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Folding graft copolymer with pendant drug segments for co-delivery of anticancer drugs

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ABSTRACT

A graft copolymer with pendant drug segments can fold into nanostructures in a protein folding-like manner. The graft copolymer is constructed by directly polymerizing γ-camptothecin-glutamate *N*-carboxyanhydride (Glu(CPT)-NCA) on multiple sites of poly(ethylene glycol) (PEG)-based main chain *via* the ring open polymerization (ROP). The "purely" conjugated anticancer agent camptothecin (CPT) is hydrophobic and serves as the principal driving force during the folding process. When exposed to water, the obtained copolymer, together with doxorubicin (Dox), another anticancer agent, can fold into monodispersed nanocarriers (with a diameter of around 50 nm) for dual-drug delivery. Equipped with a PEG shell, the nanocarriers displayed good stability and can be internalized by a variety of cancer cell lines *via* the lipid raft and clathrin-mediated endocytotic pathway without premature leakage, which showed a high synergetic activity of CPT and Dox toward various cancer cells. *In vivo* study validated that the nanocarriers exhibited strong accumulation in tumor sites and showed a prominent anticancer activity against the lung cancer xenograft mice model compared with free drugs.

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1. Introduction

Evolution of drug resistance in cancer cells has been attributed as a major factor in the failure of many forms of monotherapy [1,2]. To prevent evolutionary development, it often requires a high drug dose to kill the whole cancer cell population; whereas it always induces severe side effects [3,4]. The limitations of monotherapy can be circumvented by synergistic combination of multiple anticancer agents which allows for reduction of the drug dose and offers a potential benefit to simultaneously act on several anticancer targets, therefore preventing or delaying the emergence of drug resistance [5]. However, the traditional combination strategy, namely the drug cocktail, shows limited success in clinics due to the non-coordinated distributions of drugs after administration [6]. Pharmacokinetic interactions are also observed in these combination therapies, raising concern about the synergistic toxicity of these cocktails [7,8]. For example, a combination of rapamycin (RAPA) and cyclosporine (CsA) produces renal dysfunction, since the pharmacokinetic interaction between RAPA and CsA highly increases the concentration of CsA in kidney [7]. In addition, the difference in solubility, potency, pharmacokinetics and bioavailability between drugs makes the dose schedule extremely challenging in the cocktail therapy [9–11].

Nanocarriers have been developed as an important strategy for drug delivery due to their capabilities of enhancing drug solubility, improving pharmacokinetics and preferentially accumulating in tumor by the enhanced permeability and retention (EPR) effect [12–17]. Therefore, the nanocarrier-based combination therapy not only has the loading potency of the multiple agents, but also accommodates their biodistribution and plasma elimination, realizing an extremely simple dose optimization [18,19]. Polymeric micelle is one of the most common nanoparticle systems for co-delivery of multiple drugs due to their physicochemical stability and low toxicity [12,20,21].

Topoisomerase (Top) represents one of the most popular targets in cancer chemotherapy. Its inhibitors can block the DNA ligation step during cell cycling, and induce irreversible single and double stranded DNA breaks during transcription and replication, thereby leading to apoptosis and cell death [22]. Both camptothecin (CPT) and doxorubicin (Dox) are potent Top inhibitors, but they interfere

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with the action of Top I and II, respectively. CPT as the Top I inhibitor has shown potent anti-tumor efficacy in a broad spectrum of cancers in clinic [23,24]. However, certain clinical limitations such as resistance of cancer cells impair its clinical application [25,26]. It has been postulated that the reduced Top I activity following the CPT treatment could be compensated by the Top of the other class (Top II), because the actions of both enzymes are partially overlapping [27]. It has been proven that the increase of Top II activity occurs in cancer cells resistant to CPT [28], and Top II inhibitor Dox shows a collateral cytotoxicity on the CPT adapted cancer cells [29]. Therefore, the concomitant use of both Top I and Top II inhibitors might elicit synergistic effects and prevent the emergence of drug resistance [30]. Polymeric nanocarriers have provided a novel combination strategy for simultaneously delivering multiple agents to tumor sites *via* a single vehicle. Coordination of the solubility, pharmacokinetics and biodistribution of multiple drugs by nanocarriers allows this strategy to overcome the problems commonly associated with traditional drug cocktail methodology.

We designed a new graft copolymer, which can form a polymeric nanocarrier in a protein folding-like manner for co-delivery of two small-molecule anticancer drugs: CPT and Dox. Unlike the traditional micelle assembled from AB type of amphiphilic diblock copolymer, the current nanocarrier was folded by a structurally well-defined graft copolymer in which the side chain associated together as the hydrophobic micelle core and poly(ethylene glycol) (PEG) main chain entangled around it. As depicted in Fig. 1A, the graft copolymer was constructed from three monomers by a two-step polymerization. The "tee joint"-like monomer I and monomer II were first condensed into a linear multiblock copolymer, to which a CPT-linked monomer III γ-camptothecin-glutamate *N*-carboxyanhydride (Glu(CPT)-NCA) was subsequently grafted by an amine-initiated ring open polymerization (ROP) [31,32]. The resulting graft copolymer displayed a well-defined structure where the main chain was composed of the hydrophilic PEG and the pendant chains were polypeptide chains "purely" conjugated with hydrophobic drugs (Fig. 1B). Once reconstituted with water, this graft copolymer ("denatured" state in organic solvents, designated "S_d") folded into a uniform nanocarrier ("native" state, designated "S_n") in which the side chains were assembled into a hydrophobic core wrapped around by the main PEG chain as a shell. Unlike the traditional micelles assembled by a linear unit, this nanocarrier was folded from a grafted unit, making the final structure more compact and stable [33]. In addition, the hydrophobic core also provided accommodation for co-encapsulating other hydrophobic anticancer drugs through physical interactions such as π−π stacking [9,34,35]. In our co-delivery system, Dox was physically encapsulated in the hydrophobic core, while the CPT was covalently linked on side chains which can be released *via* the degradation of ester linkage by the intracellular esterase [36,37].

2. Materials and methods

2.1. Materials

All chemicals were obtained from commercial sources and used without further purification. Tris(2-aminoethyl)amine, di-t-butyl dicarbonate (Boc_2O), trifluoroacetic acid (TFA), *N*,*O*-bis(trimethylsilyl) acetamide (BSA) and triphosgene were purchased from Sigma Aldrich. *N*,*N*′-diisopropylcarbodiimide (DIPC), triethylamine (Et_3N) and 4-dimethylaminopyridine (DMAP) were obtained from Acros Organics. N-hydroxylsuccinimide (NHS) bifunctionalized PEG_{5000} (NHS-PEG_{5000}-NHS) was purchased from Nanocs Inc. CPT and Dox were purchased from Alfa Aesar. Boc-L-glutamic acid 5-*tert*-butyl ester (Boc-Glu-OtBu) was ordered from Chem-Impex International Inc. All the organic solvents for synthesis and analysis were ordered from Fisher Scientific Inc. and used as received.

2.2. Synthesis of graft copolymer ***5***

2.2.1. Synthesis of monomer I (***2***)

The monomer I, *N*,*N*-bis(2-aminoethyl)-*N*-[2-(tert-butylcarbamoyl)ethyl-amine (**2**), was synthesized as reported [38,39]. Briefly, a chloroform solution of Boc_2O (1 g, 4.6 mmol, 0.1 equiv) was added dropwise into a stirred solution of tris(2-aminoethyl)amine (**1**) (6.7 g, 46 mmol) in 300 mL of chloroform at 0 °C. The reaction was allowed to warm to room temperature and stirred overnight. The reaction was stopped by 15 mL of distilled deionized (DD) water. After stirred for 5 min, the organic phase was separated. The aqueous phase was re-extracted with 30 mL of chloroform. The combined organic phase was dried with Na_2SO_4, then concentrated under vacuum to give crude compound **2** which was purified by silica gel chromatography using $CHCl_3$/MeOH/concentrated aqueous NH_4OH (v/v/v, 10/5/1) as eluant. The purified monomer I was given as viscous oil (1.05 g, 92%). ^1H NMR (300 MHz, DMSO-d_6): δ 6.81 (t, 1H), 2.95 (q, 2H), 2.81 (brs, 4H), 2.52 (t, 4H), 2.38 (q, 6H), 1.39 (s, 9H). ESI-MS calcd for $C_{11}H_{26}N_4O_2$ 246.21, found 247.21 $[M + H]^+$.

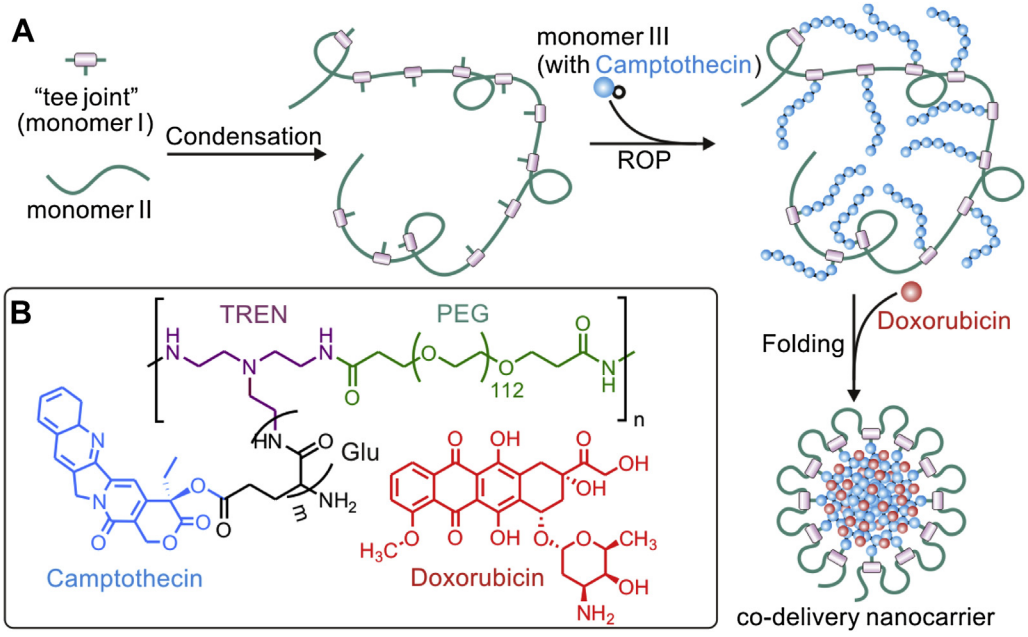

Fig. 1. Schematic design of graft copolymer synthesis and stealth nanocarrier folding. (A) Graft copolymer with pendant drug segments is synthesized from three monomers by a two-step polymerization, and then folds into nanostructure with free doxorubicin encapsulated. (B) The chemical structure of graft copolymer. The graft copolymer is comprised of a PEG-containing multiblock copolymer main chain and the polyglutamic acid-based side chains with "purely" conjugated camptothecin.