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Preparation of a multifunctional verapamil-loaded nano-carrier based on a self-assembling PEGylated prodrug

Dongping Zhao [a], Na Liu [a], Kemei Shi [c], Xiaojuan Wang [b,c,*], Guolin Wu [a,*]

[a] Key Laboratory of Functional Polymer Materials of MOE, Institute of Polymers, Collaborative Innovation Centre of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071, PR China
[b] Pain Management Center, The Second Hospital, Tianjin Medical University, Tianjin 300070, PR China
[c] Tianjin Institute of Urology, Tianjin Medical University, Tianjin 300070, PR China

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ABSTRACT

In an effort to prove the inherent side effects of doxorubicin (DOX) and potentially revoke the effects of drug resistance exhibited by cancer cells, we have designed a multifunctional DOX-delivery nano-carrier system able to encapsulate the drug resistance reversal agent Verapamil HCl (VRP·HCl). Hydrophilic short-chain polyethylene glycol (i.e., mPEG) was covalently linked to hydrophobic DOX and a benzoic imine linkage was used to form a linear amphiphilic PEGylated prodrug, namely mPEG-*b*-DOX. In aqueous solution, the amphiphilic PEG-*b*-DOX is able to self-assemble to form stable nanoparticles with a DOX loading content of approximately 40 wt% and a diameter of ~143 nm. The resulting nanoparticles can simultaneously serve as an anticancer drug conjugate and as a drug carrier system. Here, the hydrophilic VRP could be encapsulated into the nano-carriers via a conventional dialysis method. The loading efficiency in mPEG-*b*-DOX nano-carrier was determined to be 53.97% and the loading content was found to be 7.71 wt%. The VRP-loaded nano-carriers grew slightly in size, to a diameter of ~177 nm. We found that the release of DOX and VRP was much faster at a lower pH value. The biological activity of the nano-carriers were evaluated in vitro and compared with the DOX-loaded system. In doing so we found that the VRP-loaded nano-carrier features a much higher antitumor activity. Furthermore, the combined-system exhibits a significantly enhanced cytotoxicity with an elevated apoptosis rate observed for MCF-7/ADR used as a cell line in this in vitro study. This combinatory system and promising candidate for applications involving DOX chemotherapy proved to be easy to prepare and could be characterized in terms of biocompatibility, biodegradability, loading capacity, pH responsiveness and reversal of drug resistance.

1. Introduction

Doxorubicin (DOX) represents one of the first line chemotherapeutic drugs and has been used in clinical applications for years. It demonstrates anticancer activity against a wide range of cancer types, including breast cancer, ovarian and gastric cancer and acute lymphoblastic-leukemia, used either alone or in combination with other chemotherapeutics. However, the use of DOX has been somewhat limited, mostly due to a variety of shortcomings associated with the production process of the drug, poor solubility, low tumor selectivity and high systemic toxicity. Until today, a plethora of strategies have been proposed to fabricate nano-carriers intended for DOX delivery, so-called DOX-delivery systems. Examples include polymer prodrugs [1–4], dendrimers [5,6], liposomes [7] and nanoparticles [8,9]. Among biocompatible polymer-based prodrugs [10], including poly(*N*-(2-hydroxypropyl) methacrylamide) (HPMA) [11,12] and dextran [13], poly(ethylene glycol) (PEG) [14–16], also known as a PEGylated prodrug, has been widely used as a conjugation agent for DOX. PEG exhibits some critical features useful in the conjugation of DOX, e.g., excellent solubility, high flexibility, low protein absorption, ease of manufacture as well as a reduced immunogenicity and toxicity. As a consequence, the compound has been approved by the Food and Drug Administration (FDA) to be applied in humans [17,18]. Currently available PEG prodrugs are mainly based on methoxy PEG or dihydroxy PEG, containing only one or two terminal groups per polymer chain for conjugation. This low availability of functional groups in PEG results in a limited drug loading capacity. Zhou et al. [19] synthesized two freely water-soluble PEG-g-DOX prodrugs with a 2.9

* Corresponding authors. Fax: +86 88 326520/22 23502749.
E-mail addresses: nkuwxj@163.com (X. Wang), guolinwu@nankai.edu.cn (G. Wu).

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and a 3.6 DOX ratio per molecule, corresponding to a drug loading content of 5.6 and 9.0%, respectively. However, a drug loading capacity of more than 10% could never be achieved this way.

A self-assembling PEGylated prodrug is a distinct polymer generated from traditional amphiphilic block polymers. The prodrug utilizes hydrophobic DOX as the hydrophobic component of the block copolymer in order to minimize the use of other inert materials and thus increases the drug loading capacity. Due to the number of conjugation sites available, two types of self-assembling PEGylated DOX prodrugs can form. One is coined PEG-grafted-DOX formed by grafting DOX moieties on the pendant chains of the copolymer. Hu et al. [20] reported the development of PEGylated prodrugs based on an amphiphilic copolymer with pendant functional groups for drug attachment exhibiting high stability. Xu et al. [21,22] reported the preparation of a diblock copolymer, i.e., MPEG-*b*-hydrazide, via deprotection of MPEG-*b*-PMABH and further synthesis by RAFT polymerization. DOX molecules were then attached to the polymeric backbone, and the hydrazone linker of the DOX prodrug could be obtained. Meanwhile, Wang et al. [23] introduced a strategy to synthesize pH-sensitive amphiphilic oligo-(ethylene glycol) (OEG)-doxorubicin (DOX) conjugates. Here, the DOX moieties were attached to the polymeric backbone via benzoic imine linkages to obtain the OEG-DOX conjugates. For these systems, the loading capacity and stability could be increased through side functionalization. However, the preparation of the copolymers proves to be challenging. Moreover, since the DOX moieties are attached to the pendant functional groups, an unbalanced grafting ratio may result in a difficult controllability of the drug loading content.

The other type of a self-assembling DOX prodrug is represented by PEG-block-DOX. The scaffold can be prepared by conjugating DOX molecules to the terminal chain of methoxy PEG (mPEG). Gou et al. [24] reported that a high drug loading content could be achieved by PEGylating the prodrug via conjugation of various anticancer drugs to an oligo-(ethylene glycol) (OEG) terminal chain using an acid-cleavable hydrazone linkage. Compared with the grafting-conjugation system, the preparation of PEG-DOX only involves a terminal functionalization so that the polymer structure proves to be well-defined, with the drug loading content being constant and independent from preparation methods and nanoparticle size. These characteristics prove to be crucial for facile, cost efficient scale-up processes. Since the DOX moiety was conjugated via a hydrazone bond, the release of DOX is mainly influenced by the equilibrium between the DOX molecules released in solution and the conjugated DOX moieties. The PEG-DOX nanoparticles presented in Sui's study also exhibit pH-sensitive properties; however, with an accumulated release rate of the prodrug found to be only 13% at pH = 5.0 and less than 25% at pH = 4.0. These values are lower than those reported for most of the pH-sensitive DOX-delivery systems. Moreover, the release profiles indicate that DOX molecules are not able to be released intracellularly once inside the tumor cells, resulting in a low efficiency of this system.

To utilize the advantageous behavior of PEG-*b*-DOX as a prodrug and in order to dissipate the release behavior defect, a new pH-sensitive PEGylated prodrug has been designed in this study. A hydrophilic short-chain polyethylene glycol (PEG) was reacted with the hydrophobic DOX unit via an acid-cleavable benzoic imine linkage to furnish PEG-*b*-DOX. A benzoic imine bond proves to be another type of suitable covalent bond for this system. However, the steric demand of the benzaldehyde moiety is believed to be able to suppress the re-conjugation of already released DOX molecules, therefore resulting in a more efficient DOX release profile.

Although, the delivery systems for DOX are able to reduce some of the inherent defects exhibited by the compound, another issue arises as the delivery system enters a cancer cell. Generally, the effects caused by multidrug resistance (MDR) have been found to impede the activity of DOX in chemotherapeutic treatments [25]. Among a variety of mechanisms of MDR [26] studied to date, the overexpression of drug efflux transporters such as the *P*-glycoprotein (P-gp) has been found to be one of the most critical resistance parameters, causing an efflux of anticancer drugs [27]. Verapamil hydrochloride (VRP·HCl), a calcium channel blocker commonly used in the treatment of hypertension, angina and myocardial infarction [28], has been reported to be able to entirely reverse the resistance caused by P-gp in vitro at concentrations of approximately 5–10 μM [27]. VRP represents a first-generation P-gp inhibitor [29] able to potentially reverse drug resistance effects in MDR tumor cells over-expressing P-gp [30]. However, severe side effects limit the use in clinical applications. In general, two strategies are used to overcome P-gp based MDR. One strategy involves the delivery of various stimuli-responsive nano-drugs to MDR cells via mechanisms designed to evade P-gp efflux by receptor mediated endocytosis [31]. The other strategy involves the delivery of a given anticancer drug with a P-gp inhibiting agent, like Verapamil [32], siRNA [33] and Tariquidar [34]. This work involves the co-delivery of an anticancer drug with a P-gp inhibiting agent, i.e., VRP, using a self-assembled, stimuli-responsive prodrug. In doing so, a more efficient system for the reversal of drug resistance effects may be formed.

It has been shown before that an amphiphilic copolymer is able to form vesicles in aqueous solution as the weight ratio of the hydrophilic component (f) is equivalent to $35 \pm 10\%$ [35]. The hydrophobic interactions and π–π stacking interactions between DOX molecules may further trigger the formation of amphiphilic copolymers via self-assembled vesicles [36]. Since such vesicle can serve as a carrier system for the delivery of the hydrophilic Verapamil/hydrochloride salt (VRP·HCl), a higher anticancer efficacy may be achieved through the use of this reversing multidrug resistance effects exhibited by VRP·HCl. We demonstrate in this study that PEG-*b*-DOX can self-assemble into nanoparticles under physiological conditions, taking advantage of the EPR effect exhibited by targeting drug delivery systems. Furthermore, the self-assembling nanoparticles have the ability to encapsulate VRP·HCl. The nanoparticles formed have been fully characterized and the corresponding morphologies as well as the in vitro bioavailability of the nano-carriers have also been investigated. This novel mPEG-*b*-DOX nano-carrier can act as a DOX-delivery system featuring a high drug loading capacity and may therefore potentially find use as a nano-carrier for VRP in combinatory anticancer therapy.

2. Experimental

2.1. Materials

Methoxy polyethylene oxide (mPEG, Mn = 736 g/mol) was purchased from Fluka. Benzene methanesulfonyl chloride, 4-hydroxybenzaldehyde and dimethyl sulfoxide (DMSO) were obtained from Tianjin Chemical Reagent Company and were dried by distillation before use. Doxorubicin hydrochloride (DOX·HCl) was purchased from the Beijing Huafeng United Technology company. Verapamil hydrochloride (VRP·HCl) was purchased from the DingGuo Reagent company. Other reagents were of analytic grade and used as received. Deionized (DI) water was used in the experiments detailed in subsequent sections.

2.2. Synthesis of mesylate-terminal mPEG (mPEGOTS)

A modified procedure reported in the literature [37] was adopted with little modification. First, mPEG (Mn = 736 g/mol) (7.36 g, 10 mmol) was dried in toluene. After most of the toluene was distilled from the flask, 20 mL methylene chloride was added.