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Acetal-linked PEGylated paclitaxel prodrugs forming free-paclitaxel-loaded pH-responsive micelles with high drug loading capacity and improved drug delivery

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ABSTRACT

Endosomal pH-responsive micellar nanoparticles were prepared by self-assembly of an amphiphilic poly(ethylene glycol)-acetal-paclitaxel (PEG-acetal-PTX) prodrug, and free PTX could be encapsulated in the hydrophobic core of the nanoparticles. These nanoparticles exhibited excellent storage stability for over 6 months under normal conditions, but disassembled quickly in response to faintly acidic environment. Incorporating physical encapsulation and chemical conjugation, the PTX concentration in the nanoparticles solution could reach as high as 3665 μg/mL, accompanying with a high drug loading capacity of 60.3%. Additionally, benefitting from the difference in drug release mechanism and rate between encapsulated PTX and conjugated PTX, a programmed drug release behavior was observed, which may result in higher intracellular drug concentration and longer action time. CCK-8 assays showed that the nanoparticles demonstrated superior antitumor activity than free PTX against both HeLa and MDA-MB-231 cells. These prodrug-based nanomedicines have a great potential in developing translational PTX formulations for cancer therapy.

1. Introduction

Over the past decades, nanomedicines based on polymers have been intensively studied for cancer therapy, regarding their potential to increase drug solubility, enhance therapeutic effect and reduce side effects [1–8]. Comparing to small molecular anticancer drugs, nanomedicines have shown many advantages including prolonged circulation time by evading glomerular filtration, improved pharmacokinetic properties, as well as enhanced tumor accumulation *via* the enhanced permeation and retention (EPR) effect [9–14]. Among various nanomedicines such as polymeric nanoparticles, prodrugs, micelles, vesicles, nanogels and liposomes [15–23], prodrug-based nanoparticles have drawn much more attention due to the clear and simple structure and great potential in clinical translation.

Paclitaxel (PTX), a common chemotherapeutic agent isolated from *Taxus brevifolia*, shows conspicuous anticancer activity against various cancers, including ovarian, lung, and breast cancer [24–27]. On account of its extremely poor water solubility (< 2 μg/mL), the current clinical formulation of PTX (Taxol) is blended with 1:1 ratio of Cremophpr EL (polyoxyethylenated castor oil) and ethanol to improve the solubility [28]. However, Taxol has many limitations such as non-selective cytotoxicity, rapid systemic clearance, and worst of all, Cremophpr EL has caused various side effects, including hypersensitivity, neurotoxicity and nephrotoxicity [29–31]. Therefore, development of alternative PTX formulations with enhanced selective cytotoxicity and less side effects is desired.

Recently, an increasing number of nanocarriers have been developed to deliver PTX *via* physical entrapment or chemical conjugation [32–39]. Wooley and coworkers reported polyphosphoester-based shell cross-linked nanoparticles for PTX delivery, which effectively achieve high concentration of PTX and demonstrate great potential in treatment of osteosarcoma lung metastases [32]. Nevertheless, the tedious synthetic process and undefined structure of the nanocarriers as well as inevitable drug leakage make them quite challengeable for clinical translation. Conjugated PTX with polymers to form polymer-PTX prodrugs such as poly(ethylene glycol)-PTX (PEG-PTX) [40–42], poly(L-glutamic acid)-PTX (PG-PTX) [43–45], poly(*N*-(2-hydroxypropyl)-methacrylamide)-PTX (PHPMA-PTX) [46] have also been reported. Among them, PEG-PTX has been particularly studied since PEG is one of the most widely used hydrophilic polymer approved by FDA with negligible

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toxicity and immunogenicity [40,47]. PEGylation of PTX creates amphiphilic prodrugs which can self-assemble into micellar nanoparticles. The resulted PEG-PTX nanoparticles not only possess improved drug solubility and promoted plasma residence time, but also can accumulate in tumor tissues *via* EPR effect [41]. However, the reported PEG-PTX prodrugs present some inherent drawbacks, including relatively low drug loading capacity (DLC) and incomplete drug release [48,49].

To ensure successful intracellular drug release, it is important that the linkers between drugs and polymers must be cleavable in the tumor-cell environment. Due to the abnormal conditions such as higher glutathione concentration and lower pH in tumor cells, redox-sensitive disulfide and acid-labile acetal bonds have been employed to fabricate stimuli-responsive polymer-PTX prodrugs [50–54]. Chen et al. reported a disulfide-containing polymer-PTX conjugate, which demonstrated superior antitumor activity over both free PTX and a similar polymer-PTX conjugate without disulfide bonds [48]. Wang et al. reported PTX-loaded PEG-disulfide-PTX conjugate nanoparticles, showing excellent DLC and higher antitumor activity compared to their nonresponsive counterparts [51]. Zhang et al. synthesized a D-*α*-tocopherol polyethylene glycol succinate based redox-sensitive PTX prodrug, which exhibited significant tumor growth inhibition to multidrug resistance tumors [55]. However, in these systems the disulfide linker wasn't conjugated directly to PTX but *via* an ester bond, thus it was doubted that whether the PTX could be released intactly. PTX has hydroxyls which can react with vinyl ether to form acetal linkers and the acid-responsive degradation of acetal will release intact PTX, therefore, acetal linker seems to be a better choice for fabricating stimuli-responsive polymer-PTX prodrug. Zhong et al. prepared PTX prodrug micellar nanoparticles by conjugating PTX onto water-soluble poly (ethylene glycol)-*b*-poly(acrylic acid) block copolymers *via* an acid-labile acetal bond, which displayed high antitumor effect to various tumor cells [53].

DLC and drug release kinetics are of great importance to drug delivery systems. Higher DLC not only means lower cost since less carrier materials are needed, but also signifies lower risk of toxic side effect. For this reason, profuse efforts have been made to explore novel nanocarriers with high DLC [56–58]. Drug release kinetics associates with intracellular drug concentration and the time of drug action. To maximum tumor growth inhibition, it is necessary to keep effective drug concentration for a time period as long as possible. It was reported that incorporating of encapsulated PTX and conjugated PTX in one drug delivery system would achieve programmed drug release, which meant the rapid release of encapsulated PTX could enhance intracellular drug concentration within a short time, and the later released conjugated PTX could continue the treatment over a longer period, resulting in improved therapeutic effect [51].

In this study, we designed and synthesized an amphiphilic polymer-drug conjugate, poly(ethylene glycol)-acetal-paclitaxel (PEG-acetal-PTX, PAP), which could self-assemble into acid-labile micellar nanoparticles. The resulted nanoparticles could serve as drug carriers to encapsulate free PTX. Therefore, we prepared three kind of nanoparticles, which been named as PAP NPs, PAP + 20%PTX NPs, and PAP + 40%PTX NPs, respectively, according to the ratio of free PTX and PAP. These nanoparticles possessed excellent storage stability, ascribing to the low critical aggregation concentration (CAC) of PAP and protection of the outer PEG corona. Benefitting from the combination of physical encapsulation and chemical conjugation, the nanoparticles not only achieved very high DLC and PTX concentration, but also exhibited a programed drug release behavior. As illustrated in Fig. 1, the nanoparticles penetrated into tumor tissues *via* EPR effect, then they were internalized by tumor cells *via* endocytosis. Under the acidic environment of intracellular endosomal/lysosomal compartments, pH-triggered cleavage of acetal bonds led to disassembly of nanoparticles, resulting in rapid release of encapsulated PTX, subsequently the previously hidden acetal bonds were exposed to the acidic environment and finally the conjugated PTX released completely. Here, preparation of PAP prodrugs and PTX-loaded nanoparticles, pH-responsive drug release, cellular uptake and *in vitro* antitumor activity against HeLa and MDA-MB-231 cancer cells of the nanoparticles were investigated.

2. Materials and methods

2.1. Materials

2-Hydroxyethyl vinyl ether (98%, Energy Chemical), acryloyl chloride (97%, J & K), pyridinium *p*-toluenesulfonate (PPTS, 98%, Energy Chemical) and paclitaxel (PTX, 99%, Xi'an Realin Biotechnology Co., Ltd.) were used as received. Thiol-poly(ethylene glycol) (PEG-SH) was synthesized according to methods given in our previous literatures [59,60]. Dichloromethane (DCM) and triethylamine (TEA) (reagent grade, Beijing Chemical Works) were purified by stirring over calcium hydride for 24 h followed by distillation. Other solvents and compounds were purchased from Beijing Chemical Works and used without further purification.

2.2. Characterizations

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 400 MHz spectrometer and acetone-d_6 was used as solvent. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) measurements were carried out using a Bruker BIFLEX III equipped with a 337 nm nitrogen laser. Transmission electron microscopy (TEM) images were obtained on a JEM-2200FS microscope (JEOL, Japan) after the samples were negatively stained by sodium phosphotungstate. Generally, a 3 μL droplet of nanoparticles solution with a concentration of 1 mg/mL was dropped onto a copper grid (300 mesh) coated with a carbon film, then the excess liquid was wicked off and the grids were immediately placed onto individual droplets of freshly prepared and filtered, 2 wt% aqueous sodium phosphotungstate. After 2 min, excess stain was removed and the grids were allowed to dry thoroughly. Size distribution and zeta potentials of the nanoparticles were characterized by dynamic light scattering (DLS) using a zetasizer (Nano-ZS, Malvern, UK) with a 632.8 nm laser light set at a scattering angle of 173°. Each measurement was performed in triplicate, and the results were processed with Zetasizer Software version 7.02. Fluorescence spectra were recorded on a Hitachi F4600 photoluminescent spectrometer with a xenon lamp as a light source. Confocal laser scanning microscopy (CLSM) images were obtained on a Zeiss LSM 510 microscope. The concentration of PTX was measured by high performance liquid chromatography (HPLC) on a Shimadzu LC-20AT system with UV detection at 227 nm. A mixture of acetonitrile and water at 1/1 (v/v) was used as mobile phase at a flow rate of 1.0 mL/min.

2.3. Synthesis of PAP prodrugs

PAP prodrugs were prepared in three steps as shown in Fig. 2. Briefly, to a solution of 2-hydroxyethyl vinyl ether (2.64 g, 30 mmol) and TEA (3.33 g, 33 mmol) in 50 mL of dry DCM at 0 °C, acryloyl chloride (3 g, 33 mmol) was added dropwise. The mixture was then stirred at room temperature overnight. The precipitate was filtered and the filtrate was washed three times with water, then the organic layer was dried with $MgSO_4$ and evaporated under reduced pressure to obtain 2-acryloyloxyethyl vinyl ether as a colorless liquid with 94% yield.

Under a nitrogen atmosphere, to a dry DCM solution of PTX (1.71 g, 2 mmol) and PPTS (152 mg, 0.6 mmol) at 0 °C, 2-acryloyloxyethyl vinyl ether (852 mg, 6 mmol) in dry DCM was added dropwise. After being stirred at room temperature for 24 h, the reaction was quenched by adding K_2CO_3. The mixture was filtered through a pad of celite and the filtrate was evaporated under reduced pressure to obtain crude product, which was purified by silica gel chromatography using DCM/ethyl acetate (v/v = 50/1) containing 1% (v/v) TEA as eluent to obtain PTX-