International Journal of Pharmaceutics 538 (2018) 179–187

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

An efficient PEGylated gene delivery system with improved targeting: Synergism between octaarginine and a fusogenic peptide

Ikramy A. Khalil[a,b,*], Hideyoshi Harashima[a,*]

[a] Laboratory of Innovative Nanomedicine, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Kita-ku, Sapporo 060-0812, Japan
[b] Department of Pharmaceutics, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt

ARTICLE INFO

Keywords:
Octaarginine
PEG
Transferrin
GALA
MEND
Nonviral gene delivery

ABSTRACT

Because of their ability to translocate different cargos into cells, arginine-rich cell penetrating peptides (CPPs) are promising vehicles for drug and gene delivery. The use of CPP-based carriers, however, is hampered by the lack of specificity and by interactions with negative serum components. Polyethylene glycol (PEG) is used to decrease such non-specific interactions, albeit its use is associated with reduced transfection efficiency. In this study, we describe the development of PEGylated CPP-based gene carrier with an improved targeting and a high transfection activity. The system was prepared by condensing DNA with a polycation followed by coating with a lipid envelope containing the octaarginine (R8) peptide as a model CPP. R8-modified nanoparticles produced high transfection activities, but the efficiency was reduced by PEG shielding. The reduced activity could be fully restored by the addition of a targeting ligand and a pH-sensitive fusogenic peptide. The efficiency of the proposed system is quite high, even in the presence of serum, and shows improved targeting and selectivity. Surprisingly, the effect of the fusogenic peptide was dramatically reduced in the absence of R8. Although shielded, R8 augmented the activity of the fusogenic peptide, suggesting a synergistic effect between the two peptides at the intracellular level.

1. Introduction

Gene therapy is a promising approach for treating a wide range of inherited and acquired diseases that are not efficiently cured by conventional medicine (Collins and Thrasher, 2015; Mountain, 2000; Naldini, 2015). Such diseases include cancer, cystic fibrosis, diabetes, hemophilia, infections and degenerative diseases. Gene therapy implies the use of genetic materials as therapeutics to express or to interfere with the synthesis of certain proteins. Successful gene therapy requires the efficient and selective transfer of therapeutic genes, not only to specific cells but to intracellular target sites as well. Genetic materials are not efficiently transferred to target sites in the absence of an appropriate vector. An ideal gene vector should efficiently, specifically, and safely transfer genetic materials to intracellular target sites. This ideal gene vector stays speculative since currently available systems lack one or more of these attributes (Itaka and Kataoka, 2009; Kamiya et al., 2013).

Cationic lipids are commonly used to condense and protect DNA in cationic complexes (lipoplexes) that can be internalized into cells and deliver DNA to intracellular target sites (Tros de Ilarduya et al., 2010; Zhang et al., 2012a). However, the fact that lipoplexes have net positive charges renders them non-specific, since they interact with negatively charged extracellular components (Khalil et al., 2006a; Zhang et al., 2012b). In addition, lipoplexes require the use of high amounts of cationic lipids which can lead to cytotoxicity issues (Torchilin et al., 2003). The emerging use of short cell-penetrating peptides (CPPs) in drug and gene delivery represents an attractive potential alternative for replacing cationic lipids, thus increasing the safety profile (Brooks et al., 2005; Fonseca et al., 2009; Gupta et al., 2005). CPPs are generally characterized by the presence of positively charged arginine residues and even peptides comprised exclusively of arginine residues have been shown to be as efficient as common protein-derived CPPs (Futaki et al., 2001a). We previously reported on the development of liposomes modified with an octaarginine peptide (R8) that showed efficient and

Abbreviations: CHEMS, cholesteryl hemisuccinate; Chol-GALA, cholesterol GALA; DOPE, dioleoyl phosphatidylethanolamine; DSPE-PEG, distearyl phosphatidylethanolamine-polyethyleneglycol 2000; DSPE-PEG-mal, maleimidic DSPE-PEG; DTT, dithiothreitol; K8, octalysine; LF-Plus, Lipofectamine Plus reagent; MEND, multifunctional envelope-type nanodevice; PDP-Tf, 3-(2-pyridinedithio)proprioyl Tf; PEG, polyethylene glycol; PEI, polyethyleneimine; PI, propidium iodide; R8, octaarginine; R8-Lip, R8-modififed liposomes; R8-MEND, R8-modified MEND; RT-MEND, Tf-modified PEGylated R8-MEND; RTG-MEND, Tf-modified PEGylated R8-MEND with GALA; SDOC, sodium deoxycholate; SPDP, 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester; STR-R8, stearylated R8; Tf, transferrin; Tf-R, Tf receptors; T-MEND, Tf-modified PEGylated MEND

* Corresponding authors at: Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan.
E-mail addresses: ikramy@aun.edu.eg (I.A. Khalil), harasima@pharm.hokudai.ac.jp (H. Harashima).

https://doi.org/10.1016/j.ijpharm.2018.01.007
Received 30 August 2017; Received in revised form 7 December 2017; Accepted 2 January 2018
Available online 16 January 2018
0378-5173/

rapid cellular internalization through endocytosis (Khalil et al., 2006b; Khalil et al., 2008). To develop gene carriers based on R8-Lip, the simple mixing of plasmid DNA (pDNA) and liposomes was not sufficient, since the peptide should be freely expressed on the surface to efficiently interact with cell membranes (Khalil et al., 2004). A nanotechnology technique that will permit the topology of the peptide to be controlled to exert its function is clearly needed. To accomplish this, we developed an R8-modified Multi-functional Envelope-type Nano Device (R8-MEND) based on the concept of Programmed Packaging (Khalil et al., 2007; Kogure et al., 2008, 2007, 2004). The MEND is composed of a condensed pDNA core covered with a lipid envelope that is modified with the R8 peptide. This system allows the integration of multiple functional devices in single nanoparticles with appropriate topology control and permits them to exert their function. The R8 modification of the MEND resulted in a high cellular uptake and improved intracellular trafficking after endocytosis (Khalil et al., 2006b, 2007). The transfection activity of the R8-MEND encapsulating pDNA was comparable to that produced by Adenovirus-mediated transfection with reduced cytotoxicity (Khalil et al., 2007). The R8-MEND successfully transfected liver tissues after systemic administration (Hayashi et al., 2012, 2011; Khalil et al., 2011). It was even more efficient than Lipofectamine reagent in transfecting hair follicles after topical administration (Khalil et al., 2007).

Despite the promise of CPPs as tools for improving gene delivery, the non-specificity issue is a common and well characterized disadvantage of cationic CPPs in general (Fonseca et al., 2009; Gupta et al., 2005). Actually, positive CPPs can be internalized with their cargos into most cell types mediated by electrostatic interactions with negative components of the cell surface. Therefore, a strategy directed at decreasing non-specific interactions and increasing the targeting ability of CPP-based gene carriers is clearly needed to extend their applications in gene therapy. While attempting to decrease the extent of non-specific interactions, it should be noted that the transfection activity in target cells must remain high. This is a general dilemma since typical methods used for decreasing non-specific interactions also frequently result in a decreased transfection activity (Hatakeyama et al., 2013). For example, hydrophilic polymers such as polyethylene glycol (PEG) are generally used to stabilize cationic systems. However, PEGylation impairs interaction with cellular membranes leading to an overall reduced activity (Deshpande et al., 2004; Hatakeyama et al., 2013; Remaut et al., 2007; Song et al., 2002). Therefore, an innovative design is needed in which target ability is combined with a high transfection activity.

The goal of this study was to improve the targeting ability of the R8-MEND system while simultaneously maintaining its transfection activity. We propose a strategy in which the system is coated with PEG, to decrease non-specific interactions, and further modified with a targeting ligand, to enhance cellular uptake and impart active targeting ability. In addition, the system is modified with a pH-sensitive fusogenic peptide to enhance the endosomal escape. The proposed system, with a net negative charge and a dense PEG coat, shows a high transfection activity that is comparable to that of non-PEGylated R8-MEND. Compared to the original system, the optimized system showed an improved targeting, a higher specificity to cancer cells and an improved serum resistance. This study shows a dual effect of a CPP and a targeting ligand where each component exerts its function at the appropriate step. In addition, CPP and fusogenic peptides show a synergistic effect at the intracellular level.

We believe that PEGylation and adding targeting ability to CPP-based carriers while retaining their high activity has significant implications in the future design of nonviral gene delivery systems. In addition, the use of CPP for the augmentation of fusogenic peptides is a novel strategy that is expected to expand the applicability of these peptides in future medicines.

2. Materials and methods

2.1. Materials

Plasmid DNA pCMV-luc (7037 bp) encoding luciferase (pDNA) was amplified in *Escherichia coli* and purified using an EndoFree Plasmid Mega Kit (Qiagen, Hilden, Germany). Cholesteryl hemisuccinate (CHEMS), human holo-transferrin (Tf), 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP), dithiothreitol (DTT), triiodothyronine (T3), L-thyroxine (T4) and linear polyethyleneimine (PEI) (average MW 750 KDa) were purchased from SIGMA-Aldrich (St. Louis, MO, USA). Dioleoyl phosphatidylethanolamine (DOPE), cholesterol (Chol), distearyl phosphatidyl ethanolamine-polyethyleneglycol 2000 (DSPE-PEG) and maleimidic DSPE-PEG (DSPE-PEG-mal) were purchased from AVANTI Polar Lipids (Alabaster, AL, USA). Stearylated R8 (STR-R8) and cholesterol-GALA (Chol-GALA) were synthesized as described previously (Futaki et al., 2001b; Kakudo et al., 2004). Propidium iodide (PI), sodium deoxycholate (SDOC), L-ascorbate phosphate magnesium salt *n*-hydrate and chloroquine diphosphate were purchased from WAKO Pure Chemicals (Osaka, Japan). Lipofectamine Plus reagent (LF-Plus), Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Luciferase assay reagent and reporter lysis buffer were obtained from Promega Co. (Madison, WI, USA). HeLa human cervix carcinoma cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan).

2.2. Cell culture

HeLa cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. 3T3-L1 cells were cultured in DMEM supplemented with glucose (4.5 g/L), HEPES (10 mM), L-glutamine (4 mM), ascorbate (0.2 mM), T3 (1 nM), T4 (30 nM), and 10% heat-inactivated FBS. H9C2 cells were cultured in DMEM supplemented with glucose (4.5 g/L), L-glutamine (4 mM), pyruvate (0.11%) and 10% heat-inactivated FBS.

2.3. Preparation of MENDs

The R8-MEND was prepared by the lipid hydration method as reported previously with minor modifications (Kogure et al., 2004). A diluted solution of PEI (100 μL) in 10 mM HEBES buffer (pH 7.4) (HB) was added drop wise under vortexing to a dilute solution of pDNA (200 μL). The solution was then allowed to stand at room temperature (RT) for 15 min. The nitrogen/phosphate (N/P) ratio in the final solution is adjusted to 0.8. Simultaneously, a lipid film was formed by the evaporation of chloroform:ethanol (3:1) solution of lipids (90 nmol) in a round bottom glass tube. The lipid composition was DOPE/Chol/STR-R8 (7:2:1 molar ratio). The core solution (300 μL) was added to the glass tube containing the dried lipid film and the tube was rotated gently to allow electrostatic binding between the negative DNA core and the positive lipid film. After 15 min incubation at RT to allow hydration of the lipid film, the tube was sonicated for ~1 min in a bath type sonicator. The resulting R8-MEND suspension was allowed to stand for 30 min at RT. In the case of a MEND without R8 modification, the lipid film was composed of DOPE/Chol/CHEMS (7:2:2 molar ratio) and the pDNA/PEI core was prepared at an N/P ratio 4. MENDs containing GALA was prepared in the same manner, except for the inclusion of Chol-GALA (2 mol% of total lipid) in the chloroform:ethanol solution of lipids. PEGylation of different MENDs was performed by a post-modification method. An HB solution containing different amounts of DSPE-PEG was added to the MEND suspension followed by incubation for 30 min at RT.