Biomaterials 35 (2014) 4368–4381

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

PEGylated liposomes with NGR ligand and heat-activable cell-penetrating peptide–doxorubicin conjugate for tumor-specific therapy

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ARTICLE INFO

Article history:
Received 4 December 2013
Accepted 30 January 2014
Available online 22 February 2014

Keywords:
Heat activation
Thermosensitive liposomes
NGR-targeted
Cell-penetrating peptides
Tumor-oriented therapy
Conjugates of cell-penetrating peptides and doxorubicin

ABSTRACT

Cell-penetrating peptides (CPPs) mediated tumor-oriented nanocarriers have been widely studied by researchers recently. However, applications of CPPs *in vivo* were usually hampered by their loss in untargeted tissues and enzymatic degradation. These shortfalls required strategies to camouflage CPPs before their arrival at the targeted site. In this work, we constructed a thermosensitive liposome (TSL) containing Asparagines–Glycine–Arginine (NGR) peptide as the targeting moiety and heat-activable cell-penetrating peptide–doxorubicin conjugate for enhancing specific cancer therapy. Different to the masking strategies of CPPs reported, CPPs existing in conjugation form of CPPs and doxorubicin (CPP-Dox) were hidden in TSL to cloak and protect CPPs. Meanwhile, NGR moiety and local tumor hyperthermia were utilized to achieve specific targeting of CPPs to the tumor. The nanocarrier (CPP-Dox/NGR-TSL) prepared in this work possessed suitable physiochemical properties such as small particle size of about 90 nm, high drug encapsulation efficiency of approximately 95%, good stability in the medium containing 10% fetal bovine serum (FBS) and so on. *In vitro* experiments on Human fibrosarcoma cells (HT-1080) and human breast adenocarcinoma cells (MCF-7) verified the specific targeting ability and enhanced intracellular drug delivery of the liposomes to HT-1080 cells. Furthermore, comparing with NGR-targeted TSL containing Dox (Dox/NGR-TSL), the results of intravenous administration showed CPP-Dox/NGR-TSL significantly inhibited tumor growth in nude mice xenografted HT-1080 tumors and excellent body safety. In conclusion, the nanocarrier constructed in this study would be a safe and efficiently drug delivery system for specific cancer treatment.

1. Introduction

Cell-penetrating peptides (CPPs), facilitating the intracellular delivery of various cargos without causing any cellular injury, have been widely studied by researchers recently. CPPs are capable of penetrating into a wide variety of mammalian cells independent of tissue or organism types [1]. Furthermore, some certain CPPs can even facilitate targeted delivery to subcellular structure such as cell nucleus, mitochondria, lysosome and cytoplasm [2]. Cargos that have been successfully delivered by CPPs include small molecules, peptides, proteins, nucleic acids, quantum dots, polysaccharides, liposomes and nanoparticles [3]. Although of considerable clinical potential, CPPs are also a double-edged sword and have a few important drawbacks. Firstly, they have undesirable characteristic of nonspecificity and can enter any cell they come in contact with [4], which increases the risk of drug-induced toxic effect on normal tissues. Secondly, the stability *in vivo* of these peptides is at risk until they reach the target [5]. These peptides can be enzymatically cleaved by plasma enzymes and thus need to be well protected before they accessed the targeting point.

How to improve the specific targeting of CPP-mediated payload? One approach proposed to construct carriers protecting (shielding) the nonspecific function of CPPs before their arrival at the target sites [6]. In this way, CPPs were usually immobilized on the surface of the nanocarriers and their functions were conventionally shielded via two methods: 1. electrostatic attraction between the positive CPPs and negative polyanions [7]; 2. steric protection by the long polymer chains such as polyethylene glycol (PEG) chains [8].

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http://dx.doi.org/10.1016/j.biomaterials.2014.01.076
0142-9612/

Upon accumulation in the target, the CPPs could be activated through internal or external stimulus. Gao et al. [9] reported nanostructured lipid carrier modified with tumor microenvironment-sensitive polypeptides (TMSP) showed enhanced anti-tumor efficiency to the tumor, which overexpressed matrix metalloproteinases (MMPs) in microenvironment. By using different pH-levels between tumor tissue and healthy tissue, Torchilin's group [10] developed long-circulating pH-detachable PEG shielded TAT-liposomes to improve the site-specific drug delivery. However, due to intrinsic limitations of the tumor microenvironment such as the limited number of MMPs and different intensity of pH value from the margin to the interior of the targeted tumor, the methods utilizing internal stimulus may lead to insufficient activation of CPPs. Therefore, external stimulus was developed to improve the activation efficiency of CPPs. By utilizing UV irradiation-stimulus, the exposure of the constrained CPPs could be controlled for intracellular delivery of liposomes [11]. Nevertheless, UV damage to the surrounding tissue may limit its broad clinical application. A mild thermal stimulus used to control "shielding/deshielding" of CPPs to facilitate drug delivery via temperature was also reported [12], in which biotinylated CPPs and poly (*N*-iso-propylacrylamide) (PNIPAAm) was linked on the carrier by co-immobilizing. The thermosensitive characteristics of PNIPAAm chain length, which could stretch or shrink as the temperature varied was used to activate CPPs. Although the mild hyperthermia did not cause the surrounding tissue damage, the *in vivo* safety of the polymer "PNIPAAm" introduced in the system needs to be further investigated. Furthermore, as the CPPs were usually immobilized on the surface of the nanocarriers in currently reported masking strategies, the CPPs may not well be protected from enzymatic degradation *in vivo* before approaching the targeting sites.

Thermosensitive liposomes (TSL), whose lipid materials will have a phase transition upon heating in a mild-hyperthermia range and thus lead to the formation of liquid–solid boundaries in the lipid bilayer, enabling a liposome-based drug delivery system to immediately release encapsulated drugs in the heating tissues or organs [13]. Comparing with the conventional long-circulating PEGylated liposomes, TSL showed more advantages: 1. Thermal-sensitive drug delivery approach favors a formulation that is stable at 37 °C, which could maintain the drug within the liposome during blood transport [14]; 2. TSL releases the drug at hyperthermic temperatures (40–42 °C) in such an ultrafast manner that it can counteract rapid blood passage time and washout of the encapsulated drug from a tumor [14]; 3. TSL in combination with hyperthermia could selectively increase the local drug concentration in tumors and lead to an evidently improvement in therapeutic efficacy [15]. Therefore, by utilizing the ascendancies of TSL, a new strategy by encapsulating CPPs into TSL was hypothesized to camouflage CPPs function as well as improve their *in vivo* stability in the circulation in this work.

Small molecule doxorubicin (Dox) is one of the most important anticancer agents for solid tumor. However, the secondary effect of this drug, especially the cardiotoxicity, is a major drawback. Moreover, some tumors may be Dox-resistant. Conjugation (CPP-Dox) of CPPs with Dox was reported displayed more excellent therapeutic efficacy and less cytotoxicity than Dox alone [16–18]. The reason can be due to the synergistic effects of CPPs' cell penetration ability and anticancer effect of Dox. It was also found that efficient induction of apoptosis by CPP-Dox could overcome the Dox-resistant property found in human breast cancer cell line MDA-MB-231 [19]. Recently, various CPP-drug conjugates have been developed to improve anti-tumor efficacy [20,21]. In this work, CPPs would be encapsulated into TSL in the form of CPP-Dox to enhance intracellular delivery efficiency of Dox.

Although TSL could improve the targeting drug delivery to some extent, modifications on the surface of TSL using active targeting ligands could elicit cell surface binding and receptor-mediated endocytosis. Thus boost the targeting effect of TSL, especially for some specific tumors. Asparagines-Glycine-Arginine (NGR) peptide motif has been used to modify drug loaded liposomes to target tumors vascular antigen aminopeptidase N (APN/CD13) and tumor cells highly expressed CD13, resulting in improved biodistribution and tumor therapy [22]. So NGR ligands would be used to modify the surface of TSL to further elevate the targeting ability of TSL to tumor cells highly expressed CD13 in this study.

Based on the above background, in this work, we hypothesized to construct a drug delivery system (named as CPP-Dox/NGR-TSL) combining triple targeting effects of NGR, TSL and CPPs (shown in Fig. 1): (1) CPPs (CKRRMKWKK), derived from Penetratin [23], being reported to have increased membrane translocation efficiency [24] would be first conjugated with Dox to form a conjugation (CPP-Dox) via chemical reactions, then CPP-Dox would be encapsulated into TSL; (2) TSL would be used as drug delivery carrier to improve the *in vivo* stability and cloak the penetration function of CPPs; (2) NGR would be conjugated to the water-exposed tips of the PEG chains on the surface of the TSL as the targeting moiety positioning to the specific tumor cells; (3) CPPs would be activated via heat stimulus and used as the secondary targeting moiety to deliver drugs intracellular more efficiently. Accordingly, the action mechanisms of the constructed CPP-Dox/NGR-TSL would be investigated. Moreover, the evaluations on the anticancer efficiency of the carrier both *in vitro* and *in vivo* were also the objectives of this work.

2. Materials and methods

2.1. Materials

Dipalmitoyl phosphatidylcholine (DPPC), Monostearoylphosphatidylcholine(MSPC),1,2-distearyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000](DSPE-PEG$_{2000}$)and1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-maleimide(polyethyleneglycol)(DSPE-PEG$_{2000}$-Mal) were purchased from Avanti Polar Lipids (Alabaster, USA) and used without further purification. DOX (≥99%) was provided by Synbias Pharmaceutical Co, Ltd, (Zhejiang, China). *N*-Succinimidyl 3-maleimidopropionate (SMP, ≥98%) was purchased from Haofan Biomatrix Co, Ltd, (Suzhou, China). NGR motif peptide (CYGGRGNG) and CPPs (CKRRMKWKK) were custom synthesized by Shanghai GL Biochem Co, Ltd, (Shanghai, China). Trypsin was obtained from Solarbio. Dulbecco's Modified Eagle's Medium (DMEM), Modified Eagle's Medium (MEM) and fetal bovine serum (FBS) were obtained from GIBCO, Invitrogen Corp. (Carlsbad, USA). 6-coumarin (Cou-6, purity >99%), Hoechst 33258 and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St Louis, MO). Penicillin and streptomycin were provided by North China Pharmaceutical Co., Ltd, (Hebei, China). All other chemicals were analytical or high performance liquid chromatography (HPLC) grade.

Female Nu/Nu nude mice (weighing 18–20 g) were purchased from Vital River Laboratories (Beijing, China). All animals were handled according to the code of ethics in research, training and testing of drugs as laid down by the Animal Care and Use Ethics Committee of Academy of Military Medical Sciences.

2.2. Synthesis of CPP-Dox conjugate

CPP-Dox conjugate was synthesized by coupling CPPs with Dox via a linker of SMP [25]. As the general synthetic scheme depicted in Fig. 2a, Dox-SMP was prepared firstly, Dox (43.5 mg), SMP (22.0 mg), and triethylamine (TEA, 21 μL) were dissolved in 8 mL of dimethylformamide (DMF) and kept stirring for 2 h at room temperature. The reaction solution was precipitated by cold anhydrous diethyl ether and washed twice with anhydrous diethyl ether for purification. The red solid obtained was separated from the solvent by centrifugation and vacuum dried to give a 71.2% yield. Secondly, the CPP-Dox was synthesized by mixing solution of Dox-SMP (3.9 mg) dissolved in 0.25 mL DMF, solution of CPPs (6.75 mg) dissolved in 0.25 mL DMF, TEA (10 μL) and stirring at ambient temperature for 2 h. The resulting product was also purified with cold anhydrous diethyl ether and separated from the solvent by centrifugation. At last, the product with a 83.5% yield in red crystals was obtained through vacuum drying and verified by MALDI-TOF MS.

2.3. Synthesis of NGR-PEG$_{2000}$-DSPE

DSPE-PEG$_{2000}$-NGR was synthesized according to the Ref. [26], with slight modifications. Briefly, cysteine-modified NGR (6.8 mg) was dissolved in 4.5 mL of HEPES buffer (20 mM HEPES, 10 mM EDTA-2Na, pH 6.5). Dried lipid film containing