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Constrained and UV-activatable cell-penetrating peptides for intracellular delivery of liposomes

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ABSTRACT

Herein we report on the development of a novel method of constraining a cell-penetrating peptide, which can be used to trigger transport of liposomes into cells upon in this case radiation with UV-light. A cell-penetrating peptide, which was modified on both termini with an alkyl chain, was anchored to the liposomal surface in a constrained and deactivated form. Since one of the two alkyl chains was connected to the peptide *via* a UV-cleavable linker, disconnection of this alkyl chain upon irradiation led to the exposure of the cell-penetrating peptide, and mediated the transport of the entire liposome particle into cells.

1. Introduction

Over the past decades, numerous drug carriers have been developed from a wide range of materials [1–12]. A common goal for most of these carriers is to address some of the inherent problems associated with administration of drugs, such as toxicity, instability and unfavorable biodistribution. To deal with these issues, encapsulation of the drug in a carrier is often the method of choice, as this serves to protect the drug from the body environment and *vice versa*, and to shift the biodistribution towards the target site hence the stability, safety and targeting efficiency of the drug can be increased. In this respect, liposomes have received a fair amount of attention as carriers of encapsulated drugs [13–16], because of their easy preparation (at lab scale), their composition of natural and relatively inexpensive starting materials, as well as approval of several liposome products by the FDA [17–19]. Of particular interest are the so-called Stealth liposomes, which are coated with poly(ethylene glycol) (PEG) to evade immune system interactions and thereby achieve a long circulation time in the bloodstream [20–24].

However, while encapsulation provides a solution for unfavorable biodistribution, toxicity and instability of drugs, encapsulated drugs on the other hand have to escape from the interior of the carrier to exert their activity. To gain control over this release, a plethora of triggers has been employed such as reduction [25,26], ultrasound [27,28], light [29–34], pH [6,35–40], temperature [41–43], magnetic [44] and enzymatic activity [2,45–47] to mediate drug release. Of these triggers, pH, temperature and enzymatic triggers have been used to target drug carrier systems specifically to diseased tissues, as the latter areas often exhibit a decreased pH, elevated temperature or express specific enzymes [48]. The most precise control, however, is achieved when external triggers such as ultrasound, magnetism and light are employed since these can be applied to defined localized areas to induce drug release [49]. The disadvantage is that most of these externally applied triggers are restricted to superficial tissues, though deep-seated tissue may be reached with the aid of laparoscopy [49,50].

Up to now, most triggered-release carrier systems (including light-triggered systems) work *via* a destabilization of the carrier causing release of the encapsulated drug in the extra-cellular space. This, however, may lead to poor cellular uptake of the released drugs in the case of macromolecular and/or hydrophilic drugs, which do not easily cross the cell membrane (*e.g.* oligonucleotides) [51,52]. Hence, cellular uptake of the carrier *before* releasing its contents may be desirable. Regarding uptake, this can be facilitated by functionalization of the carrier with cell-penetrating peptides (CPPs), which is a class of peptides capable of inducing cellular uptake of almost any kind of cargo to which they have been attached [53–61]. However, modification of the liposome surface with Tat-peptides leads to aspecific interaction and uptake in non-target cells [62]. Moreover, its positive charge leads to the interaction with blood components and enhances clearance *via* the reticuloendothelial system [63], thereby compromising the stealth property that is essential for prolonged circulation. In order to combine stealth behavior and Tat-mediated cellular uptake, we designed a UV-activatable cell-penetrating peptide which is inactivated by means of constraining the peptide by 'hiding' it on the surface of a liposome. Reactivation of

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the peptide can be accomplished by releasing the constrain *via* UV-irradiation.

2. Materials and methods

Breipohl resin was purchased from Novabiochem and Fmoc-L-amino acids were from Bachem (Bubendorf, Switzerland) or Novabiochem (EMD Chemicals, Gibbstown, USA). Lipids were from Avanti Polar Lipids (Alabaster, USA) or Lipoid (Steinhausen, Switzerland). Atto655 was purchased from ATTO-TEC (Siegen, Germany). All other chemicals were purchased from Baker, Fluka or Sigma Aldrich and used as received. Mass spectra were recorded on a Bruker Biflex MALDI-TOF (Bruker Daltronik, Bremen, Germany). Lyophilization was achieved using an ilShin Freeze Dryer (ilShin, Ede, The Netherlands).

Hepes (99%) was obtained from Acros Organics BVBA (Geel, Belgium). Phosphate-buffered saline (PBS) was purchased from B. Braun (Melsungen AG, Melsungen); fetal bovine serum (FBS) was from Integro, Zaandam, The Netherlands, Trypsin/EDTA, Plain DMEM (Dulbecco's modification of Eagle's medium, with 3.7 g/L sodium bicarbonate, 1 g/L L-glucose, L-glutamine) and antibiotics/antimycotics (penicillin, streptomycin sulfate, amphotericin B) were all from PAA Laboratories (Pasching, Austria).

Human epithelial ovarian carcinoma (HeLa) cells were kindly given by the Institute of Biomembranes (Utrecht University, The Netherlands).

2.1. General peptide synthesis

Peptides were synthesized on Breipohl resins [64,65] using a Labortec640 peptide synthesizer (Labortec, Bubendorf, Switzerland) and employing a standard Fmoc solid-phase peptide synthesis (SPPS) protocol [66]. Briefly, the resin was swollen in DMF for 20 min prior to use. The first and subsequent Fmoc groups were removed by washing the resin with piperidine in DMF (20%, v/v) and then shaking for 25 min with another portion of piperidine in DMF. The desired sequence of amino acids was coupled to the resin using Fmoc-L-amino acids (3.0 eq), diisopropylcarbodiimide (DIPCDI, 3.3 eq) and *N*-hydroxy benzotriazole (HOBt, 3.6 eq). Peptide couplings were followed to completion using the Kaiser test [67]. After the final Fmoc removal the resin was washed with DMF, CH_2Cl_2, *i*-PrOH, CH_2Cl_2, and Et_2O and air-dried for at least 2 h.

2.2. Synthesis of Tat

The following sequence was synthesized by standard SPPS, as described above: YGRKKRRQRRRGC. Tat on Wang resin (204 mg, loading 0.59 mmol/g, 35.4 μmol peptide) was suspended in cleavage mixture (2 mL, trifluoroacetic acid/water/triisopropyl-silane/thioanisole 90:5:2.5:2.5) for 18 h. The free peptide was precipitated from Et_2O, redissolved in water and lyophilized yielding the crude peptide as a white powder. The crude peptide was purified by semi-preparative HPLC affording Tat as a white powder after lyophilization (20 mg, 33%). MALDI-TOF $[M+H]+m/z$: 1718.3 (calcd. 1718.0).

2.3. Synthesis of Tat (R>A)

The following sequence was synthesized by standard SPPS and cleaved off the resin as described above: YGAKKARQRRAGC. Tat (R>A) resin (140 mg, loading 0.59 mmol/g, 29.7 μmol peptide) afforded Tat (R>A) as a white powder after HPLC purification (6 mg, 13%). MALDI-TOF $[M+H]+m/z$: 1463.3 (calcd. 1462.8).

2.4. C12-UV-Tat (**7a**)

Tat on Breipohl resin (200 mg, loading 0.59 mmol/g, 34.7 μmol peptide) was suspended in DMF for 5 min, then DMF was removed and the resin re-suspended in fresh DMF. DMAP (5 mg, 40.9 μmol) and activated nitro-benzyl alcohol **4a** (106 mg, 50.3 μmol) were added and the suspension was shaken in the dark over night. Then the resin was washed with DMF, CH_2Cl_2, *i*-PrOH, CH_2Cl_2 and Et_2O and suspended in the cleavage mixture (2 mL, trifluoroacetic acid/water/triisopropyl-silane/thioanisole 90:5:2.5:2.5) for 18 h. The free peptide was precipitated from Et_2O, redissolved in water and lyophilized yielding the crude peptide as a white powder. The crude peptide was purified by semi-preparative HPLC affording C12-UV-Tat **7a** as a white powder after lyophilization (20 mg, 27%). MALDI-TOF $[M+H]+m/z$: 2109.9 (calcd. 2108.2), fragment at 1778.4 and 1718.9 (the UV-cleavable linker was cleaved by the MALDI laser).

2.5. C16-UV-Tat (**7b**)

The title compound was prepared as described above for C12-UV-cleavable anchor Tat **7a** from Tat resin (333 mg, loading 0.59 mmol/g, 57.8 μmol peptide), activated benzyl alcohol **4b** (186 mg, 317 μmol) and DMAP (5 mg, 40.9 μmol) affording C16-UV-Tat **7b** as a white powder after lyophilization (81 mg, 65%). MALDI-TOF $[M+H]+m/z$: 2165.3 (calcd. 2164.3), fragment at 1787.7 and 1718.2 (the UV-cleavable linker was cleaved by the MALDI laser).

2.6. C12 Tat (**8a**)

Tat on Breipohl resin (300 mg, loading 0.59 mmol/g, 52.1 μmol peptide) was suspended in DMF for 5 min, then DMF was removed and the resin re-suspended in fresh DMF. Dodecanoic acid (75 mg, 337 μmol), DIPEA (120 μL, 1.25 mmol) and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP; 155 mg, 350 μmol) were added and the suspension was shaken over night. Then the resin was washed with DMF, CH_2Cl_2, *i*-PrOH, CH_2Cl_2 and Et_2O and suspended in cleavage mixture (2 mL, trifluoroacetic acid/water/triisopropylsilane/thioanisole 90:5:2.5:2.5) for 18 h. The free peptide was precipitated from Et_2O, redissolved in water and lyophilized affording a white powder. The crude peptide was purified by semi-preparative HPLC affording C12-Tat **8a** as a white powder after lyophilization (10 mg, 10%). MALDI-TOF $[M+H]+m/z$: 1900.3 (calcd. 1900.2).

2.7. C16 Tat (**8b**)

The title compound was prepared as described above for C12 Tat **8a** from Tat on Breipohl resin (300 mg, loading 0.59 mmol/g, 52.1 μmol peptide), hexadecanoic acid (87 mg, 339 μmol), DIPEA (120 μL, 1.25 mmol) and BOP (155 mg, 350 μmol) affording C16 Tat **8b** as a white powder (20 mg, 19%). MALDI-TOF $[M+H]+m/z$: 1957.4 (calcd. 1956.2).

2.8. Optimization of irradiation time

A solution of Atto655 (22.5 μg/mL) was prepared in HEPES buffer. This corresponds to the expected concentration in the liposome preparations assuming a statistical encapsulation of the dye. This solution was irradiated with a Bluepoint2 UV-lamp (Dr. Hönle, Münich, Germany) placed directly above the quartz cuvette containing the solution for the following time intervals: 0 s, 15 s, 30 s, 1 min, 2 min, 3 min, 4 min and 5 min. After each irradiation step a fluorescence spectrum was acquired on a LS55 fluorescence spectrometer (PerkinElmer, Groningen, The Netherlands) using the following settings: excitation at 600 nm, excitation slit 5.0 nm, emission slit 7.0 nm, scan range 620–850 nm, and scan speed 100 nm/min. Unless stated otherwise irradiation, when required, was performed for 2 min throughout this work.