugate) x100%; and the conjugating rate was calculated as follows: Conjugating rate (%)=(weight of DOX in conjugate/weight of DOX input) x100%.

Characterization of PMLA-Hz-DOX and PMLA-ami-DOX. The chemical structures of the synthesized PMLA-Hz-DOX and PMLA-ami-DOX were determined by FT-IR using a KBr disc (Shimadzu FTIR-8400S) and ^{1}H NMR using Varian 400 mHz spectrometer (Avance, Bruker Corporation, Billerica, MA, USA). The samples were prepared from freeze-dried products by dissolving in DMSO-d6 (20 mg/ml) at 25°C. Chemical shifts were determined in δ units relative to the tetramethyl silane signal as an internal reference.

The ζ potential of these two conjugates was determined by dynamic light scattering (DLS) following dissolving in water.

In vitro release of DOX from the conjugates. The *in vitro* drug release profiles were obtained by a dynamic dialysis method (30). The release experiments were performed at 37°C. Typically, the conjugate solution of 200 μg/ml equivalent DOX concentration was dialyzed into 100 ml of 0.1 M phosphate-buffered saline (PBS; pH 5.6, 6.0, 6.8, and 7.4) with magnetic stirring at 200 rpm. At hourly intervals, 0.5 ml was removed from the release medium for each sample, and the same volume and temperature of PBS was added to the release medium. The released DOX was determined by HPLC (Agilent 1260), according to Chinese Pharmacopoeia 2015 (ChP 2015) (31). The results of the triplicate tests were used to calculate the accumulated drug release.

In vitro cytotoxicity. HT1080 fibrosarcoma cells, which were provided by Shanghai Zhongqiaoxinzhou Biotechnology Co., Ltd. (Shanghai, China), were used as *in vitro* models. The cells were cultured in MEM containing 10% heat-activated FBS and 100 IU/ml penicillin and 100 μg/ml streptomycin. They were incubated in a 37°C water-jacketed incubator equilibrated with 5% CO_2 and maintained at ~99% relative humidity. The medium was replenished every other day until confluence was achieved. The cells were then washed with PBS and harvested with 0.125% trypsin-EDTA solution.

The HT1080 fibrosarcoma cells were seeded at a density of $5x10^4$ cells/well in a 96-well transparent plate and incubated
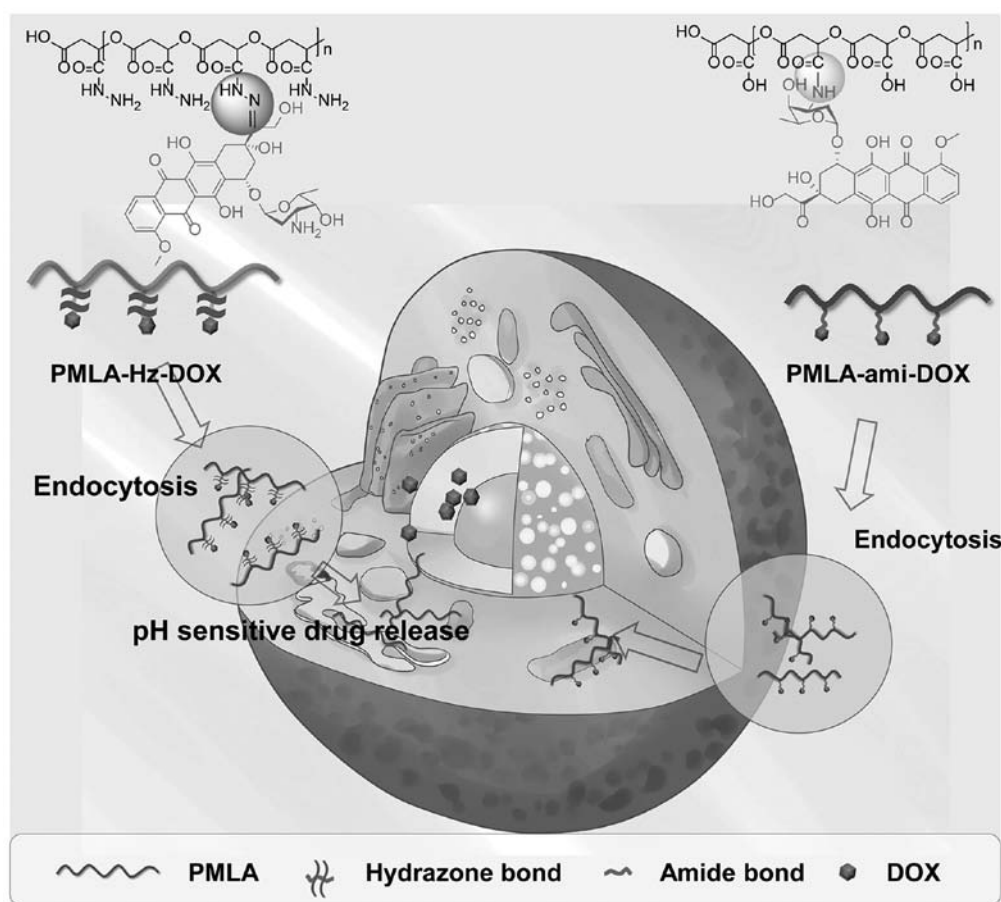
Figure 1. Schematic illustration of tumor cell uptake of nanoconjugates and pH-triggered drug release. PMLA, poly (β-malic acid); DOX, doxorubicin.

for 24 h. All growth medium was prepared by supplementing MEM with 10% FBS and sterilized with a 0.2 μm filter prior to use. The medium was then replaced with PMLA-Hz-DOX, PMLA-ami-DOX or free DOX at various drug concentrations (0.005, 0.01, 0.05, 0.1, 0.5 and 1 μg/ml) in medium at pH 6.0 or 7.4. The cells were then incubated for another 48 h prior to replacing the medium with 0 1 ml of fresh growth medium containing 10% CCK-8. Following incubation for another 2 h, the plates were vigorously shaken prior to measuring the relative color intensity at 450 nm using a microplate reader. Cell viability was determined as a percentage of the intensity of the controls ± standard deviation. Each experiment was repeated five times at each polymer concentration.

The cell viability with PMLA alone was also investigated at various concentrations equivalent to those for the conjugate.

Confocal laser scanning microscopy (CLSM) observation. The HT1080 cells were maintained in a 6-well plate at 5x10^{5} cells/well for 24 h and treated with PMLA-Hz-DOX, PMLA-ami-DOX or free DOX for 2 h at 37˚C. Following incubation, the cells were washed with PBS three times to remove excess conjugates. The concentration of DOX was 5 μg/ml. The cells were the washed with PBS three times, fixed in precooled 4% paraformaldehyde for 20 min, and stained with DAPI nuclear stain (5 μg/ml) for 5 min at 4˚C in the dark. The cells were washed with PBS, and fluorescent images of cells were analyzed using an FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan).
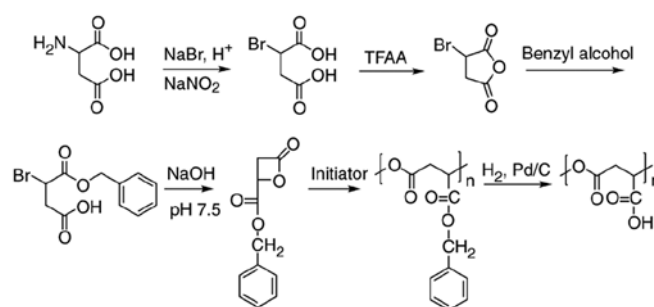

Figure 2. Synthesis of poly (β-malic acid).

Flow cytometric analysis. As described in detail previously (32), flow cytometry was used to confirm the uptake of conjugates by HT1080 cells. Similar to the confocal study, the HT1080 cells were seeded in a 6-well plate at 5x10^{5} cells/well for 24 h and treated with PMLA-Hz-DOX, PMLA-ami-DOX or free DOX for 2 h at 37˚C. Following incubation, the cells were washed with PBS three times. The cells were then harvested by trypsinization, centrifuged at 352 x g for 5 min, at 4˚C, resuspended in 500 μl PBS medium and examined by flow cytometry using a FACScan instrument (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis. All experiments were performed in triplicate and the obtained data were processed using GraphPad

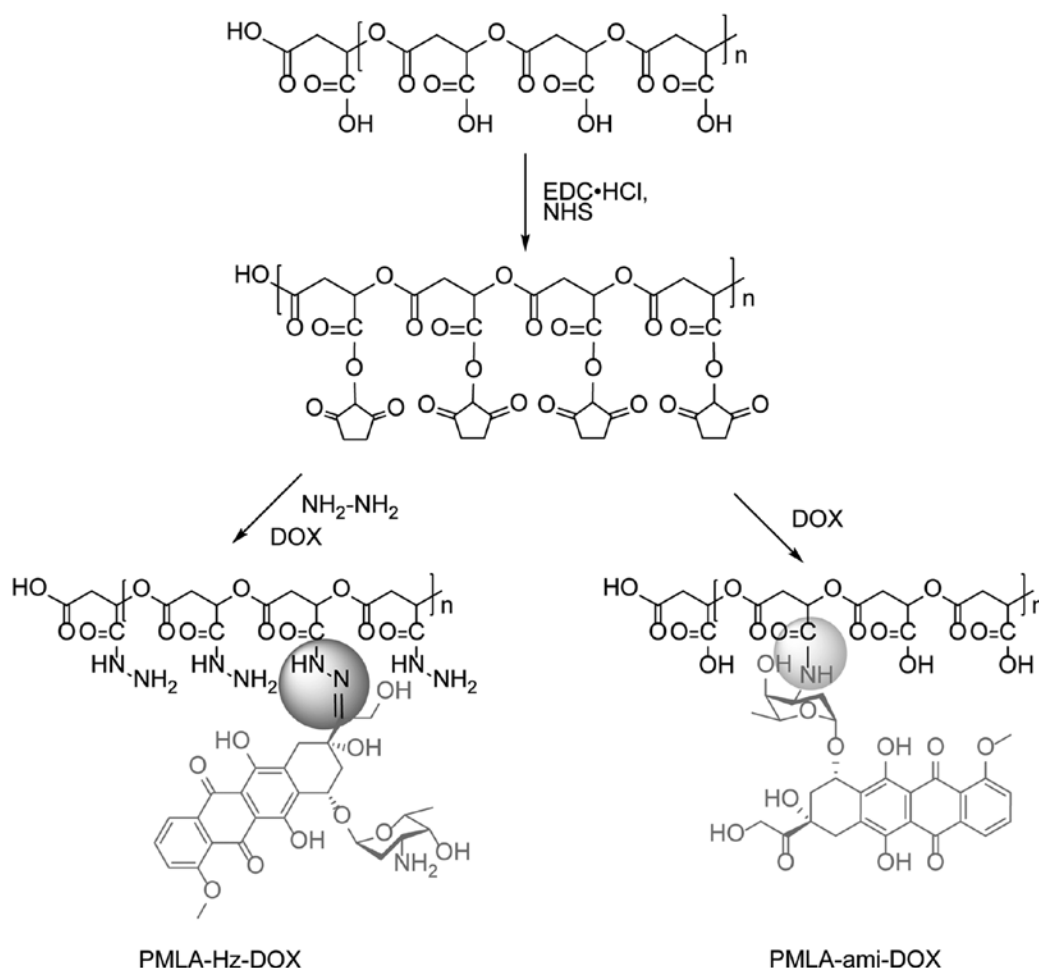Figure 3. Synthesis of PMLA-Hz-DOX and PMLA-ami-DOX. PMLA, poly (β-malic acid); DOX, doxorubicin.

Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) and Origin 2018 (OriginLab, Northampton, MA, USA) software. Differences between the two groups were analyzed using the unpaired t-test in these software programs. $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Drug loading and conjugation rate of PMLA-Hz-DOX and PMLA-ami-DOX. The DOX loading rates of the PMLA-Hz-DOX and PMLA-ami-DOX conjugates were 20.09±2.64 and 19.13±3.30 wt%, respectively, and the DOX conjugating rates were 69.28±9.11 and 65.96±11.39%, respectively. A large number of suspended carboxyl groups on the PMLA backbone makes it a high drug loading carrier compared with other polymers, including PEG, PLA, PCL and other polyesters that have no active groups on the main chains, meaning drugs may only be encapsulated with a loading rate of <5% (1,2).

Characterization of PMLA-Hz-DOX and PMLA-ami-DOX. In PMLA-Hz-DOX, the carbonyl group at the C-13 position of DOX reacts with the hydrazide of the PMLA side chain to form a hydrazone bond whereas the amino group at the 3' position of the DOX pyran ring in PMLA-ami-DOX forms an amide bond with the carboxyl group on PMLA. ^{1}H NMR
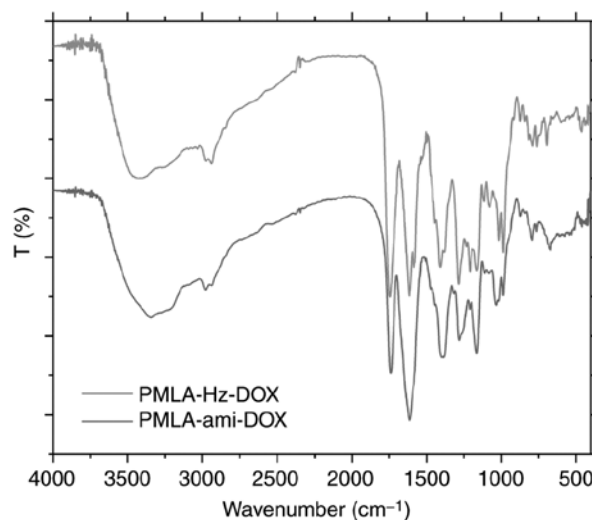

Figure 4. IR spectra of PMLA-Hz-DOX and PMLA-ami-DOX. PMLA, poly (β-malic acid); DOX, doxorubicin; T, transmittance.

in DMSO-d_6 was used to confirm the product identity. The IR spectra of these two conjugates are shown in Fig. 4. IR (v, cm^{-1}): 1,585 (-NH$_2$, δ), 1,381 (C-N, st). The peak close to 1,540 cm^{-1} in the IR spectrum of PMLA-Hz-DOX was δ_{NH} (-CO-NH-). The ^{1}H-NMR spectrum of PMLA-Hz-DOX and
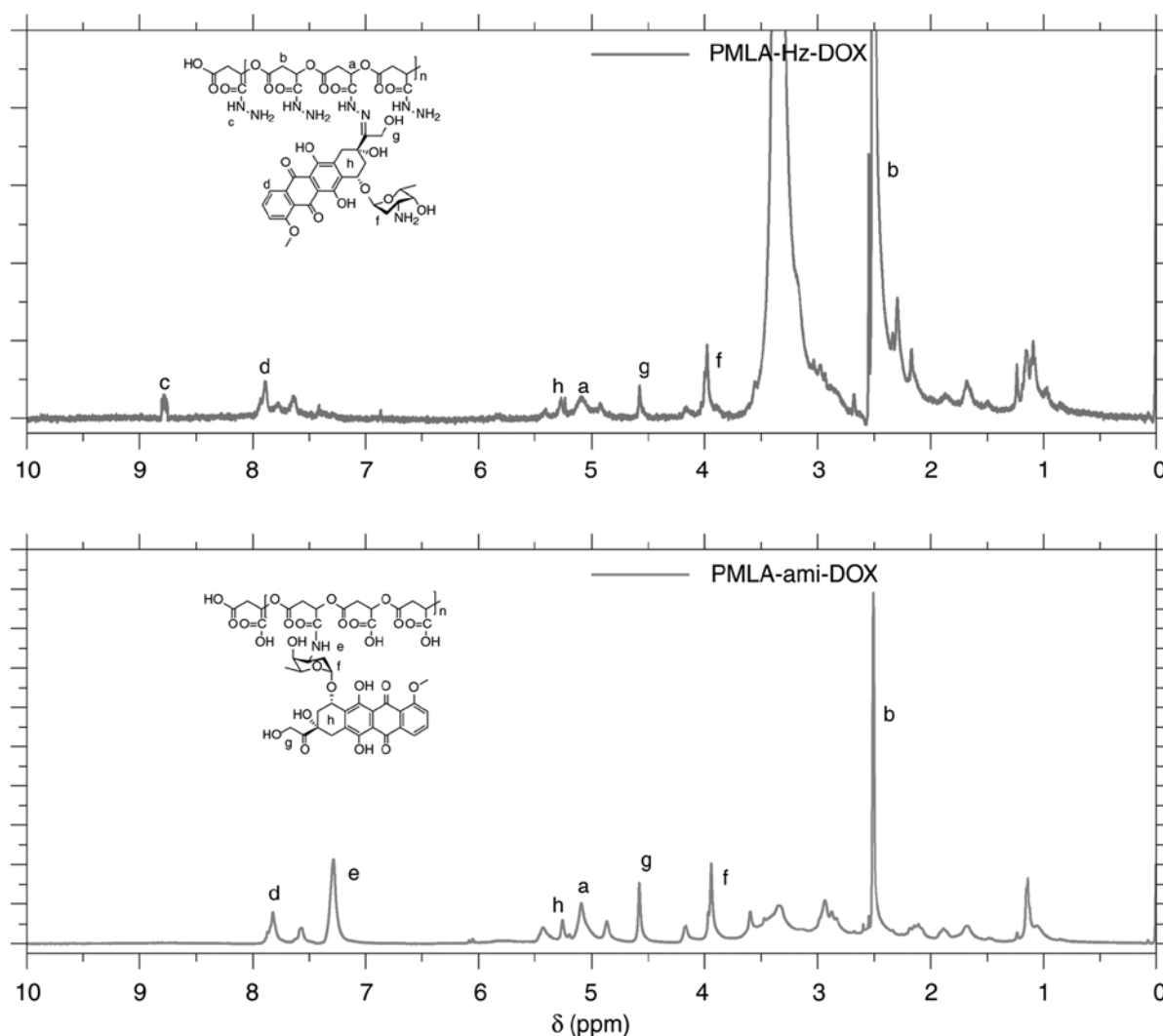
Figure 5. ¹H-NMR of PMLA-Hz-DOX and PMLA-ami-DOX. PMLA, poly (β-malic acid); DOX, doxorubicin. a-h, position of protons on DOX.

PMLA-ami-DOX structures are shown in Fig. 5, in which the peaks have been identified and marked. The peaks at different positions of a-h correspond to different protons on DOX.

The ζ potential of these two conjugates was determined by DLS (Table I). PMLA without the DOX connection had negative ζ potential and, once DOX was conjugated, it showed positive ζ potential. The ζ potential of PMLA-Hz-DOX was 20.25±0.36 mV due to the modification of the amino group on the carboxyl group and the introduction of DOX, whereas the ζ potential of PMLA-ami-DOX was 10.57±0.42 mV. The positively charged nanoconjugates effectively interact with the negatively charged cell membrane by electrostatic attraction, triggering efficient cell internalization (33,34).

In vitro release of DOX from the conjugates. The *in vitro* release of the drug from PMLA-Hz-DOX and PMLA-ami-DOX conjugates was measured at various pH conditions. As shown in Fig. 6, the DOX release showed no marked initial burst in PMLA-Hz-DOX. It was, however, significantly pH-dependent; the lower the pH was, the faster the drug released. Specifically, the drug released rapidly from the PMLA-Hz-DOX conjugate at pH 5.6 and pH 6.0, reaching

Table I. ζ potential of PMLA-ami-DOX and PMLA-Hz-DOX.

Conjugate	ζ potential (mV)
PMLA-Hz-DOX	20.25±0.36
PMLA-ami-DOX	10.57±0.42

DOX, doxorubicin; PMLA, poly (β-malic acid).

70.0 and 54.9%, respectively, at 24 h, whereas DOX release at pH 6.8 and pH 7.4 was markedly slower at 16.87 and 9.93% in the same period, respectively. For PMLA-ami-DOX, DOX release was slower than that of PMLA-Hz-DOX, and there was no pH dependence. Using this polymeric drug design, the conjugates can stably preserve drugs under physiological conditions and selectively degrade and release them by responding to the tumor extracellular pH (pH_e), endosomes (pH 5-6) or lysosomes (pH 4-5) (25,35,36). The *in vitro* drug release experiments showed that the release of DOX was pH-dependent. When the conjugate reached the tumor tissue, DOX was released, and the free and grafted DOX entered the
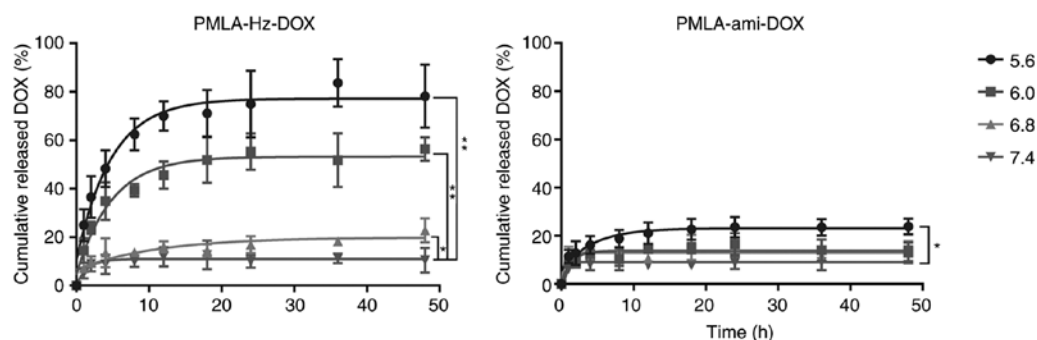
Figure 6. Cumulative release profile of PMLA-Hz-DOX and PMLA-ami-DOX at different pH values at 37˚C. PMLA, poly (β-malic acid); DOX, doxorubicin. **P<0.01 and *P<0.05 vs. pH 7.4.

tumor cells by diffusion or endocytosis (Fig. 1). In the tumor cell endosome, in which pH was as low as 4-5, DOX release was increased, although the release rate of DOX in tumor tissues requires further improvement.

In vitro cytotoxicity. The *in vitro* antitumor activities of various DOX formulations were evaluated using a CCK-8 assay of HT1080 fibrosarcoma cells, and the 50% inhibitory concentration (IC_{50}) was determined. PMLA did not show cytotoxicity up to a concentration of 1 mg/ml, making it safe to use as a drug carrier (Fig. 7). The *in vitro* cytotoxicity at different pH values exhibited pH-dependent cytotoxic effects (Fig. 8). At pH 7.4, the two conjugates showed lower IC_{50} values than free DOX, suggesting that DOX grafted to PMLA is likely to have lower side effects in systemic circulation. At pH 6.0, the pH of cell endosomes, PMLA-Hz-DOX had a higher cell cytotoxicity (IC_{50}=0.026 μg/ml); however, the cell viability of PMLA-ami-DOX was markedly higher (IC_{50}=0.31 μg/ml) (P<0.05). According to the structure-activity association of DOX, the carbonyl of C-13 may interact with the DNA double helix by hydrogen bond, which is crucial for maintaining its activity. Following linking to PMLA via a hydrazone bond, the antitumor activity reduced. These results suggested that the cytotoxicity of polymeric conjugates against HT1080 cells was lower than free DOX under physiological pH.

Cellular uptake measured by CLSM and flow cytometry. CLSM and flow cytometry were performed to investigate the effects of pH-sensitivity on the cellular uptake of nanoconjugates. As shown in Fig. 9, the PMLA-ami-DOX conjugate showed the weakest intracellular fluorescence of DOX at pH 7.4 and 6.0, indicating the DOX grafted to PMLA via an amide bond had reduced cell uptake. Compared with PMLA-ami-DOX, PMLA-Hz-DOX showed higher intracellular fluorescence of DOX at pH 6.0 than that at pH 7.4, suggesting that PMLA-Hz-DOX was more efficient following uptake by cells.

Flow cytometry was used to further examine the cellular uptake of the DOX-loaded nanoconjugates. As shown in Fig. 10, following incubation with HT1080 cells for 2 h, the two nanoconjugates showed relatively low cell internalization at pH 7.4. When the pH decreased to 6.0, PMLA-ami-DOX showed similar cellular uptake, however, the cellular uptake of PMLA-Hz-DOX was increased (P<0.05). These results were consistent with the CLSM analysis.

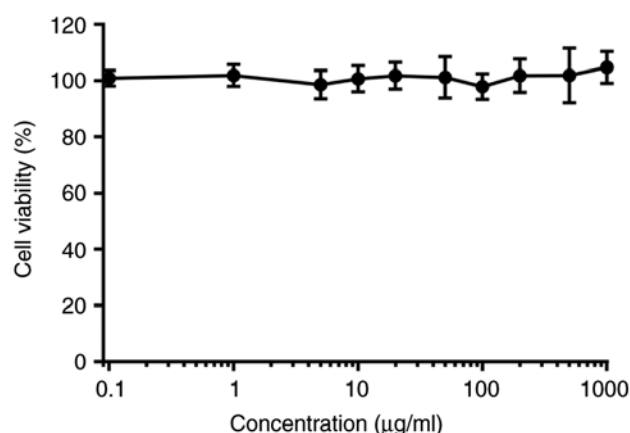
Figure 7. HT1080 cell viability following 48 h of treatment with poly (β-malic acid).

PMLA has preferable biocompatibility and biodegradability and is non-toxic, therefore, it is considered a promising drug carrier material. In the present study, the antitumor drug DOX was attached to PMLA via a hydrazine bond to obtain a pH-sensitive drug delivery conjugate. Drug release experiments at different pH conditions showed that the release of DOX was pH-dependent in PMLA-Hz-DOX, whereas the release behavior of PMLA-ami-DOX did not alter with the decrease of pH. The *in vitro* biological experiments showed that grafting DOX onto PMLA reduced the toxicity of DOX. However, following uptake by cancer cells, DOX released from the PMLA-Hz-DOX conjugate due to the tumor-specific pH environment. PMLA-Hz-DOX, with its pH-responding drug delivery properties, is expected to become a novel type of controlled release drug platform.
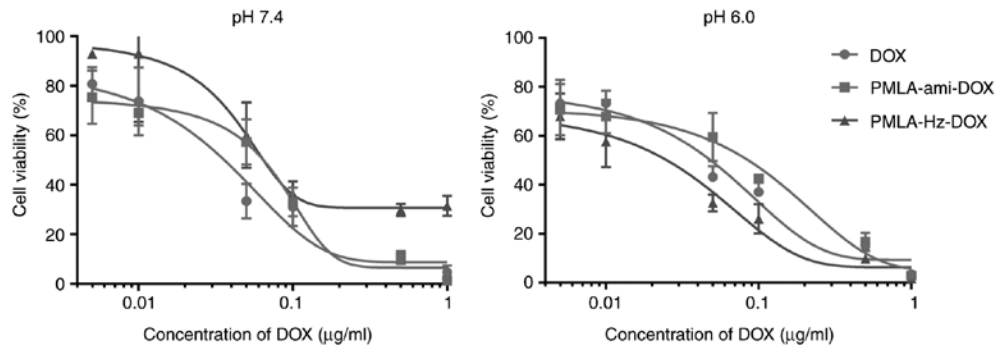
Figure 8. Viability of HT1080 cells following exposure to PMLA-Hz-DOX, PMLA-ami-DOX, and free DOX at various drug concentrations at 37°C for 48 h. PMLA, poly (β-malic acid); DOX, doxorubicin.
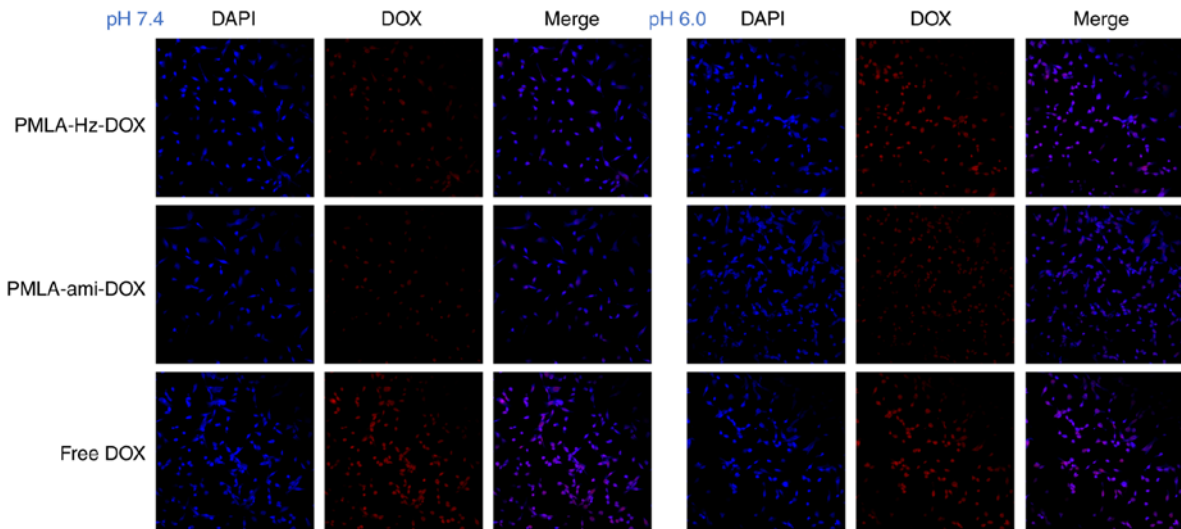

Figure 9. Internalization of different nanoconjugates in HT1080 cells at pH 7.4 and pH 6.8 imaged by confocal laser scanning microscopy at 2 h. Magnification, x20. PMLA, poly (β-malic acid); DOX, doxorubicin; DAPI, 4',6-diamidino-2-phenylindole.
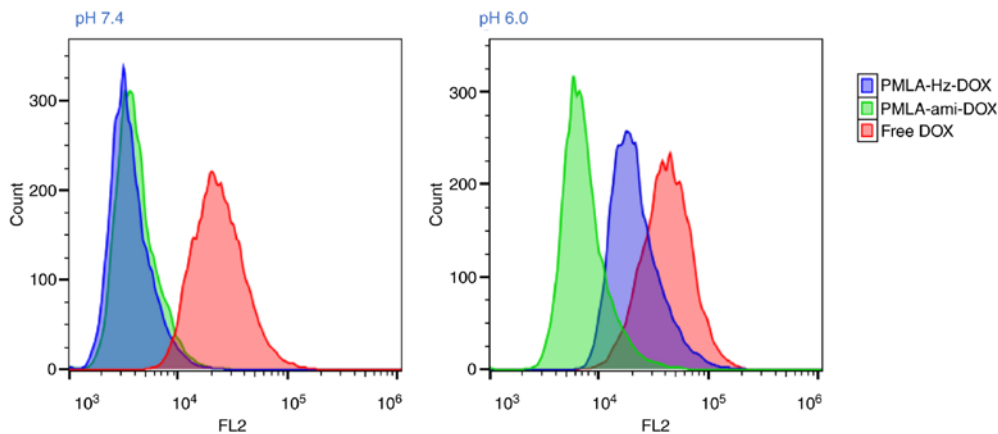

Figure 10. Cellular uptake of DOX-loaded nanoconjugates in HT1080 cells at pH 7.4 and pH 6.8, analyzed by flow cytometry at 2 h. PMLA, poly (β-malic acid); DOX, doxorubicin.

Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BL and YP performed the experiments and drafted the manuscript. EJ analyzed the data. YQ performed the experiments and analysed the data. HW made substantial contributions to

the design of the present study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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