International Journal of Pharmaceutics 468 (2014) 83–90

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

New amphiphilic *N*-phosphoryl oligopeptides designed for gene delivery

Yunfei Sun [1], Long Chen [1], Fude Sun, Xibo Tian, Shi-Zhong Luo *

Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, PR China

ARTICLE INFO

Article history:
Received 27 December 2013
Received in revised form 13 March 2014
Accepted 3 April 2014
Available online 5 April 2014

Keywords:
Gene delivery
Non-viral vector
Cationic lipopeptide
Transfection

ABSTRACT

Gene therapy is a potent tool for the treatment of cancer and other gene defect diseases, which involves using DNA that encodes a functional, therapeutic gene to replace a mutated gene. However, the DNA transfection efficiency is restricted by its negative charges and low susceptibility to endonucleases which prevent them penetrating tissue and cellular membranes. Both viral and non-viral vectors have been used for gene delivery, but the former are limited by their immunogenicity, while the latter are less efficient than their viral counterpart. Cationic amphiphilic lipopeptides whose structures can be easily modified and transformed have been used as non-viral vectors in gene delivery system due to their low cytotoxicity and high transfection efficiency. In this study, a series of cationic amphiphilic *N*-phosphoryl oligopeptides with varied lengths of hydrophobic tails and oligopeptide headgroups (C12-K6, C14-K6, C16-K6, Chol-K6 and C12-H6) were synthesized and used as gene delivery vectors. The affinities, abilities to condense pDNA and transfection efficiencies of the K6-lipopeptides were better than those of the H6-lipopeptides. In addition, the hydrophobic chains of the lipopeptides also affected their transfection efficiencies. The K6-lipopeptide with a hydrophobic chain of twelve carbons (C12-K6) showed the highest transfection efficiency in all these synthetic lipopeptides. At an optimal P/N ratio of 20, C12-K6 showed comparable pDNA transfection efficiency to PEI-25k, a well-defined gene delivery vector, but the cytotoxicity of C12-K6 was much lower. With acceptable gene transfection efficiency and low cytotoxicity, this cationic amphiphilic lipopeptide will have promising applications in gene therapy.

1. Introduction

Many diseases, such as cancer and hemophilia, are associated with the acquired and inherited defects of related genes. For example, about 30% of tumors, including lung, colon, thyroid, and pancreatic carcinomas, have a mutation in RAS gene. Another example is hemophilia, which is associated with defects of clotting factors IIX, IX and XI. Gene therapy has attracted much attention in the treatment of this kind of diseases as it is accomplished by transporting a therapeutic gene (pDNA, siRNA, ODNA, etc.) into target cells to replace a deficient gene or lead to a modulation of the expression of genes, thereby restoring production of a functional protein (Friend et al., 1996).

However, the DNA transfection efficiency is restricted by its negative charges and low susceptibility to endonucleases which prevent them penetrating tissue and cellular membranes. Thus, gene delivery vectors are used to facilitate genes transfection and protect them from being degraded. Depending on the vectors used for nucleic acid transfer, gene delivery is usually divided into two main systems: viral and non-viral gene delivery. Although viral vectors are widely studied and have some advantages, their immunogenicity and potential oncogenicity may cause serious problems in terms of safety and limited their application *in vivo* (Luo and Saltzman, 2000). A broad range of non-viral delivery systems including mechanical, electrical and chemical methods have been established. For example, naked DNA can be directly injected into a cell's nucleus by microinjection (Harland and Weintraub, 1985). Cellular uptake of exogenous DNA can be also realized by electrical pulses which are able to transiently permeabilize cell membranes (Heiser, 2000). The first chemical method for DNA delivery was introduced in the late 1950s, and high salt concentration and polycationic proteins were used to improve the ability of nucleic acids to enter cells (Felgner, 1990). DEAE-dextran (Pagano, 1970) and calcium phosphate (Schenborn and Goiffon, 2000) were also used for interaction with DNA to form DEAE-dextran-DNA and calcium phosphate-DNA complexes, respectively, which were internalized via endocytosis after being deposited onto cells. So far, synthetic gene delivery

* Corresponding author. Tel.: +86 10 64438015; fax: +86 10 64438015.
E-mail address: luosz@mail.buct.edu.cn (S.-Z. Luo).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ijpharm.2014.04.007
0378-5173/

chemicals, including cationic lipids (Martin et al., 2005), cationic polymers (Boussif et al., 1995; Furgeson et al., 2003; Suh et al., 1994; Xu and Yang, 2011), cell-penetrating peptides (CPPs) (Furgeson et al., 2003; Parente et al., 1990; Suh et al., 1994; Trabulo et al., 2010) and some dendrimers (Furgeson et al., 2003; Kukowska-Latallo et al., 1996; Suh et al., 1994; Zhou et al., 2006) have become the most widely used vectors in biological studies and pre-clinical gene therapies.

The basic principle of non-viral vectors mediated transfection is as follows: (i) Non-viral vectors compact large DNA molecules into small particles by electrostatic interactions between the positively charged vectors and negatively charged DNA molecules (Furgeson et al., 2003; Gershon et al., 1993; Ma et al., 2007; Suh et al., 1994). The formation of vector/DNA complex can also protect DNA from degradation by enzymes in the external environment. (ii) An excess addition of cationic vectors equip the complex surfaces with positive charges, which is presumed to facilitate subsequent cellular uptake via interaction with the negative cell surface structures such as heparin sulphates and other proteoglycans (Mislick and Baldeschwieler, 1996). These complexes are taken up by cells through clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis (Conner and Schmid, 2003; Kirkham and Parton, 2005; Nichols, 2003; Suh et al., 1994). (iii) Once in the endocytic pathway, the plasmids may be degraded when reaching the lysosomes. Accordingly, for effective transfections, the plasmids need to acquire cytosolic access at an earlier stage by escaping from the endosomes (Mukherjee et al., 1997). Different mechanisms such as pore formation in the endosomal membranes (Huang et al., 2004), pH-buffering effect of protonable groups (Moreira et al., 2009; Pack et al., 2000; Suh et al., 1994; Varkouhi et al., 2011) and fusion into the lipid bilayer of endosomes (Xu and Szoka, 1996) have been proposed to facilitate the endosomal escape.

Felgner et al. (Felgner et al., 1987) reported the first cationic lipid named DOTMA (*N*-(1-(2,3-dioleyloxy) propyl)-*N*,*N*,*N*-trimethyl-ammonium chloride), which consists of a quaternary amine connected to two unsaturated aliphatic hydrocarbon chains via ether groups. DOTMA was 5 to over 100 folds more effective than either the DEAE-dextran or the calcium phosphate transfection technique. Cationic lipids are amphiphilic molecules that consist of the following three structural segments: (i) a hydrophilic headgroup which is positively charged, usually via the protonation of monovalent or multivalent amine groups; (ii) a hydrophobic region composed of a steroid or alkyl chains (saturated or unsaturated); (iii) a linker connecting the cationic headgroup with the hydrophobic anchor, whose nature and length may impact on the stability and biodegradability of the vector.

Cell-penetrating peptides (CPPs) are short peptides with potential abilities to translocate across the plasma membrane of living cells. Many CPPs are derived from the transduction domains of viral proteins involved in interaction with cell membranes (Vivès et al., 1997). A typical example of CPPs is HIV Tat (Ignatovich et al., 2003) consisting of 6 arginines and 2 lysines in its 13 amino acid residues, which is effective in binding to plasmid DNA through charge interaction and condensing it. CPPs have been studied during the last decades as materials for drug delivery vehicles due to their biodegradability, biocompatibility, low toxicity and ease of synthesis (Torchilin et al., 2003).

In this study, a series of cationic amphiphilic *N*-phosphoryl oligopeptides (C12-K6, C14-K6, C16-K6, Chol-K6 and C12-H6) were designed and synthesized using the phosphite method (Ma and Zhao, 1992; Moreira et al., 2009; Musiol et al., 1994; Suh et al., 1994) and Fmoc solid-phase synthesis. Results showed that the length and structures of the *N*-terminal phosphoryl ester chains could dramatically affect the transfection efficiency of lipopeptide/pDNA complexes. At an optimal P/N ratio of 20, C12-K6 showed best pDNA transfection efficiency which is comparable to the commercial gene delivery vector PEI-25k, but its cytotoxicity was much lower. With acceptable gene transfection efficiency and low cytotoxicity, this cationic amphiphilic lipopeptide could be potentially applied in gene therapy.

2. Materials and methods

2.1. Materials

Dodecanol, tetradecanol, hexadecanol and cholesteryl chloroformate were purchased from Alfa Aesar Co. (Ward Hill, MA). Fmoc-Lys(Boc)-Wang resin, H-His(Trt)-2-Chlorotrityl resin, Fmoc-Lys(Boc)-OH, Fmoc-His(Trt)-OH, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetra-methyluroniumhexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), piperdine, diisopropylethylamine (DIEA), thioanisole, dithioglycol and trifluoroacetic acid (TFA) were obtained from GL Biochem (Shanghai) Ltd. (Shanghai, China). Dimethylformamide (DMF) and dichloromethane (DCM) were provided by DiKMA Technologies Inc. (Beijing, China). Branched poly(ethylene imine) (PEI-25k, Mw = 25,000) was supplied by Sigma–Aldrich Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin were purchased from Gibco (Grand Island, NY). Dimethylsulfoxide (DMSO) and thiazoyl blue tetrazolium bromide (MTT) were purchased from BioDee (Beijing, China). pEGFP-N2 and 293T cell line were generously provided by the laboratory of Prof. Yanmei Li (School of Life Science, Tsinghua University).

2.2. Synthesis of the lipopeptides

The *N*-phosphoryl oligopeptides were synthesized from phosphites and oligopeptides, which could be divided into two major steps: synthesis of phosphites and its incorporation to the oligopeptides (Ma and Zhao, 1992; Moreira et al., 2009; Musiol et al., 1994; Suh et al., 1994). The synthesis of phosphites with long dialkoxyl chains was obtained through direct esterification of phosphorus trichloride by 3 equivalents of alcohol in benzene (Fig. 1). Separation of the products was accomplished by reduced pressure distillation.

Peptides were synthesized on resin utilizing standard Fmoc solid phase procedure. Phosphites (Fig. 1A) and cholesteryl chloroformate (Fig. 1B) were incorporated as the last amino acid. The resulting dry resin bound lipopeptides were cleaved and side-chains were deprotected using a cocktail of TFA:H_2O:thioanisole:phenol:ethanedithiol (82.5:5:5:5:2.5). The resin was then removed by filtration, and TFA was evaporated with a slow stream of N_2. The lipopeptides were precipitated from the filtrate with cold diethyl ether. The resulting crude lipopeptides were then purified by RP-HPLC (Cosmosil C18 & C4 peptide/protein column). Purified lipopeptide solutions were evaporated to remove organic phase and then lyophilized, obtaining a pure lipopeptide powder utilized for all subsequent experiments. The purity (>90%) of each lipopeptide was assessed by analytical HPLC and ESI-MS.

2.3. Agarose gel retardation assay

To examine the ability of each cationic lipopeptide to bind pDNA, an agarose gel retardation assay was performed (Weyland et al., 2013). The complex solution was prepared under a predetermined N/P charge ratio (the molar ratio of the amine groups in the lipopeptides to the phosphates in pDNA) by mixing pDNA (0.8 μg pDNA in 8 μl TE buffer) with an equal volume of the lipopeptides in PBS buffer solution (pH 7.4). Each mixture was vortexed for 30 s and incubated for 30 min at room temperature. Then, the mixture was analyzed on a 0.8% agarose gel containing