Acta Biomaterialia 58 (2017) 146–157

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Manipulating the membrane penetration mechanism of helical polypeptides via aromatic modification for efficient gene delivery

Nan Zheng [a,b,1], Ziyuan Song [a,1], Jiandong Yang [c,1], Yang Liu [a], Fangfang Li [c], Jianjun Cheng [a,*], Lichen Yin [c,*]

[a] Department of Materials Science and Engineering, University of Illinois at Urbana–Champaign, 1304 W Green Street, Urbana, IL 61801, USA
[b] State Key Laboratory of Fine Chemicals, Department of Polymer Science and Engineering, School of Chemical Engineering, Dalian University of Technology, Dalian 116024, PR China
[c] Institute of Functional Nano & Soft Materials (FUNSOM), Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Collaborative Innovation Center of Suzhou Nano Science and Technology, Soochow University, Suzhou 215123, PR China

ARTICLE INFO

Article history:
Received 24 January 2017
Received in revised form 12 April 2017
Accepted 1 May 2017
Available online 3 May 2017

Keywords:
Helical polypeptide
Aromatic domain
Gene delivery
Cell penetration mechanism
Direct translocation

ABSTRACT

The delivery performance of non-viral gene vectors is greatly related to their intracellular kinetics. Cationic helical polypeptides with potent membrane penetration properties and gene transfection efficiencies have been recently developed by us. However, they suffer from severe drawbacks in terms of their membrane penetration mechanisms that mainly include endocytosis and pore formation. The endocytosis mechanism leads to endosomal entrapment of gene cargos, while the charge- and helicity-induced pore formation causes appreciable cytotoxicity at high concentrations. With the attempt to overcome such critical challenges, we incorporated aromatic motifs into the design of helical polypeptides to enhance their membrane activities and more importantly, to manipulate their membrane penetration mechanisms. The aromatically modified polypeptides exhibited higher cellular internalization level than the unmodified analogue by up to 2.5 folds. Such improvement is possibly because aromatic domains promoted the polypeptides to penetrate cell membranes via direct transduction, a non-endocytosis and non-pore formation mechanism. As such, gene cargos were more efficiently delivered into cells by bypassing endocytosis and subsequently avoiding endosomal entrapment, and the material toxicity associated with excessive pore formation was also reduced. The top-performing aromatic polypeptide containing naphthyl side chains at the incorporated content of 20 mol% revealed notably higher transfection efficiencies than commercial reagents in melanoma cells *in vitro* (by 11.7 folds) and *in vivo* (by 9.1 folds), and thus found potential utilities toward topical gene delivery for cancer therapy.

Statement of significance

Cationic helical polypeptides, as efficient gene delivery materials, suffer from severe drawbacks in terms of their membrane penetration mechanisms. The main cell penetration mechanisms involved are endocytosis and pore formation. However, the endocytosis mechanism has the limitation of endosomal entrapment of gene cargos, while the charge- and helicity-induced pore formation causes cytotoxicity at high concentrations. To address such critical issues toward the maximization of gene delivery efficiency, we incorporated aromatic domains into helical polypeptides to promote the cell membrane penetrations via direct transduction, which is a non-endocytosis and non-pore formation mechanism. The manipulation of their membrane penetration mechanisms allows gene cargos to be more efficiently delivered by bypassing endocytosis and subsequently avoiding endosomal entrapment.

1. Introduction

Gene therapy has emerged as a promising strategy for the treatment of various genetic diseases, such as cancer, neurodegenerative diseases, and immunodeficiency [1–5]. The key challenge toward successful gene therapy is the development of effective delivery systems because nucleic acids themselves cannot penetrate into cells and suffer from rapid degradation by the nucleases in the body [6–8]. While viral vectors afford high transfection efficiencies, they often suffer from serious clinical risks such as oncogenicity, immunogenicity, and insertional mutagenesis [9]. Non-viral vectors, mainly exemplified by cationic polymers

* Corresponding authors.
E-mail addresses: jianjunc@illinois.edu (J. Cheng), lcyin@suda.edu.cn (L. Yin).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.actbio.2017.05.001
1742-7061/

(polycations) and cationic lipids, have been considered as ideal alternatives to viral vectors because of their minimal immunogenicity, desired biocompatibility, and chemical flexibility [10–15]. However, the clinical application of non-viral vectors has been greatly hampered by their low transfection efficiencies due to the various extracellular and intracellular barriers that prevent the effective delivery of nucleic acid cargos to the target cells/organelles [16]. Among the various barriers, the biological membranes serve as a major delivery barrier against effective gene transfection. For instance, the cell membrane prevents the internalization of nucleic acids; the endosomal/lysosomal membrane prevents effective delivery into cytoplasm and thus leads to degradation of gene cargos in the lysosome; the nucleus membrane prevents DNA entry into the nuclei and thus prevents gene transcription in the nuclei [17]. As such, development of gene carriers with potent membrane activities is highly necessitated toward effective gene transfection.

Cell penetrating peptides (CPPs) are sequence-specific short oligopeptides which can mediate effective membrane penetration and translocation via either energy-dependent endocytosis or energy-independent transduction [18]. They often consist of 10–30 amino acid residues and contain a large number of positively charged amino acids such as lysine and arginine [19]. Well-known CPPs include HIV-TAT, Arg9, penetratin, and melittin. Most of the CPPs adopt inherent α-helical secondary structures, or can form trans-membrane α-helix after interacting with cell membranes, which can stabilize the membrane interactions and facilitate the cellular internalization [20,21]. Due to their excellent membrane activities, CPPs are widely utilized to facilitate the cellular delivery of a variety of exogenous materials [22]. However, when used for gene delivery, the short backbone length and insufficient cationic charge density of CPPs greatly impede their capabilities to condense and deliver genes independently [23,24]. Particularly, after complexing the gene cargos, the cationic charges and α-helical secondary structures of the short CPPs will be shielded, and thus the charge- and helix-dependent membrane activities are greatly compromised. To address such critical challenges of traditional CPPs, we recently developed cationic polypeptides with sufficiently long backbone length and stable helical structures that afford both strong membrane penetration properties and superb gene transfection efficiencies [25–28].

While the cationic helical polypeptides display excellent performance as a new category of cell penetration and gene delivery materials, they suffer from undesired drawbacks that are related to their intracellular kinetics. Particularly, the stiff helical structure allows the polypeptide to puncture pores on cell membranes, thus facilitating the direct transduction of gene cargos into cells [25–27]. However, only part of the gene cargos is internalized via such "pore formation" mechanism, while the rest of them is internalized via endocytosis, a process that leads to endosomal/lysosomal entrapment against effective gene transfection. Additionally, the charge- and helicity-induced "pore formation" mechanism will cause irreversible damage to cells when polypeptides were used at excessively high concentrations [25–27,29]. With the attempt to maximize the transfection efficiencies of helical polypeptides while minimize their toxicities, we thus seek a strategy to manipulate the membrane penetration mechanisms of helical polypeptides by promoting the non-endocytic cell uptake yet alleviating the "pore formation" mechanism.

It has been reported that hydrophobic counterions could promote the interactions of CPPs with phospholipids by coating cationic structures with lipophilic moieties as activators [30,31]. Recently, the hydrophobic domain has also been introduced into the side chains or backbones of CPPs and polymeric CPP mimics to "self-activate" their membrane penetration properties [25,32–34]. Among various hydrophobic motifs, aromatic activators have been demonstrated to outperform aliphatic domains in enhancing membrane activities, mainly due to their flat-rigid shapes and π-electronic structures, specific interactions such as π-cation interactions, and the favorable ability to anchor proteins in the membranes [30,31]. For instance, sodium 4-(pyren-1-yl)butane-1-sulfonate exhibited better penetration enhancement than sodium dodecane-1-sulfonate [32,35]. More importantly, the direct membrane translocation of CPPs, a non-endocytic and "non-pore formation" mechanism, is closely related to the presence of aromatic domains. For instance, the incorporation of tryptophan residue into the CPP building blocks will promote their cell internalization via an energy-independent non-endocytosis pathway [36].

Based on the above understandings on the advantages of aromatic motifs, we in the current study incorporated various aromatic groups (phenyl, naphthyl, and anthryl) into the design of cationic, α-helical polypeptides, attempting to strengthen their membrane activities and potentially manipulate the membrane penetration mechanisms. To this end, random co-polypeptides bearing both charged amine groups and aromatic groups on the side chain terminals were synthesized, and we hypothesized that the aromatic domain will allow more polypeptides to penetrate cell membranes via direct transduction by bypassing the endocytic pathways. As such, they would be able to mediate effective gene delivery by minimizing the endosomal/lysosomal entrapment (Scheme 1). Additionally, due to the partial replacement of amine groups by aromatic groups, the cationic charge density of the polypeptides could be diminished, such that the excessive pore formation on cell membranes could be restricted to reduce the material toxicity. To demonstrate such hypothesis, the effect of aromatic groups on the membrane penetration potency, cell penetration mechanisms, *in vitro* and *in vivo* gene delivery efficiencies, and cytotoxicities were mechanistically explored.

2. Materials and methods

2.1. Materials and cell lines

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Tetrahydrofuran (THF), hexane, and dimethylformamide (DMF) were dried by a column packed with 4Å molecular sieves and stored in a glovebox. Dry nitrobenzene (NB) was prepared by treating regular NB with CaH_2 followed by distillation under reduced pressure. Hexamethyldisilazane (HMDS) and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) were dissolved in anhydrous DMF in a glovebox. γ-(4-Vinylbenzyl)-L-glutamate *N*-carboxyanhydride (VB-L-Glu-NCA) was prepared as previously reported [25–27]. Pierce BCA assay kit was purchased from ThermoFisher Scientific (Rockford, IL, USA). Plasmid DNA (pDNA) encoding luciferase (pCMV-Luc) was purchased from Elim Biopharm (Hayward, CA, USA). 3-(4,5-Dimethylthiahiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and YOYO-1 were purchased from Invitrogen (Carlsbad, CA, USA).

HeLa (human cervix adenocarcinoma cells) and B16F10 (murine melanoma cells) were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Female C57BL/6 mice (8–10 week old) were obtained from Charles River Laboratory (Wilmington, MA, USA) and were housed in a germ-free environment with four mice per cage. Mice were given access to food and water and exposed to a 12:12 h light–dark cycle (7:00 am–7:00 pm) at 25 ± 1 °C. The animal experimental protocol was approved by the Institutional Animal Care and Use