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ARTICLE

Synthesis of an acid-labile polymeric prodrug DOX-*acetal*-PEG-*acetal*-DOX with high drug loading content for pH-triggered intracellular drug release

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In this study, three PEGylated doxorubicin (DOX) prodrugs with acid-labile acetal and carbamate linkages have been prepared *via* the combination of Cu(I)-catalyzed Huisgen 1, 3-dipolar cycloaddition of alkynes and azides (CuAAC) "click" reaction and ammonolysis reaction. The chemical structures of the prodrugs and the drug contents were characterized by ^{1}H NMR, FT-IR and HPLC analyses. To avoid some side effects caused by the acidic degradation products from conventional hydrophobic polymers, DOX was directly linked to the PEG chain. These prodrugs could self-assemble into micelles in aqueous solution with DOX as the core and PEG chains as the corona. The dissociation of prodrug micelles was confirmed by monitoring the size change as a function of time through DLS analysis. Compared with free DOX, the pH-triggered DOX release of prodrug exhibited a well-controlled and faster release behavior at pH 5.0 than pH 7.4. *In vitro* cytotoxicity tests against HeLa cells by MTT assay demonstrated that these prodrugs displayed the desirable antitumor activity. The intracellular drug release was observed by a live cell imaging system at different DOX dosages. This work provides a strategy for the preparation of a new type of pH-cleavable water-soluble antitumor prodrugs for cancer chemotherapy.

INTRODUCTION

Cancer therapy has become extremely important in recently decades. With the improvement of science and technology, several effective antitumor drugs have been developed. As a widely used chemotherapeutic drug of anthracycline, doxorubicin (DOX) has attracted numerous attentions. However, its application in clinical treatment was seriously limited due to the high toxicity to heart and liver tissues.[1-3] What's more, the poor water-solubility of DOX also lead to a low bioavailability. To overcome these disadvantages, many efforts have been taken to improve the therapeutic efficacy of DOX,[4-9] among which the polymer-drug conjugates would be one of the most promising species.

Since proposed by Ringsdorf in 1975, the polymer-drug conjugates system (or polymeric prodrug) has been recognized and considered to use in cancer therapy.[10-13] Polymeric prodrug is a new kind of therapeutic, whose bioactive agent is not encapsulated but linked to a biocompatible polymer carrier.[14-19] Through conjugating onto the polymer chains, the water-solubility of hydrophobic drugs can be significantly enhanced, which will benefit the pharmacokinetic profile of drugs a lot.[20-23] The polymer carrier possesses the ability to protect the conjugated drug from the degradation process as well as the clearance by kidney and liver, so that it can increase the plasma half-life and biodistribution of anti-tumor drugs.[24-26] Moreover, after the discovery of the enhanced permeability and retention (EPR) effect,[27] various polymer-protein and polymer-drug conjugates have been prepared and studied, which would become the efficient method for cancer treatment.[28]

An ideal prodrug should offer great water-solubility and stability in physiological environment while it can rapidly release the conjugated drugs *via* the cleavage of linker under the microenviornment of tumor tissue. Several natural or synthetic hydrophilic and biocompatible polymers have been used as the carriers. For example, poly[*N*-(2-hydroxypropyl) methacrylamide] (HPMA),[29-31] poly-L-glutamic acid (PGA),[32] polyphosphoesters (PPEs),[33-36] dextran,[37] poly(ethylene glycol) (PEG) and PEGylated copolymers.[38-43] Particularly, as a FDA-proved material, PEG owns excellent biocompatibility, high flexibility and water-solubility.[44,45] Kataoka et al. reported a series of pH-sensitive prodrugs based on PEG-polyaspartate block copolymers with DOX grafted *via* hydrazone bond.[46-48] They studied the DOX release behavior at different pH values and the results showed that the prodrugs exhibited a higher bioavailability than free drugs, while the intracellular delivery of drugs would be more effective for cancer chemotherapy with the enhanced therapeutic efficacy and lower toxicity.

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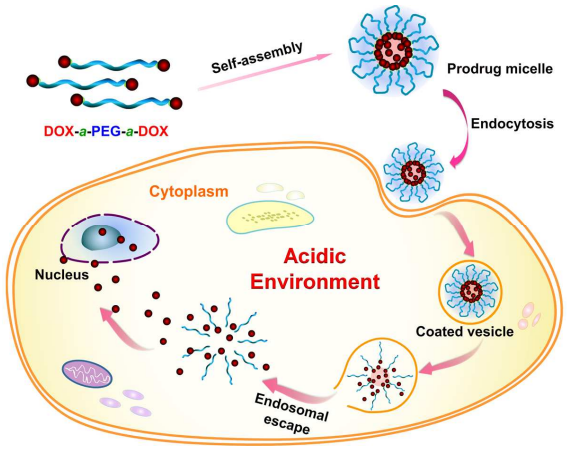


Scheme 1 Schematic illustration of acid-cleavable PEGylated produgs DOX-*acetal*-PEG-*acetal*-DOX and their drug micelles for the pH-triggered intracellular delivery of DOX.

Zhong et al. reported a kind of pH-sensitive PEG-*graft*-DOX prodrugs.[49] They prepared PEG with C=C double bond as the pendent group by anionic ring-opening polymerization of ethylene oxide, and DOX was then conjugated to the pendent *via* hydrazone linkage. The results of pH-dependent *in vitro* drug release and intracellular test showed that this PEGylated prodrug could deliver DOX into nuclei of different tumor cells. And the *in vivo* pharmacokinetics and biodistribution studies indicated this prodrug could be long-circulating and selectively accumulated in tumor tissues. Furthermore, they continued to report another kind of prodrug nanogels PEG-*b*-P(HEMA-*co*-GMA-DOX) which were investigated for triggered intracellular drug release.[50] These prodrug nanogels were sufficiently stable with minimal drug release at neutral pH condition, and fast released DOX under mildly acidic conditions.

However, there are still some challenges in the prodrug systems. For example, most of polymeric prodrugs were prepared by conjugating drugs to hydrophobic polymer chains (polyesters, etc.), and many of these amphiphilic copolymers have been found to own a potential possibility to initiate inflammatory response caused by the acidic degradation product of the hydrophobic domains.[51] Therefore, how to prepare a good water-soluble and biocompatible polymeric prodrug with stimuli-responsive linker and low cytotoxicity by means of efficient synthesis methods is still a focal issue. It is expected that when the prodrug micelles are internalized by tumor tissues, a rapid degradation or disconnection of the linkage between drugs and polymeric carriers would occur and further release the native drugs inside the tumor cells.

Herein, we report on a new kind of acid-cleavable PEGylated polymeric prodrug, namely doxorubicin-*acetal*-poly(ethylene glycol)-*acetal*-doxorubicin (abbreviated as DOX-*a*-PEG-*a*-DOX) *via* the highly efficient Cu(I)-catalyzed Huisgen 1, 3-dipolar cycloaddition of alkynes and azides (CuAAC) "click" chemistry and ammonolysis reaction. The incorporation of these linkages between PEG and DOX was anticipated to enable the cleavage of DOX derivative at the desired site under the tumor-relevant acidic condition, leading to a rapid release of the native DOX. We focus on a water-soluble and acid-cleavable DOX prodrug without any hydrophobic polymer chains, thus making the prodrug avoid some other side effects from the acidic degradation products. As shown in Scheme 1, when the polymeric prodrug micelles were internalized by tumor cells, the acetal and carbamate linkages would be cleaved in endosomal pH conditions and then DOX would be released. With the purpose of investigating the properties of prodrugs, the *in vitro* cytotoxicity, intracellular release of DOX and pH-dependent drug release behavior with free DOX as the control were studied in detail.

EXPERIMENTAL

Materials

Poly(ethylene glycol) (HO-PEG-OH, $\overline{M}_n \approx$ 2000 g mol^{-1}, Sigma-Aldrich; $\overline{M}_n \approx$ 4000 g mol^{-1}, TCI; $\overline{M}_n \approx$ 6000 g mol^{-1}, Sigma-Aldrich), 4-nitrophenyl chloroformate (97%, Alfa Aesar), pyridinium *p*-toluenesulfonate (PPTS, 98%, Acros), *N*, *N*, *N*′, *N*′′, *N*′′-pentamethyldiethylene triamine (PMDETA, 98%, Sigma-Aldrich) and 2-chloroethyl vinyl ether (CEVE, 98%, TCI) were used as received. Propargyl alcohol (98%, Alfa Aesar) and triethylamine (TEA, Sinopharm Chemical Reagent) were distilled under reduced pressure before use. Cuprous bromide (CuBr, 95%, Sinopharm Chemical Reagent) was successively washed three times with glacial acetic acid and acetone, followed by drying for 12 h under vacuum at room temperature. Doxorubicin hydrochloride (DOX·HCl, 99%, Beijing Zhongshuo Pharmaceutical Technology Development) was used as received. Toluene (AR) and dichloromethane (AR) were obtained from Sinopharm Chemical Reagent, dried over CaH_2 and distilled under vacuum before use. *N*, *N*-dimethylformamide (DMF, AR) was dried over anhydrous $MgSO_4$ and distilled before use. All the other reagents and solvents were purchased from Sinopharm Chemical Reagent and used as received. Tetrahydrofuran (THF, AR) was dried over KOH for at least two days and then refluxed over sodium wire with benzophenone as indicator until the colour turned to purple.

Synthesis of N_3-*acetal*-PEG-*acetal*-N_3 (N_3-*a*-PEG-*a*-N_3)

The mixture of HO-PEG-OH ($\overline{M}_n \approx$ 2000 g mol^{-1}, 4.01 g, 2.0 mmol) and PPTS (108 mg, 0.4 mmol) was dried by azeotropic distillation with toluene just before use. 50 mL of anhydrous CH_2Cl_2 was added to the flask after cooling to the room temperature. 2 mL of CEVE (20 mmol) was dissolved into 5 mL of anhydrous CH_2Cl_2, and the solution was added dropwise into the flask at 0 °C over 30 min under a dry nitrogen atmosphere. After stirring for 1 h, 10 mL of 10 wt% Na_2CO_3 aqueous solution was added to the flask to quench the reaction in order to avoid the cleavage of the acetal linkage. Subsequently, the mixture was diluted with 60 mL of CH_2Cl_2

and washed twice with 10 mL of basic brine (pH 10.0). The organic phase was combined and dried over $MgSO_4$ for 2 h. After filtered, the solution was concentrated and then dissolved in a mixture of CH_2Cl_2 (2 mL) and THF (2 mL), followed by precipitated into 100 mL of cold hexane, filtered and dried under vacuum at 30 °C for 24 h to obtain Cl-*acetal*-PEG-*acetal*-Cl (abbreviated as Cl-*a*-PEG-*a*-Cl, 3.15 g, 70.95% yield). ^{1}H NMR (400 MH_Z, $CDCl_3$, 25 °C, ppm, Fig. 1(B)): δ 1.34 (d, 6H, -CH(C***H***$_3$)-), δ 3.82 (t, 4H, -C***H***$_2$Cl), δ 3.85 (t, 4H, -OC***H***$_2$CH$_2$Cl), δ 4.83 (m, 2H, -C***H***(CH$_3$)-), δ 3.43-3.78 attributed to the PEG back bone.

Afterwards, Cl-*a*-PEG-*a*-Cl (2.94 g, 1.31 mmol) and NaN_3 (0.96 g, 14.7 mmol) were added into another 50 mL flask. 10 mL of DMF was added to the flask and the reaction was performed at 60 °C for 40 h. The resultant solution was passed through a basic Al_2O_3 column to remove the residual NaN_3 and diluted with 60 mL CH_2Cl_2, followed by washed twice with basic brine (pH 10.0). The organic phase was combined and dried over $MgSO_4$ for 2 h. After filtered and evaporated under reduced pressure, the solution was precipitated into a mixture of cold hexane (30 mL) and ethyl ether (30 mL). The product was collected by filtration and dried under vacuum for 24 h at 30 °C to produce N_3-*acetal*-PEG-*acetal*-N_3 (abbreviated as N_3-*a*-PEG-*a*-N_3, 1.63 g, 55.3% yield). ^{1}H NMR (400 MH_Z, $CDCl_3$, 25 °C, ppm, Fig. 1(C)): δ 1.34 (d, 6H, -CH(C***H***$_3$)-), δ 3.39 (t, 4H, -C***H***$_2$N$_3$), δ 3.75 (t, 4H, -OC***H***$_2$CH$_2$N$_3$), δ 4.83 (m, 2H, -C***H***(CH$_3$)-), δ 3.43-3.78 attributed to the PEG back bone.

Synthesis of 4-Nitrophenyl Progargyl Carbonate (4-NPC)

4-nitrophenyl chloroformate (2.03 g, 0.01 mol) was added into a 50 mL flask containing 20 mL of anhydrous THF. 0.6 mL of propargyl alcohol (0.02 mol) and 1.6 mL of TEA (0.01 mol) were dissolved in 10 mL of anhydrous THF. The solution was then added dropwise into the flask over 30 min at 0 °C. After stirring for 12 h, 10 mL of 35 wt% NH_4Cl aqueous solution was added into the flask to quench the reaction. After diluted with 80 mL of ethyl acetate, the organic phase was collected and dried over anhydrous Na_2SO_4 for 2 h and filtered. The filtrate was concentrated while the residue was subjected to column chromatography on silica gel with a mixture of hexane and ethyl acetate (v/v, 5/1). The solution was concentrated and dried under vacuum to obtain 4-nitrophenyl progargyl carbonate (abbreviated as 4-NPC, 1.37 g, 66.2% yield). ^{1}H NMR (400 MH_Z, $CDCl_3$, 25 °C, ppm, Fig. 2(A)): δ 2.61 (s, 1H, C***H***≡C-), δ 4.89 (s, 2H, CH≡CC***H***$_2$-), δ 7.41, 8.29 (d, d, 2H, 2H, phenyl).

Synthesis of NC-*acetal*-PEG-*acetal*-NC (NC-*a*-PEG-*a*-NC)

The acid-cleavable NC-*a*-PEG-*a*-NC was synthesized *via* CuAAC "click" reaction between N_3-*a*-PEG-*a*-N_3 and 4-NPC. The detailed synthesis route is described as follows. All the magnetic stirring bars and glassware were dried at 120 °C for 24 h and cooled under vacuum to eliminate the moisture before use. N_3-*a*-PEG_{45}-*a*-N_3 (489 mg, 0.22 mmol) and 4-NPC (255 mg, 1.16 mmol) were dissolved in 25 mL of anhydrous THF in a nitrogen-purged flask. Then, CuBr (14.4 mg, 0.1 mmol) and PMDETA (21 μL, 0.1 mmol) were added sequentially into the flask. After being degassed through three exhausting-refilling nitrogen cycles, the mixture was stirred for 24 h under a nitrogen atmosphere at 25 °C. Subsequently, the mixture was exposed to air to terminate the reaction and concentrated under reduced pressure. The resulting solid was dissolved in a mixed solvent of CH_2Cl_2 and anhydrous ethanol (1/1, *v*/*v*). After being passed through a basic Al_2O_3 column to remove the copper catalyst, the solution was concentrated under reduced pressure and precipitated twice in cold ethyl ether. The precipitates were then filtered and dried under vacuum for 24 h to obtain nitrophenyl carbonate-*acetal*-PEG-*acetal*-nitrophenyl carbonate (abbreviated as NC-*a*-PEG-*a*-NC, 455 mg, 78% yield). ^{1}H NMR (400 MH_Z, $CDCl_3$, 25 °C, ppm, Fig. 2(B)): δ 1.34 (d, 6H, -CH(C***H***$_3$)-), δ 3.86 (t, 4H, -OC***H***$_2$CH$_2$N-), δ 4.56 (t, 4H, -OCH$_2$C***H***$_2$N-), δ 4.83 (m, 2H, -C***H***(CH$_3$)-), δ 5.42 (s, 4H, -CC***H***$_2$O(CO)-), δ 7.38, 8.28 (d, d, 4H, 4H, phenyl), δ 7.86 (S, 2H, -NC***H***=C-),δ 3.43-3.78 attributed to the PEG back bone.

Synthesis of DOX-*a*-PEG-*a*-DOX

The PEGylated DOX prodrug was prepared by the ammonolysis reaction between DOX·HCl and NC-*a*-PEG-*a*-NC. The detailed process is described as follows. All the magnetic stirring bars and glassware used in the experiments were dried at 120 °C for 24 h and cooled under vacuum to eliminate the moisture before use. NC-*a*-PEG-*a*-NC (123 mg, 0.05 mmol), DOX·HCl (119 mg, 0.2 mmol) and TEA (0.1 mL, 0.6 mmol) were dissolved in 10 mL of anhydrous DMF in a nitrogen-purged flask. The mixture was stirred for 48 h under a nitrogen atmosphere at 60 °C. Subsequently, the mixture was concentrated under reduced pressure to obtain the crude product. The solid was then redissolved in 5 mL of anhydrous methanol, dialyzed against anhydrous methanol for 24 h and Milli-Q Water for 48 h in succession. Finally, the prodrug was obtained by lyophilization (DOX-*a*-PEG-*a*-DOX, 78 mg, 60% yield).

Structural Characterizations

^{1}H NMR spectra were recorded on a 400 MHz NMR spectrometer (INVOA-400) at 25 °C with $CDCl_3$ as solvent and tetramethylsilane (TMS) as the internal standard. Fourier transform infrared spectroscopy (FT-IR) spectra were determined with a Nicolet 6700 Fourier transform infrared spectrometer using the KBr disk method.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired on an UltrafleXtreme MALDI TOF mass spectrometer equipped with a 1 kHz smart beam-II laser. The compound *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]-malononitrile (DCTB, Aldrich, >98%) served as the matrix and was prepared in $CHCl_3$ at a concentration of 20 mg mL^{-1}. The cationizing agent sodium trifluoroacetate was prepared in ethanol at a concentration of 10 mg mL^{-1}. The matrix and cationizing salt solutions were mixed in a ratio of 10/1 (*v*/*v*). All samples were dissolved in CH_2Cl_2 at a concentration of 10 mg mL^{-1}. After sample preparation and solvent evaporation, the plate was inserted

into the MALDI mass spectrometer. The attenuation of the laser was adjusted to minimize undesired polymer fragmentation and to maximize the sensitivity. Data analyses were conducted with Bruker's FlexAnalysis software.

Self-assembly Behavior of Prodrug

The morphologies of the aggregates self-assembled from DOX-*a*-PEG-*a*-DOX prodrug were observed on a TEM instrument (HT7700, Hitachi) at 120 kV. The solution with a concentration of 0.1 mg mL^{-1} was prepared by a direct dissolution method. Briefly, 1 mg of prodrug was directly dissolved in 10 mL of Milli-Q water and stirred for 24 h before use. The sample was then prepared by a freeze-drying method. The carbon-coated copper grid was placed on the bottom of a glass cell. Then the glass cell was immediately transferred into liquid nitrogen. Subsequently, 10 μL of the micellar solution was dripped onto the grid, and the solvent in its frozen solid state was directly removed without melting in a freeze-drier. The morphology was then imaged in a normal TEM at room temperature.

The average particle size ($\overline{D}_z$) and size polydispersity indices (size PDIs) of the aggregates were measured at 25 °C using a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern) equipped with a He-Ne laser (633 nm) and 90° collecting optics. All samples were prepared by the same method as described in the TEM analysis and filtered through a Φ = 0.45 μm Millipore microfilter before measurement. The size change of the aggregates in pH 5.0 acetate buffer solution was also measured by DLS. Briefly, 0.2 mL of micelle solution (0.5 mg mL^{-1}) was mixed with 4.8 mL of pH 5.0 acetate buffer solution and stirred at 25 °C. The sizes were determined by DLS at different time intervals.

***In Vitro* Drug Release**

The *in vitro* drug release behavior of the prodrug was studied as following. Typically, 5 mg of DOX-*a*-PEG_{45}-*a*-DOX prodrug was dissolved in 50 mL of Milli-Q water. After stirring for 24 h, 5 mL of the prodrug micellar solution was placed in a dialysis membrane (MWCO 1000) and dialyzed against 30 mL of pH 5.0 acetate buffer solution or pH 7.4 PB buffer solution. At the desired intervals, 5 mL of the release solutions was withdrawn for fluorescence measurements and replaced by 5 mL of fresh buffer solution. The concentration of DOX was determined by fluorescence spectroscopy (FLS920, Edinburgh) with excitation at 480 nm and emission wavelength at 560 nm, with a slit width of 5 nm. The release of free DOX was conducted as a control by using the same method. All the release experiments were carried out in the dark.

To determine the DOX content of the prodrug, 1 mg of DOX-*a*-PEG-*a*-DOX was dissolved in 5 mL of 1 M HCl and stirred for 24 h, the amount of DOX was determined using fluorescence spectrometer. The same method was used to measure the DOX release.

HPLC Analysis

The ratio of loaded DOX and conjugated DOX of prodrugs were determined by reverse phase high performance liquid chromatography (HPLC) (Agilent 1260 infinity series) using a C18 reversed phase column (4.6 × 150 mm, 5 μm) at 30 °C and UV detector was conducted at 254 nm. The mobile phase consisted at acetonitrile (HPLC grade, Sinopharm Chemical Reagent)-water (Mill-Q water) (50/50, *v*/*v*) with a flow rate of 1.0 mL min^{-1}. The data was analyzed by Chem-Station software.

MTT Assay

The *in vitro* cytotoxicity of the prodrugs against human cervical cancer HeLa cells was evaluated by the MTT assay using free DOX as the control. The HeLa cells were obtained from American Type Culture Collection (ATCC) and cultured in 10 % heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin contained culture medium at 37 °C under a 5% CO_2 atmosphere. The cells were seeded in a 96-well plate at a density of about 5×10^4 cells per well for 24 h. The sample solutions with a series of concentrations were added to the wells and incubated for another 24 h. And then, 25 μL of the MTT stock solution (5 mg mL^{-1} in PBS) was added into each well. After incubation for additional 4 h, 150 μL of DMSO was added to dissolve the resulted purple formazan. The optical density (OD) was measured on a microplate reader (Bio rad 680, USA) at 490 nm. The absorbance values were normalized to the wells where cells were not treated with samples and the data are presented as the average values with standard deviations.

Cellular Uptake

The cellular uptake of prodrug micelles by HeLa cells was investigated by the live cell imaging system (Cell'R, Olympus). Cells were cultured in the same medium with MTT assay at 37 °C under a 5% CO_2 atmosphere. The culture medium was removed after 24 h incubation. Cells were washed with pH 7.4 PBS buffer solution and stained with Hoechst 33342 (10 mg L^{-1}). During observation, the cells were incubated with RPMI-1640 culture medium and samples at 37 °C under a 5% CO_2 atmosphere. The images were captured with the excitation wavelength of 340 nm (blue) and 480 nm (red) per 30 min.

RESULTS AND DISCUSSION

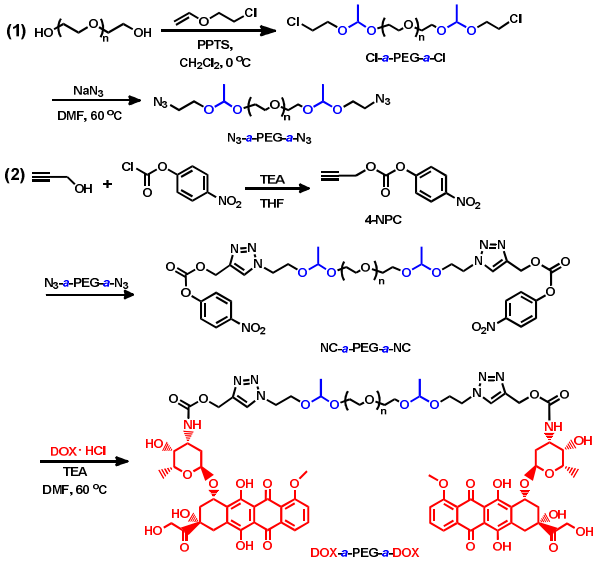
Structural Characterization

As shown in Scheme 2, the PEGylated pH-sensitive prodrug DOX-*a*-PEG-*a*-DOX was synthesized *via* the following steps. Acetal-containing PEG with two terminal azide groups (N_3-*a*-PEG-*a*-N_3) was synthesized according to the previously published method.[52-55] Briefly, HO-PEG-OH was used to react with 2-chloroethyl vinyl ether (CEVE) under the catalysis of pyridinium *p*-toluenesulfonate (PPTS) followed by reacting with NaN_3. Subsequently, propargyl alcohol was used to react with 4-nitrophenyl chloroformate (4-NC) to yield the alkynyl-modified 4-nitrophenyl progargyl carbonate (4-NPC) which was then used to react with N_3-*a*-PEG-*a*-N_3. Finally, DOX was

conjugated to the two ends of the PEG *via* the ammonolysis reaction.

For simplicity, P45, P90 and P135 were used to represent the three polymeric prodrugs DOX-*a*-PEG_{45}-*a*-DOX, DOX-*a*-PEG_{90}-*a*-DOX and DOX-*a*-PEG_{135}-*a*-DOX, respectively. Fig. 1 shows the ^{1}H NMR spectra of HO-PEG_{45}-OH, Cl-*a*-PEG_{45}-*a*-Cl and N_3-*a*-PEG_{45}-*a*-N_3, respectively. Comparing with the original HO-PEG_{45}-OH, the proton signals attributed to acetal methine (peak b) at δ 4.83 ppm and the adjacent methyl (peak c) at δ 1.34 ppm were exhibited in Fig. 1(B), demonstrating the successful introduction of acetal groups. On the other hand, the chemical shift of the methylene group adjacent to the terminal -Cl or -N_3 group moved from δ 3.84 ppm (Fig. 1(B)) to δ 3.38 ppm (Fig. 1(C)), also indicating the successful transfer from chloro groups to azide.

To improve the efficiency of conjugation, alkynyl-modified 4-nitrophenyl progargyl carbonate (4-NPC) was used to react with N_3-*a*-PEG_{45}-*a*-N_3 *via* the highly efficient CuAAC "click" reaction. The ^{1}H NMR spectra of 4-NPC and NC-*a*-PEG_{45}-*a*-NC were shown in Fig. 2. Peak f' (δ 7.88 ppm) attributed to the protons of triazole ring can be clearly detected, while the peak b (δ 4.74 ppm) and peak c (δ 1.27 ppm) attributed to the protons of acetal group can be still found, indicating that the acetal-containing activated PEG had been successfully



Scheme 2 Synthesis of the acid-cleavable DOX-*a*-PEG-*a*-DOX prodrug.

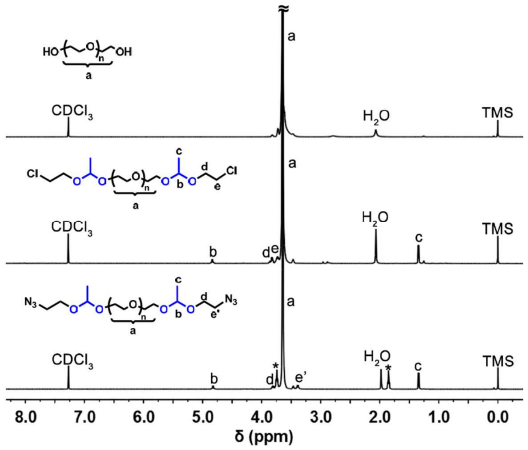


Fig. 1 ^{1}H NMR spectra of (A) HO-PEG_{45}-OH, (B) Cl-*a*-PEG_{45}-*a*-Cl and (C) N_3-*a*-PEG_{45}-*a*-N_3 in $CDCl_3$. The asterisks represent the residual THF.

synthesized *via* the CuAAC reaction. Also, because of the different chemical environment of alkynyl group and triazole ring, the chemical shift of the adjacent methylene shifted from δ 4.89 ppm (peak g) to δ 5.41 ppm (peak g').

FT-IR analysis is a well-known effective method to confirm the appearance of azide group and successful CuAAC "click" reaction. As shown in Fig. 3(C), the absorption peak of azide group at 2105 cm^{-1} corresponding to azide groups can be found, demonstrating the successful modification of the PEG chain. In addition, the FT-IR spectrum of NC-*a*-PEG_{45}-*a*-NC (Fig. 3(E)) shows that the absorption peak of azide group and alkynyl group were totally disappeared while the absorption peak of carbonyl group was maintained after the CuAAC "click" reaction, which further confirmed the successful preparation of NC-*a*-PEG_{45}-*a*-NC.

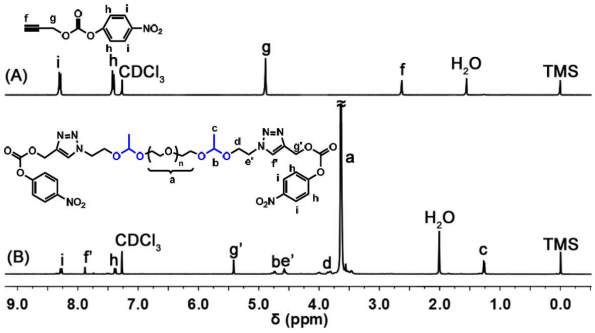


Fig. 2 ^{1}H NMR spectra of (A) 4-NPC and (B) NC-*a*-PEG_{45}-*a*-NC in $CDCl_3$.

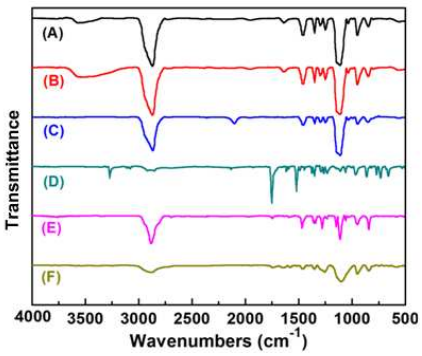


Fig. 3 FT-IR spectra of (A) HO-PEG$_{45}$-OH, (B) Cl-*a*-PEG$_{45}$-*a*-Cl, (C) N_3-*a*-PEG$_{45}$-*a*-N_3, (D) 4-NPC, (E) NC-*a*-PEG$_{45}$-*a*-NC and (F) P45.

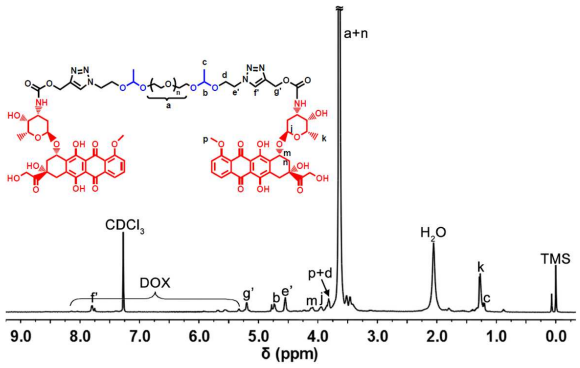


Fig. 4 ^{1}H NMR spectrum of P45 prodrug in $CDCl_3$.

Table 1. DOX loading content, self-assembly properties and IC_{50} values of DOX-*a*-PEG-*a*-DOX prodrug micelles.

Samples	DOX content [a] (Theor. *wt*%)	DOX content [b] (Act. *wt*%)	$\overline{D}_z$ (nm)	Size PDI	IC_{50} (mg DOX equiv. L^{-1})
P45	30.56	37.09	115 ± 3	0.270	0.96
P90	19.52	24.06	157 ± 4	0.286	1.08
P145	14.34	15.65	177 ± 6	0.289	6.36

[a] Theoretical DOX content was calculated based on the molecular weight content of DOX in the prodrug structure.

[b] Actual DOX content was determined by fluorescence test after treating with 1 M HCl.

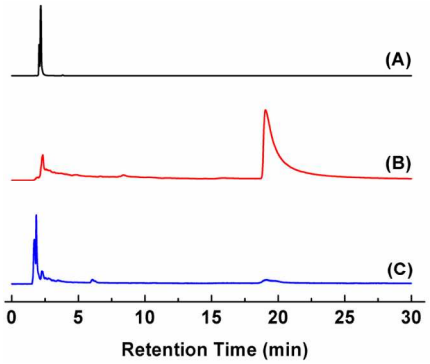


Fig. 5 HPLC spectra of (A) free DOX, (B) P45 and (C) released DOX from P45 prodrug.

The chemical structure of polymeric prodrugs DOX-*a*-PEG-*a*-DOX was confirmed by several methods. Fig. 4 shows the ^{1}H NMR spectrum of P45. We can find that the peak b (δ 4.74 ppm) and peak c (δ 1.27 ppm) asscribed to the proton of acetal group were maintained while the proton peaks of nitrophenyl were disappeared and the proton peaks of DOX were also appeared, indicating that the acetal-containing polymeric prodrug DOX-*a*-PEG-*a*-DOX had been successfully synthesized. In addition, the MALDI-TOF spectra of HO-PEG$_{45}$-OH, NC-*a*-PEG$_{45}$-*a*-NC and P45 were shown in Fig. S1 in ESI†. Compared to the original HO-PEG$_{45}$-OH, the spectrum of NC-*a*-PEG$_{45}$-*a*-NC exhibited a symmetric distribution while the representative peak *m/z* value of 2690.42 g mol^{-1} corresponding to the NC-*a*-PEG$_{45}$-*a*-NC with sodium was in good agree with the calculated mass ([M+Na]$^+$, cal.: 2690.37 g mol^{-1}), indicating the successful synthesis of NC-*a*-PEG$_{45}$-*a*-NC. On the other hand, one could also find the shift between each interval is regular, ca. 44.0, which was in good agreement with the molecular weight of one PEG repeating unit (44.03) in Fig. 1(C), illustrating the successful synthesize of PEGylated prodrugs.

The DOX loading capacity of prodrugs was determined by fluorescence measurement after treating with 1 M HCl for 24 h. As shown in Table 1, with the increasing molecular weight of PEG, the DOX content is decreased, in which P45 exhibits the highest DOX loading content up to 37.09 wt%. This result indicated that this type of prodrug structure could provide high drug contents comparing to the conventional method using polymeric micelles as the carriers.

To compare the theoretical DOX content with the actual DOX content, HPLC was employed to determine the contents of encapsulated DOX and conjugated DOX. Fig. 5(A) and (B) show the HPLC spectra of free DOX and P45, from which one can find that the P45 prodrug possesses a very different retention time comparing to free DOX, indicating the successful conjugation of DOX and PEG. The integral area of main peak attributed to the conjugated DOX was up to 87%, while the value of the peak attributed to the encapsulated DOX was only about 13%. In addition, the major peak in Figure 5(C) represents the released DOX from the *in vitro* release experiment of P45 prodrug after 70 h, which was similar to the retention time with free DOX. This result confirmed the hydrolysis process of acetal group and carbamate groups.

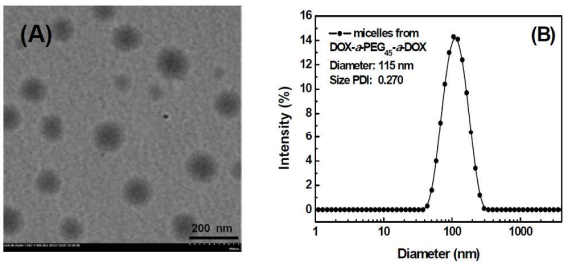


Fig. 6 (A) TEM image of micelles self-assembled from P45 prodrug and (B) the corresponding particle size distribution plot. The concentration was 0.1 mg mL^{-1}.

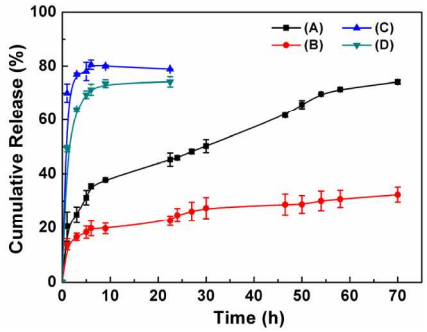


Fig. 7 The pH-dependent release of DOX from P45 prodrug at (A) pH 5.0 and (B) pH 7.4, as well as the release of free DOX at (C) pH 5.0 and (D) pH 7.4.

Self-assembly Behavior of DOX-*α*-PEG-*α*-DOX

Since the prodrug DOX-*α*-PEG-*α*-DOX consists of hydrophilic PEG and hydrophobic DOX, it possesses the possibility of self-assembly into prodrug micelles in aqueous solution with DOX as the core and PEG as the corona. Fig. 6(A) shows the morphologies of assembled P45 prodrug micelles observed by TEM, while the corresponding particle size distribution curve with an average diameter of 115 nm and the size PDI of 0.27 are displayed in Fig. 6(B). The difference of particle size between TEM and DLS could be ascribed to the existence of the hydrophilic PEG shell, which can extend to the aqueous solution in DLS measurement, but difficult to be observed by TEM. From the results in Table 1, one can find that the average particle size of the micelles increased with the increase of molecule weights of PEG, which may due to the larger hydrophilic shell consisted of PEG chains. According to the literatures, an optimum size (<200 nm in diameter) is necessary to minimize the uptake by a reticuloendothelial system (RES) and maintain the advantage of the enhanced permeability and retention (EPR) effect for passive targeting.[56] Therefore, our prodrug micelles could meet the requirement for the particle size and have potential application in biomedicine.

***In vitro* Drug Release**

Considering that DOX was conjugated to PEG *via* the acidic-cleavable linkers, it is predictable that this prodrug may possess a pH-dependent drug release behavior. Thus the *in vitro* release of DOX from P45 prodrug was studied at pH 5.0 and 7.4 respectively, using free DOX as a control. As shown in Fig. 7, compared with the burst release of free DOX, the P45 prodrug exhibits a sustained release process and the controlled release behavior. Moreover, the pH-dependent release was also shown in Fig. 7(A) and (B), from which one can find that there are nearly 75% of DOX was released at pH 5.0 after 70 h, while less than 35 % of DOX was released at pH 7.4 under the same condition. This phenomenon may be attributed to the fast hydrolysis of acetal and carbamate groups, leading to the further dissociation of polymeric prodrug to release native DOX.

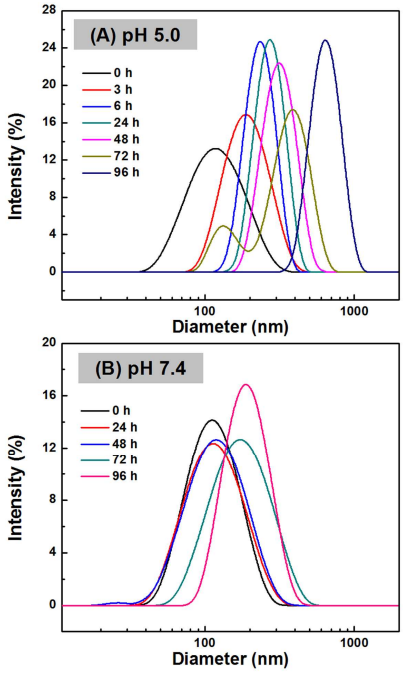


Fig. 8 The particle size change of the P45 prodrug micelles in (A) pH 5.0 and (B) pH 7.4 buffer solutions at 25 °C.

Hydrolysis of DOX-*α*-PEG-*α*-DOX

To investigate the hydrolysis behavior of the pH-sensitive polymeric prodrug under acidic condition, DLS measurement was used to determine the particle size change of micelles with increase of hydrolysis time at pH 5.0 and 7.4. As shown in Fig. 8(A), the particle size increased steadily along with the increasing time, changing from 115 nm within 24 h to 300 nm in 48 h, which may be the consequence of partially cleavage of the acetal and carbamate groups to induce the unfolding of PEG chains. It is noticeable that the particle size increased to over 700 nm in 96 h. The possible reason for this phenomenon is the shedding of PEG chains and aggregation of hydrophobic

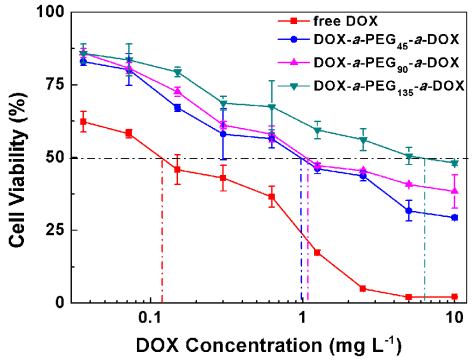


Fig. 9 Cell viability of HeLa cells incubated with polymeric produgs and free DOX at different concentrations for 48 h.

DOX initiated by the complete cleavage of acetal and carbamate linkages. In addition, the protonation of DOX at low pH also made DOX itself more polar which affected the micellar size a lot. However, there is no obvious size change in 48 h at pH 7.4, even in 96 h, the size of nanoparticles was changed a little.

***In vitro* Cytotoxicity**

The *in vitro* cytotoxicity of prodrugs against HeLa cells were investigated by the MTT assays to evaluate their antitumor activities with free DOX as a control. As shown in Fig. 9, the tumor cells which incubated with the three prodrugs exhibit better viabilities than those cells treated with free DOX at the same DOX dosages. On the other hand, with the increasing of molecular weight of PEG chains, the cell viability also increased at the same DOX dosages. These results may be caused by the prolonged release of DOX from prodrug micelles, making the longer time to militate with tumor cells. The IC_{50} values (inhibitory concentration to produce 50% cell death) are shown in Table 1, in which all the prodrugs possess higher IC_{50} values than free DOX (0.12 mg DOX equiv. L^{-1}), which can be observed in many polymeric prodrug systems.[57,58] The reason could be also ascribed to the longer time for DOX to be dissociated and released from prodrugs to tumor cells.

Cellular Uptake

To further investigate the intracellular drug release of the polymeric prodrugs, the live cell imaging system was used to observe the fluorescence intensity of DOX to estimate the release of DOX with free DOX as a control. Fig. 10 shows the fluorescence images of HeLa cells incubated with P45 and free DOX at the DOX concentration of 10 mg L^{-1}. Obviously, the fluorescence intensity of DOX (red) became stronger with the increasing of incubation time, indicating the gradually release of DOX from prodrugs. Furthermore, from Fig. 10(A) we can observe the red fluorescence in the cytoplasm after 3 h of incubation, while the DOX was dispersed into cytoplasm after

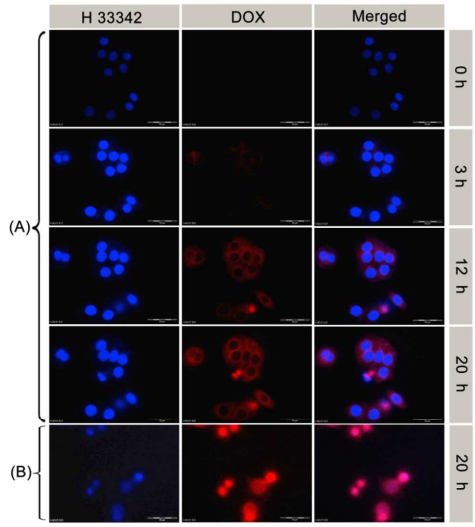


Fig. 10 Fluorescence images of HeLa cells incubated with (A) P45 prodrug and (B) free DOX for different times. The DOX dosage was 10 mg L^{-1}. From left to right: Hoechst 33342 (blue), DOX (red), and overlays of the two images. The scale bars correspond to 50 μm in all images.

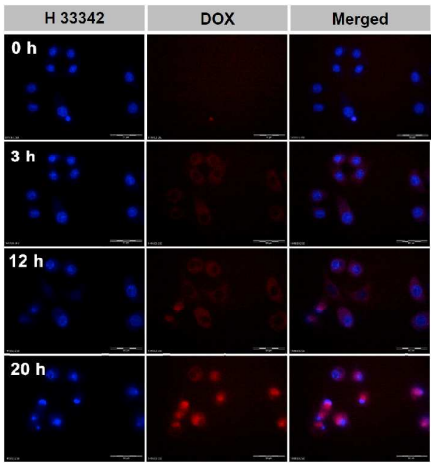


Fig. 11 Fluorescence images of HeLa cells incubated with P45 prodrug for different times.The DOX dosage was 0.2 mg L^{-1}. From left to right: Hoechst 33342 (blue), DOX (red), and overlays of the two images. The scale bars correspond to 50 μm in all images.

12 h of incubation; after 20 h of incubation, several blue fluorescence parts were overlapped by the red fluorescence, demonstrating the successful delivery of DOX into the nuclei. In contrast, HeLa cells incubated with free DOX showed higher fluorescence intensity in the nuclei after 20 h as shown in Fig. 10(B). This phenomenon is consistent with the reports of previously literatures[5,59] and the result of *in vitro* DOX release experiments and the MTT assays, which could be attributed to

the prolonged circulation time of the prodrug micelles and the controlled release behavior. Besides, the cellular uptake of free DOX is a diffusion process, depending on the concentration gradient across the cell membrane of tumor cells, this process would lead to a fast accumulation of DOX in the nuclei at a high concentration condition. These results demonstrated that the polymeric prodrug have several advantages including micellar stability, pH-triggered hydrolysis of acetal and carbamate groups and controlled release of DOX in the cellular environment.

In addition, it is interesting for us to notice that the prodrug micelles could deliver and release DOX to tumor cells at a very low DOX concentration (0.2 mg L^{-1}). As shown in Fig. 11, the regularly enhanced red fluorescence of DOX can still be observed with the incubation time increased. According to the results of our previous reports,[53,55] the cellular uptake of free DOX at the low concentration is not as efficient as this consequence. As stated by Maysinger and Eisenberg,[60,61] the polymeric micelles are internalized into cells *via* an endocytosis process, while a passive diffusion process is taken by free DOX to entered into the cells. Hence, although it is fast for free DOX to enter into cells in the beginning, some free DOX could be pumped out of the cells quickly. However, the micellar DOX prodrugs may not be pumped out of the cells after they entered into the cells based on the endocytosis mechanism.

CONCLUSIONS

In summary, a novel PEGylated pH-sensitive water-soluble DOX prodrug with high DOX loading content has been prepared and evaluated. This new kind of prodrug could self-assemble into micelles with DOX as the core and PEG as the corona. The results of pH-sensitive degradation and pH-dependent *in vitro* drug release indicated that the prodrug possesses an obvious pH-response and sustained release behavior. The consequence of *in vitro* cytotoxicity suggested that this PEGylated prodrug could decrease the cytoxicity of the native drug, while the antitumor ability was maintained. On the other hand, the intracellular drug release observed by a live cell imaging system implied that these prodrug micelles could be internalized into HeLa cells through endocytosis and DOX could be released because of the acidic environment inside the tumor cells. This research provides a new strategy to prepare the pH-responsive polymeric prodrugs with tailor-made structure, good water-solubility and well-controlled drug release property.

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REFERENCES AND NOTES

1 S. Y. Zhou, A. Starkov, M. K. Froberg, R. L. Leino and K. B. Wallace, *Cancer Rev.,* 2001, **61**, 771-777.
2 Y. Shizukuda, S. Matoba, O. Y. Mian, T. Nguyen and P. M. Hwang, *Mol. Cell Biochem.,* 2005, **273**, 25-32.
3 S. V. Kalivendi, E. A. Konorev, S. Cunningham, S. K. Vanamala, E. H. Kaji, J. Joseph and B. Kalyanaraman, *Biochem. J.,* 2005, **389**, 527-539.
4 A. M. Nowicka, A. Kowalczyk, A. Jarzebinska, M. Donten, P. Krysinski and Z. Stojek, *Biomacromolecules, 2013,* **14**, 828-833.
5 M. Prabaharan, J. J. Grailer, S. Pilla, D. A. Steeber and S. Q. Gong, *Biomaterials,* 2009, **30**, 5757-5766.
6 J. A. Johnson, Y. Y. Lu, A. O. Burts, Y. H. Lim, M. G. Finn, J. T. Koberstein, N. J. Turro, D. A. Tirrell and R. H. Grubbs, *J. Am. Soc. Chem.,* 2011, **133**, 559-566.
7 X. J. Chen, S. S. Parelkar, E. Henchey, S. Schneider and T. Emrick, *Bioconjugate Chem.,* 2012, **23**, 1753-1763.
8 J. W. Cui, Y. Yan, Y. J. Wang and F. Caruso, *Adv. Funct. Mater.,* 2012, **22**, 4718-4723.
9 J. Q. Wang, X. R. Sun, W. W. Mao, W. L. Sun, J. B. Tang, M. H. Sui, Y. Q. Shen and Z. W. Gu, *Adv. Mater.,* 2013, **25**, 3670-3676.
10 H. Ringsdorf, *J. Polym. Sci.: Polym. Symp.,* 1975, **51**,135-153.
11 J. Khandare and T. Minko, *Prog. Polym. Sci.,* 2006, **31**, 359-397.
12 K. Ulbrich and V. Šubr, *Adv. Drug Delivery Rev.,* 2010, **62**, 150-166.
13 X. L. Hu, Z. G. Xie, Y. B. Huang and X. B. Jing, *Acta Polym. Sin.,* 2013, **6**, 733-749.
14 R. Duncan, *Nat. Rev. Drug Discov.,* 2003, **2**, 347-360.
15 R. Duncan, *Nat. Rev. Cancer,* 2006, ***6***, 688-701.
16 R. Haag, F. Kratz, *Angew. Chem. Int. Ed.,* 2006, **45**, 1198-1215.
17 R. Duncan and M. J. Vicent, *Adv. Drug Delivery. Rev.,* 2010, **62**, 272-282.
18 Z. G. Xu, K. L. Zhang, C. L. Hou, D. D. Wang, X. Y. Liu, X. J. Guan, X. Y. Zhang and H. X. Zhang, *J. Mater. Chem. B*, 2014, **2**, 3433-3437.
19 J. W. Li, Y. X. Zhou, C. W. Li, D. S. Wang, Y. J. Gao, C. Zhang, L. Zhao, Y. S. Li, Y. Liu and X. R. Li, *Bioconjugate Chem.*, 2015, **26**, 110-119.
20 G. Pasut and F. M. Veronese, *Prog. Polym. Sci.,* 2007, **32**, 933-961.
21 A. Nori and J. Kopeček, *Adv. Drug Delivery Rev.,* 2005, **57**, 609-636.
22 Y. S. Lin, R. Tungpradit, S. Sinchaikul, F. M. An. D. Z. Liu, S. Phutrakul and S. T. Chen, *J. Med. Chem.,* 2008, **51**, 7428-7441.
23 Y. D. Gu, Y. N. Zhong, F. H. Meng, R. Cheng, C. Deng and Z. Y. Zhong, *Biomacromolecules,* 2013, **14**, 2772-2780.
24 A. V. Yurkovetskiy and R. J. Fram, *Adv. Drug Delivery Rev.,* 2009, **61**, 1193-1202.
25 C. Li and S. Wallace, *Adv. Drug Delivery Rev.,* 2008, **60**, 886-898.
26 X. L. Hu and X. B. Jing, *Expert Opin. Drug Delivery,* 2009, **6**, 1079-1090.

27 Y. Matsumura and H. Maeda, *Cancer Res.,* 1986, **46**, 6387-6392.
28 M. J. Vicent and R. Duncan, *Trends Biotechnol.,* 2006, **24**, 39-47.
29 J. Callahan, P. Kopečkováand J. Kopeček, *Biomacromolecules,* 2009, **10**, 1704-1714.
30 M. Talelli, K. Morita, C. J. F. Rijcken, R. W. M. Aben, T. Lammers, H. W. Scheeren, C. F. van Nostrum, G. Storm and W. E. Hennink, *Bioconjugate Chem.,* 2011, **22**, 2519-2530.
31 R. Satchi-Fainaro, H. Hailu, J. W. Davies, C. Summerford and R. Duncan, *Bioconjugate Chem.,* 2003, **14**, 797-804.
32 S. D. Chipman, F. B. Oldham, G. Pezzoni and J. W. Singer, *Int. J. Nanomed.,* 2006, **1**, 375-383.
33 J. Z. Du, X. J. Du, C. Q. Mao and J. Wang, *J. Am. Chem. Soc.,* 2011, **133**, 17560-17563.
34 C. Y. Sun, S. Dou, J. Z. Du, X. Z. Yang, Y. P. Li, J and Wang, *Adv. Healthcare Mater.,* 2014, **3**, 261-272.
35 J. Zou, F. W. Zhang, S. Y. Zhang, S. F. Pollack, M. Elsabahy, J. W. Fan and K. L. Wooley, *Adv. Healthcare Mater.,* 2014, **3**, 441-448.
36 G. Y. Zhang, M. Z. Zhang, J. L. He and P. H. Ni, *Polym. Chem.,* 2013, **4**, 4515-4525.
37 R. Mehvar, *J. Controlled Release,* 2000, **69**, 1-25.
38 X. F. Zhng, Y. X. Li, X. S. Chen, X. H. Wang, X. Y. Xu, Q. Z. Liang, J. L. Hu and X. B. Jing, *Biomaterials,* 2005, **26**, 2121-2128.
39 R. B. Green, *J. Controlled Release,* 2001, **74**, 159-171.
40 H. S. Yoo and T. G. Park, *J. Controlled Release,* 2001, **70**, 63-70.
41 H. S. Yoo, E. A. Lee and. G. Park, *J. Controlled Release,* 2002, **82**, 17-27.
42 H. S. Yoo and T. G. Park, *J. Controlled Release,* 2004, **96**, 273-283.
43 Z. G. Xie, H. L. Guan, X. S. Chen, C. H. Lu, L. Chen, X. L. Hu, Q. Shi and X. B. Jing, *J. Controlled Release,* 2007, **117**, 210-216.
44 M. J. Hwang, J. M. Suh, Y. H. Bae, S. W. Kim and B. Jeong, *Biomacromolecules,* 2005, **6**, 885-890.
45 Y. Zhang and R. X. Zhuo, *Biomaterials,* 2005, 26, 6736-6742.
46 Y. Bae, S. Fukushima, A. Harada and K. Kataoka, *Angew. Chem. Int. Ed.,* 2003, **42**, 4640-4643.
47 Y. Bae, N. Nishiyama, S. Fukushima, H. Koyama, M. Yasuhiro and K. Kataoka, *Bioconjugate Chem.,* 2005, **16**, 122-130.
48 Y. Bae, N. Nishiyama and K. Kataoka, *Bioconjugate Chem.,* 2007, **18**, 1131-1139.
49 L. Zhou, R. Chen, H. Q. Tao, S. B. Ma, W. W. Guo, F. H. Meng, H. Y. Liu, Z. Liu and Z. Y. Zhong, *Biomacromolecules,* 2011, **12**, 1460-1467.
50 F. X. Zhan, W. Chen, Z. J. Wang, W. T. Lu, R. Cheng, C. Deng, F. H. Meng , H. Y. Liu and Z. Y. Zhong, *Biomacromolesules,* 2011, **12**, 3612-3620.
51 M. Hakkarainen, A. Höglund, K. Odelius and A. C. Albertsson, *J. Am. Soc. Chem.,* 2007, **129**, 6308-6312.
52 K. Satoh, J. E. Poelma, L. M. Campos, B. Stahl, C. J and Hawker, *Polym. Chem.,* 2012, **3**, 1890-1898.
53 H. R. Wang, J. L. He, M. Z. Zhang, Y. F. Tao, F. Li. K. C. Tam and P. H. Ni, *J. Mater. Chem. B,* 2013, **1**, 6596-6607.
54 Y. Zhang, J. L. He, D. L. Cao, M. Z. Zhang and P. H. Ni, *Polym. Chem.,* 2014, **5**, 5124-5138.
55 J. Hu, J. L. He, M. Z. Zhang and P. H. Ni, *Polym. Chem.,* 2015, **6**, 1533-1556.
56 G. Gaucher, M. H. Dufresne, V. P. Sant, N. Kang, D. Maysinger and J. C. Leroux, *J. Controlled Release,* 2005, **109**, 169-188.
57 K. Ulbrich, T. Etrych, P. Chytil, M. Jelínkováand B. Říhová, *J. Controlled Release,* 2003, **87**, 33-47.
58 V. Cuchelkar, P. Kopečková and J. Kopeček, *Macromol. Biosci.,* 2008, **8**, 375-383.
59 Y. Jin, L. Song, Y. Su, L. J. Zhu, Y. Pang, F. Qiu, G. S. Tong, D. Y. Yan, B. S. Zhu and X. Y. Zhu, *Biomacromolecules,* 2011, **12**, 3460-3468.
60 L. B. Luo, J. Tam, D. Maysinger and A. Eisenberg, *Bioconjugate Chem.*, 2002, **13**, 1259-1265.
61 R. Savić, L. Luo, A. Eisenberg and D. Maysinger, *Science*, 2003, **300**, 615-618.

Synthesis of acid-labile polymeric prodrug DOX-*acetal*-PEG-*acetal*-DOX with high drug loading content for pH-triggered intracellular drug release

PEGylated doxorubicin (DOX) prodrugs with acid-labile acetal and carbamate linkages have been prepared *via* the combination of CuAAC "click" reaction and ester-amide exchange reaction. The chemical structures, self-assembly behavior, hydrolysis under acidic environment, pH-triggered DOX release of prodrug were investigated in detail. The intracellular drug release was investigated by a live cell imaging system, demonstrating that the polymeric prodrug could deliver native drug into HeLa cells. This work provides a facile strategy for the preparation of a new type of water-soluble antitumor prodrugs for cancer chemotherapy.