Colloids and Surfaces B: Biointerfaces 128 (2015) 427–438

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

A near-infrared two-photon-sensitive peptide-mediated liposomal delivery system

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ARTICLE INFO

Article history:
Received 6 October 2014
Received in revised form 7 February 2015
Accepted 19 February 2015
Available online 26 February 2015

Keywords:
Near-infrared (NIR) two-photon photolysis
Photo-sensitive peptides
Cell-penetrating peptides
Drug delivery
Liposomes

ABSTRACT

Tumour-oriented nanocarrier drug delivery approaches with photo-sensitivity have been drawing considerable attention over the years. However, due to its low penetrability and ability to induce tissue damage, the use of UV light for triggered nanocarrier release in *in vivo* applications has been limited. Compared with UV light, near-infrared (NIR) light deeply penetrates tissues and is less damaging to cells. Here, we report on the development of a novel method employing photo-sensitive cell-penetrating peptides (CPPs), which can be used to trigger the transport of liposomes into cells following stimulation, which was irradiation with NIR light in this case. The positive charges of the lysine residues on the CPP were temporarily caged by a NIR two-photon excitation-responsive protective group (PG), thereby forming photo-sensitive peptides (PSPs). The PSP was connected with DSPE *via* a polyethylene glycol (PEG) spacer to prepare the modified liposomes (PSP-L). Once illuminated by NIR light in tumour tissues, these PGs were cleaved, and the positively charged CPP regained its activity and facilitated rapid intracellular delivery of the liposomes into cancer cells. The PSP-L carrying vinorelbine bitartrate prepared in this work possessed suitable physiochemical properties. In addition, strong cellular uptake and cytotoxic activity of PSP-L in MCF-7 cells were correlated with NIR illumination. Furthermore, triggered NIR activation of PSP-L led to higher antitumour efficacy in the MCF-7 tumour model in nude mice compared with the unmodified liposomes (N-L). In conclusion, the application of PSP modifications to drug-carrying liposomes may provide an approach for the targeted delivery of antitumour agents.

1. Introduction

Many current chemotherapeutic drugs often cause severe side effects because they are similarly cytotoxic to both cancerous and healthy cells. To overcome this challenge, researchers have developed a number of strategies to target drugs to tumours [1,2]. One of such approaches is microenvironment-sensitive nanostructures, which respond to changes in environmental conditions, such as light [3], temperature [4], pH [5] and enzymatic activity [6]. Recently, this strategy has received extensive attention for the unique advantages of these nanostructures for targeted drug delivery.

For the aforementioned endogenously triggered release mechanisms (enzyme or pH), accurate controlled release in a complex physiological environment at the appropriate moment without interference still poses a considerable challenge [7]. Thus, it would be preferable to develop a common induced-release methodology that is independent of the characteristics of the extracellular tumour microenvironment. Photo-irradiation is quite advantageous due to its non-invasive nature, desirable modulability and high spatial resolution [8]. However, one disadvantage is that the externally applied trigger is typically restricted to superficial tissues, although deep tissues may be reached with the aid of laparoscopy.

To date, a variety of photo-responsive modalities have been utilized, such as switchable cis-trans isomerization [9], reversible photo-dimerization [10] and cleavable covalent linking [11]. Compared with the first two approaches, the presence of a specific cleavable linkage between a photo-sensitive protective group (PG) and the pharmacological agent provides excellent control of the on/off switch *via* complete rupture. Recently, the irreversible photoactivation of many promising caged biomolecules has been described, including cell-penetrating peptides (CPP) [12], amino acids [13], proteins [14] and nucleic acids [15]. Such

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http://dx.doi.org/10.1016/j.colsurfb.2015.02.041
0927-7765/

photo-uncaging has been applied *in vitro* for payload release from nanocarriers upon ultraviolet (UV) light illumination. However, due to its low penetrability and potential to harm tissue, the use of UV light for adjustable payload release in *in vivo* applications has been limited [16].

Compared with UV one-photon photolysis, near-infrared (NIR) two-photon photolysis can overcome the aforementioned problems. Radiation below 650 nm cannot penetrate deeper than 1 cm into tissue due to high scattering and absorption by haemoglobin, oxy-haemoglobin and water. Conversely, NIR light of 650–900 nm can penetrate up to 10 cm into living tissue and causes minimal tissue damage at the site of application [17]. For the development of a PG which can be cleaved *via* NIR two-photon absorption, multiple studies have reported that the 4,5-dimethoxy-2-nitrobenzyl group is easily cleaved by NIR radiation (wavelength 740 nm), and it its application for an NIR two-photon excitation (2PE)-responsive caged compound used to control the function of living cells has recently been reported [18–21].

Inspired by these results, a rational strategy was employed to take advantage of a photo-sensitive peptide (PSP) to generate a more selective and efficient system for drug delivery to tumour cells in this study. The PSP includes two units: the CPP (CGRRMKWKK) and the PG (1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene). The CPP (CGRRMKWKK), derived from penetratin, is a novel cell-penetrating peptide that enhances the delivery of molecules across biological barriers to achieve intracellular access [22]. The PG (1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene) is a NIR 2PE-responsive group, which can be cleaved by NIR two-photon absorption. In our study, the positive charges of the lysine residues on the CPP were temporarily masked by the PG, thus forming the PSP. The PSP enhances specific cellular uptake *via* the CPP, which is generated *via* selective removal of the PG at the target site upon illumination with NIR light.

To develop a NIR two-photon-sensitive peptide-mediated liposomal delivery system (PSP-L), the PSP was conjugated with DSPE-PEG_{2000}-MAL. Here, we relied firstly upon passive targeting of PSP-L to the cancer cell microenvironment, due to the enhanced permeability and retention (EPR) effect, and secondly upon the use of NIR light for selective binding and penetration into the cells, leading to increased cytotoxicity. While in the circulating fluid, the cell-penetrating ability of the CPP is blocked, but upon arriving at the target tissue, the uncaging of the PSP is triggered by NIR light, causing the nonfunctional PSP to be converted to an activated CPP. The peptide then regains sufficient positive charges, and its cell membrane-translocating activity is subsequently restored. Finally, with the aid of activated CPP, the nanocarriers will enter into tumour cells efficiently. In this work, we describe the physicochemical and biological characterization of PSP-L for vinorelbine bitartrate (VB)-based cancer therapy at the cellular level; the *in vivo* antitumour activity of PSP-L was also explored.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethyleneglycol) (ammonium salt) (DSPE-mPEG_{2000}) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-maleimide(polyethylene glycol) (DSPE-PEG_{2000}-MAL) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Egg lecithin (EPC) and cholesterol (Chol) were purchased from Lipoid GmbH (Mannheim, Germany). Vinorelbine bitartrate (VB) was provided by Min Sheng Pharmaceutical Co. (Zhejiang, China). Navelbine® (vinorelbine bitartrate injection) was purchased from Pierre Fabre Medicament (Boulogne, France). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DIR) and 6-coumarin (COU) were purchased from Biotium. All chemicals were of reagent grade and were obtained from Sigma–Aldrich, unless otherwise stated.

Human breast adenocarcinoma cells (MCF-7 cells) purchased from the Cell Resource Centre of IBMS (Beijing, China) were maintained in culture medium consisting of Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained in a 37 °C humidified incubator in a 5% CO_2 atmosphere.

Female BALB/c 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 stated by the Animal Care and Use Ethics Committee of the Academy of Military Medical Sciences.

2.2. Synthesis of the PSP

The CPP (CGRRMKWKK) was synthesized by a standard solid phase Fmoc-protocol using Rink amide resin (0.44 mmol/g; Nankai Hecheng, Tianjin, China) on a CEM Liberty peptide synthesizer (CEM, Matthews, NC, USA). The amino-termini of amino acids were acetylated and the carboxyl-termini were amidated. The synthesis used the following amino acids: Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Met-OH, Fmoc-Lys(Mtt)-OH and Fmoc-Trp-OH, with Trt, Pbf and Mtt representing trityl, 2,2,4,6,7-pentamethyldihydro benzofuran-6-sulfonyl and 4-methyl trityl groups, respectively. After synthesis of the fully protected peptide, the Mtt groups (orthogonal protecting groups) were selectively removed with 1% trifluoroacetic acid (TFA) in dry dichloromethane (DCM), a process that was repeated 10 times, for 2 min each time. The free ε-amine of the lysine side chain was reacted with 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene (PG) at 4 mol equivalents relative to the resin loading using K_2CO_3 (2.5 equiv.) in dry DCM for 12 h. The PSPs, equipped with the PGs at the desired positions, were then cleaved from the resin and globally deprotected with Reagent K (TFA/*m*-cresol/water/ethanedithiol at a ratio of 85:10:2.5:2.5).

2.3. NIR-induced uncaging of the PSP

A solution containing the PSP (400 μM) was prepared in MOPS buffer at pH 7.4 and was illuminated using a NIR pulsed laser ($\lambda = 740$ nm, 3.48×10^{12} photon s^{-1}). Aliquots (50 μL) were removed at various time points and immediately frozen in the dark until they could be analyzed *via* reverse-phase HPLC (Agilent 1211, Agilent Technologies, USA). For HPLC separations, an Akasil C18 column (4.6 mm × 250 mm, 5 μm, Agela Technologies, flow rate 1 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of a 0.1% aqueous solution of TFA (v/v, solvent A) and 0.1% TFA in acetonitrile (v/v, solvent B) was used for HPLC elution [23].

2.4. Synthesis of functional conjugates

The DSPE-PEG_{2000}-peptides were synthesized as previously described by Gao et al. [24], with slight modifications. Briefly, PSP or CPP were conjugated with DSPE-PEG_{2000}-MAL (1.2:1 molar ratio) in DMF containing triethylamine (TEA, 5 equiv.) at room temperature (20–25 °C) for 24 h under stirring. The reaction mixture was dialyzed (molecular weight cut-off (MWCO) 25 kDa) in distilled water for 48 h to remove the DMF and excess peptides. The final solution was lyophilized and stored at −20 °C until use. The conjugations were confirmed by determining the molecular weights of the resulting DSPE-PEG_{2000}-PSPs or DSPE-PEG_{2000}-CPPs *via* matrix-assisted laser desorption/ionization-time-of-flight mass