



pH-responsive polymeric micelles based on poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) for tumor-targeting and controlled delivery of doxorubicin and P-glycoprotein inhibitor



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ABSTRACT

The combination of a chemotherapeutic drug with a P-glycoprotein (P-gp) inhibitor has emerged as a promising strategy for treating multidrug resistance (MDR) cancer. To ensure that two drugs can be co-delivered to the tumor region and quickly released in tumor cells, tumor-targeted and pH-sensitive polymeric micelles were designed and prepared by combining cationic ring-opening polymerization of 2-ethyl-2-oxazoline (EOz) with anionic ring-opening polymerization of D,L-lactide (LA), and then encapsulating doxorubicin (DOX) and D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS1000) into the micelles self-assembled by poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) (PEOz-PLA) and DSPE-PEG-folate. PEOz-PLA exhibited a low critical micelle concentration and negligible cytotoxicity. The micelles enabled the rapid release of DOX when pH decreased from 7.4 to 5.0. The targeting ability of the micelles was demonstrated by *in vitro* flow cytometry in KBv cells and *in vivo* real time near-infrared fluorescence imaging in KBv tumor-bearing nude mice. The efficiency of MDR reversion for the micelles was testified by enhancement of intracellular DOX accumulation and cytotoxicity. The efficient drug delivery by the micelles was attributed to synergistic effects of folate-mediated targeting, pH-triggered drug release and TPGS1000-aroused P-gp inhibition. Therefore, the designed multifunctional polymeric micelles may have significant promise for therapeutic application of MDR cancer.

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1. Introduction

The resistance of cancer cells to multiple structurally unrelated chemotherapeutic drugs termed “multidrug resistance (MDR)” is one of the main obstacles to successful cancer chemotherapy and limits the effectiveness of anticancer drugs [1]. Over-expression of drug efflux transporter P-glycoprotein (P-gp) in tumor cells has been recognized as one of the key factors resulting in the development of tumor MDR [2,3]. Therefore, alleviating the drug efflux mediated by P-gp may be a promising method to overcome MDR. The combination of a chemotherapeutic drug with a MDR modulator has been proved to be a promising strategy for treating MDR cancer. Unfortunately, the difference in pharmacokinetics, biodistribution and membrane transport ability for the two drug molecules makes dosing and scheduling optimization extremely difficult. Many nanotechnology strategies have emerged to circumvent P-gp-based MDR, in which nanocarrier systems co-

encapsulating anticancer agent and P-gp inhibitor [4–8] are prominent approaches with advantages of high therapeutic effectiveness and minimal side effects of cancer chemotherapy. Among them, polymeric micelles have presented their great potential in reversion of MDR in recent years due to their good biocompatibility, high stability *in vitro* and *in vivo*, small size and convenient decoration with tumor-specific targeting ligands or antibodies [4,9].

Notably, an important concern is that the difference in physico-chemical properties of the two drugs may result in a difference in their release behavior from micelles, especially at tumor site and in tumor cells. Consequently, to guarantee the synergistic delivery of the two drugs to the tumor site and sufficient drug concentration for optimal synergy and to reduce side effects, nanocarriers are required to remain stable in the blood circulation and quickly release drugs into tumor cells. This might be achieved by using a targeted polymeric micelle system with a triggered release mechanism which enables the nanocarriers to release their cargos in response to the stimuli of tumor extracellular matrix or intracellular compartment, such as temperature, pH, or enzymes [10,11]. pH-responsive polymeric micelles appeared to be the most

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attractive candidate due to the intrinsic difference between various solid tumors and the surrounding normal tissues in their relative acidity. Typical extracellular pH (pHe) ranges from 7.2 to 6.5 in both tumor xenograft animal models and in clinical tumors [12]. This acidic pH is thought to be a tumor phenotype caused by anaerobic respiration and subsequent glycolysis [12,13]. The low pH in the tumor extracellular matrix provides a tissue-specific stimulus that may be exploited for targeting applications. Moreover, polymeric micelles are typically taken up by targeted cells via endocytosis and trafficked through the endosomal-lysosomal pathway. The pH value of endo/lysosomal vesicles was gradually decreased to 5.5–6.5 and 4.5–5.0 because protons were pumped into the vesicles [10]. These prompted us to design tumor specific ligand-modified pH-responsive polymeric micelles to co-deliver the anticancer drug and P-gp inhibitor to tumor sites by relying on enhanced permeation and retention (EPR) and receptor recognizing effect, and control their release by the pH gradient subjected by pH-responsive polymeric micelles in their endocytic pathway.

Up to now, in the published reports on pH-responsive polymeric micelles, pH-responsive polyacids or polybases were generally used as hydrophobic building blocks for polymeric micelles that convey pH-sensitivity to drug release. For example, protonation of a polybase poly(L-histidine) being used frequently in the hydrophobic core of mixed PEG-poly(L-histidine)/PEG-poly(L-lactic acid) micelles in the tumor cells results in destabilization of micelle cores and expedient drug release [14]. But pH-sensitive micelles based on amphiphilic copolymers with pH-responsive hydrophilic blocks have rarely been reported. Poly(2-ethyl-2-oxazoline) (PEOz) has been shown to be a pH-sensitive polymer with a favorable pK_a value and low toxicity [15]. It can be ionized at endo/lysosomal pH. Hence, in this work, PEOz was selected as a functional hydrophilic segment, and pH-responsive diblock copolymer poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) (PEOz-PLA) was synthesized to fabricate multifunctional mixed micelles with DSPE-PEG-folate to concurrently deliver doxorubicin (DOX) and TPGS1000, a potent P-gp inhibitor [16], to the tumor region, and to ensure their quick release in tumor cells for reversion of tumor MDR. We hypothesized that our designed multifunctional micelles may show the combined and synergistic effects of folate-mediated targeting, pH-triggered drug release and TPGS1000-aroused P-gp inhibition to induce an improved anticancer efficacy.

2. Materials and methods

2.1. Materials

D,L-Lactide obtained from Daigang Biological Technology Co. Ltd. (Jinan, China) was purified by recrystallization three times from ethyl acetate before use. 2-Ethyl-2-oxazoline and methyl *p*-toluenesulfonate supplied by Sigma–Aldrich (St. Louis, MO, USA) were dried by vacuum distillation over calcium hydride. Acetonitrile and toluene purchased from Beijing Chemical Works (Beijing, China) were dried over CaH_2 and distilled. Stannous octoate obtained from Sigma (St. Louis, MO, USA), D- α -tocopherol polyethylene glycol 1000 succinate (TPGS1000) obtained from Sigma (St. Louis, MO, USA) and DSPE-PEG5000-folate obtained from Sunlights Technology Ltd. (NANOCS, USA) were used as received. mPEG5000-PLA5000 was synthesized by our laboratory as reported previously [17]. Sulforhodamine B sodium salt (SRB) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Doxorubicin-hydrochloride (DOX HCl) was provided by the Haizheng Pharmacy (Zhejiang, China). Bis Benzimide Hoechst 33258 was obtained from Biodee Biotechnology Co. Ltd. (Beijing, China). Near-infrared lipophilic carbocyanine dye 1, 10-dioctadecyltetramethyl indotricarbocyanine iodide (DiR) was purchased from Biotium, Inc. (Hayward, CA, USA). RPMI 1640 medium, Penicillin–

Streptomycin 100 \times and trypsin–EDTA were all obtained from MAC Gene Technology (Beijing, China). Two kinds of fetal bovine serum (FBS) were purchased from GIBCO and HYCLONE. (Ontario, USA). All other chemicals and reagents were of analytical grade or better.

25 cm² and 75 cm² plastic culture flasks, 12-well and 96-well tissue culture plates were obtained from Costar (Corning Incorporated, USA).

2.2. Synthesis and characterization of PEOz-PLA copolymer

PEOz-PLA diblock copolymer was prepared using a two-step reaction procedure as reported previously by our group [18]. Monohydroxyl poly(2-ethyl-2-oxazoline) (PEOz-OH) was first synthesized by cationic ring-opening polymerization of 2-ethyl-2-oxazoline (EOz) using methyl *p*-toluenesulfonate (MeOTs) as an initiator. The resulting PEOz-OH was subsequently polymerized with D,L-lactide to synthesize PEOz-PLA copolymers. The obtained PEOz-OH and PEOz-PLA were dissolved in $CDCl_3$ and then ¹H NMR spectra were recorded on a Bruker MSL2300 spectrometer (400 MHz, Germany) using tetramethylsilane (TMS) as an internal reference at room temperature. The molecular weight and molecular weight distribution of the products were measured by gel permeation chromatography (GPC, Spectra System P100) with a refractive index detector (RefractoMonitor IV) by using polystyrene as standards.

2.3. Determination of critical micelle concentration of PEOz-PLA

The critical micelle concentration (CMC) of PEOz-PLA copolymer was determined by a fluorescence technique with pyrene as a hydrophobic probe as previously described [19].

2.4. Determination of pK_a of PEOz-OH and PEOz-PLA

The pK_a of PEOz-OH and PEOz-PLA was determined by acid-base titration with NaOH [20]. Briefly, the polymer was first dissolved in HCl aqueous solution (0.01 mol/L, 20 mL) at a concentration of 10 mg/mL. The resulting polymer solution was titrated against NaOH aqueous solution (0.02 mol/L) with constant stirring, and changes in pH values following incremental addition of NaOH were monitored using a pH meter (Thermo Orino). The pH values were plotted versus the NaOH volume added to the polymer solution. The pK_a of the polymer corresponds to the pH value at which the ionization degree was 0.5.

2.5. Preparation of polymeric micelles

DOX-loaded PEOz-PLA polymeric micelles (denoted as DOX-PP-PM, as described in Table 1) were prepared by the dialysis method. DOX-HCl was first neutralized with a quintuple mol equiv of triethylamine in DMSO under stirring overnight in the dark [21]. DOX solution (2 mg/mL, 1 mL) in DMSO was then added to PEOz-PLA solution (5 mg/mL, 4 mL) in DMSO, and the mixture was then dialyzed using a dialysis membrane (MWCO 3500, Spectrum Laboratories) against 1 L of deionized water, which was replaced every 3 h in the course over 12 h, followed by filtration through a 0.45 μ m filter and lyophilization for storage.

DOX-loaded PEOz-PLA/DSPE-PEG-FA mixed polymeric micelles (denoted as DOX-PP/FA-PM, as shown in Table 1) were prepared by the dialysis method as described above except that PEOz-PLA was replaced by a mixture of PEOz-PLA and DSPE-PEG-FA in the mass ratio of 10:1.

DOX/TPGS1000-loaded PEOz-PLA polymeric micelles (denoted as DOX-PP/T-PM, as described in Table 1) and DOX/TPGS1000-loaded PEOz-PLA/DSPE-PEG-FA mixed polymeric micelles (denoted

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