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Gene delivery from supercharged coiled-coil protein and cationic lipid hybrid complex

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ABSTRACT

A lipoproteoplex comprised of an engineered supercharged coiled-coil protein (CSP) bearing multiple arginines and the cationic lipid formulation FuGENE HD (FG) was developed for effective condensation and delivery of nucleic acids. The CSP was able to maintain helical structure and self-assembly properties while exhibiting binding to plasmid DNA. The ternary CSP·DNA(8:1)·FG lipoproteoplex complex demonstrated enhanced transfection of β-galactosidase DNA into MC3T3-E1 mouse preosteoblasts. The lipoproteoplexes showed significant increases in transfection efficiency when compared to conventional FG and an mTat·FG lipopolyplex with a 6- and 2.5-fold increase in transfection, respectively. The CSP·DNA(8:1)·FG lipoproteoplex assembled into spherical particles with a net positive surface charge, enabling efficient gene delivery. These results support the application of lipoproteoplexes with protein engineered CSP for non-viral gene delivery.

1. Introduction

A central challenge for gene therapy is the effective delivery of highly labile nucleic acids that are susceptible to nucleases [1]. While there are examples of successful nucleic acid delivery *in vitro* and *in vivo* by viral and non-viral vectors, achieving high transfection efficiency while maintaining low toxicity remains a significant challenge [2,3]. Although virus-mediated vehicles are very efficient in gene transduction [4–6], they exhibit severe immunogenic properties and can cause detrimental mutagenic responses rendering them problematic [7,8].

Considerable effort has been made to develop non-viral vectors such as cationic lipids [9,10], cationic polymers [3,11], and cell-penetrating peptides (CPPs) [12,13]. Cationic lipids form non-covalent complexes with nucleic acids to generate lipoplexes. However, as the condensation ability of lipids alone is not effective, the resulting lipoplexes do not protect genes against nucleases *in vivo* [14,15]. Cationic polymers such as polyethylenimine (PEI) [16–18], poly (L-lysine) (PLL) [19,20], polyamidoamine (PAMAM) dendrimers [21] and polymethacrylates [22] form particulate complexes with DNA producing polyplexes that can deliver genes [16,23]. Although such polyplexes demonstrate higher transfection ability, they exhibit high cytotoxicity. Moreover, further chemical modifications to the cationic polymers are required to reduce their cytotoxicity. The resultant chemically modified polymers demonstrate decreased transfection ability [24,25]. While CPPs have been explored for their ability to deliver nucleic acids, delivery remains a major challenge [13] due to entrapment into endocytic vesicle and lysosomal degradation [26,27]. Recently, lipopolyplexes composed of a cationic lipid and cationic peptide-based ternary complex have been introduced to enhance transfection of nucleic acids [28–34]. While lipopolyplexes have been successfully employed for gene delivery, it depends on the development of branched systems carrying a net positive charge [35]; in such cases, identifying optimal branching, charge and sequence will require various synthetic design strategies.

Protein engineered systems have emerged as an alternative to synthetic counterparts due to the unique advantages of programmed specificity in terms of structure and assembly, environmentally friendly production, non-toxic contaminants and biodegradability [36,37]. In this study, we have engineered supercharged coiled-coil protein (CSP), derived from cartilage oligomeric matrix protein coiled-coil (COMPcc). The solvent exposed residues are mutated into arginine for effective binding to plasmid DNA and cationic lipids are introduced in conjunction with CSP to produce what we term "lipoproteoplexes" for enhanced gene delivery

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(Fig. 1). The CSP is expressed, purified and assessed for its secondary structure and binding ability to DNA. The optimal ratio of FG to CSP is developed for *in vitro* delivery of β-galactosidase gene into MC3T3-E1 mouse preosteoblasts. The CSP and lipoproteoplex are evaluated for cytotoxicity against MC3T3-E1 cells. Also, CSP·DNA complex and lipoproteoplexes are further characterized for their size, surface charge and morphology.

2. Materials and method

2.1. Materials

Primers were purchased from Eurofin MWG Operon (Huntsville, AL), *pfu* Ultra DNA polymerase from Stratagene (Santa Clara, CA) and *DpnI* restriction enzyme from New England Biolabs (Ipswich, MA). Tris base, isopropyl β-D-1-thiogalactopyranoside (IPTG), tryptone, ampicillin, sodium chloride, imidazole and urea were obtained from VWR. Ni-NTA beads were purchased from Sigma–Aldrich, β-galactosidase plasmid DNA from Genlantics (San Diego, CA) and Beta-Glo assay kit from Promega, (Madison, WI). Gibco alpha minimal essential medium (αMEM), Gibco fetal bovine serum (FBS), 5000 U/mL penicillin and 5000 μg/mL streptomycin were purchased from Invitrogen (Carlsbad, CA). FG was obtained from Roche (Branchburg, NJ) and the HIV-1-Tat (RKKRRQRRRR) modified (mTat) with ten histidine residues and two cysteine residues (C-5H-Tat-5H-C) was purchased from Biomatik Corporation (Cambridge, Canada) [38]. The MC3T3-E1, subclone 14 (CRL-2594) mouse preosteoblasts was gift from Dr. Mani Alikhani (New York University College of Dentistry).

2.2. Site-directed mutagenesis (SDM) and PCR assembly

The COMPcc gene in pQE9 vector was used as a template to perform multiple mutations. The residues at D28, A30, E39, Q45, F60, M66, E67 and D69 were mutated to arginine by using following primers and their complementary sequences. **D28R and A30R**/5′-CAT CAC GGA TCC GGT CGT CTG CGT CCG CAG ATG-3′; **E39R**/5′-GAA CTG CAG CGT ACC AAC-3′; **Q45R**/5′-GCG CTG CGT GAC GTT CG-3′; **F60R**/5′-GAA ATC ACC CGT CTG AAA-3′; **M66R, E67R and D69R**/5′-C ACC GTT CGT CGT TCT CGT GCG TCT GGT AAG CTT AAT TAG-3′

The DNA fragments with required mutations were synthesized by PCR by using forward primer of one mutant and reverse primer of the following mutant [39,40]. The resulting gene bearing all 24 base pairs mutations was used as a megaprimer for mutagenesis of pQE9/COMPcc [41,42] to produce pQE9/CSP. Site directed mutagenesis SDM was performed using a standard protocol and the resulting sample was digested with *Dpn*I enzyme (New England Biolabs) for 3 h at 37 °C. The *Dpn*I digested sample was transformed into XL-1 blue cells.

2.3. Protein expression and purification

To express the CSP and COMPcc proteins, the *Escherichia coli* strains AF-IQ [43,44] and XL-1 blue were used for transformation of CSP and COMPcc, respectively. Starter culture of CSP and COMPcc were made in 5 mL of LB containing ampicillin (200 μg/mL) and chloramphenicol (35 μg/mL) and LB containing ampicillin (200 μg/mL), respectively and incubated overnight at 37 °C and 350 rpm. The starter cultures were used to inoculate 800 mL of LB media with the aforementioned respective antibiotics and incubated for 6 h at 37 °C and 250 rpm for large scale expression. After 6 h, OD_{600} was measured (≈0.8–1.0) and the protein expression was induced by the addition of 200 μg/mL IPTG and incubated under the same conditions for 3 h. Cells were harvested after overexpression by centrifugation and stored at −80 °C until purification. Overexpression was confirmed by SDS-PAGE (Fig. S1a). The cells pellets were thawed and resuspended into 50 mM Tris–HCl buffer pH 8.0 with 0.5 M NaCl, 20 mM imidazole and 6 M urea and lysed via sonication. Whole cell lysates were clarified by centrifugation and purified under native condition using Ni-NTA beads. Purification was performed in a 10 mL gravity column (Thermo Scientific). The beads were washed with the buffer composed of 50 mM Tris–HCl, 0.5 M NaCl and 20 mM imidazole and the protein was eluted with increase concentration of imidazole from 200 mM, 500 mM and 1 M imidazole. The purity of the proteins was confirmed by SDS-PAGE (Fig. S1b and Fig. S1c). The proteins were dialyzed against 50 mM Tris–HCl buffer pH 8.0 with 0.5 M NaCl to remove the imidazole.

2.4. CD spectroscopy

The secondary structure of CSP/COMPcc was analyzed using a Jasco J-815 spectrometer at 10 μM protein concentration in 50 mM Tris–HCl buffer pH 8.0. The wavelength scans were performed at 4 °C over a range of 200–250 nm with a 1 nm step size. Temperature scans were performed at 222 nm from 20 °C to 85 °C with temperature ramp of 1 °C/min. The observed ellipticity value (Θ) was converted into mean residue ellipticity (MRE) using the standard equation $\Theta_{MRE} = \Theta/(10\,cpl)$ where c is the molar concentration of the protein, p is the path length in centimeters and l is the number of amino acids [17]. The fraction folded was derived using equation $F = (\Theta_A - \Theta_U)/(\Theta_N - \Theta_U)$, where, Θ_A is the MRE observed at given temperature, Θ_U is MRE value for completely unfolded protein and Θ_N is the MRE value of completely folded protein that is considered at 25 °C. The first derivative of fraction folded was used to calculate melting temperature (T_m) of protein [44]. All data were represented as an average of three trials.

2.5. Electrophoretic mobility shift assay and lipoproteoplex preparation

Plasmid DNA encoding β-galactosidase (gWiz β-galactosidase) under the control of the cytomegalovirus promoter/enhancer was used to investigate protein binding and also acted as a reporter for successful transfection. The β-galactosidase plasmid DNA (5.1 kb) at a concentration of 50 ng was mixed with different concentrations of

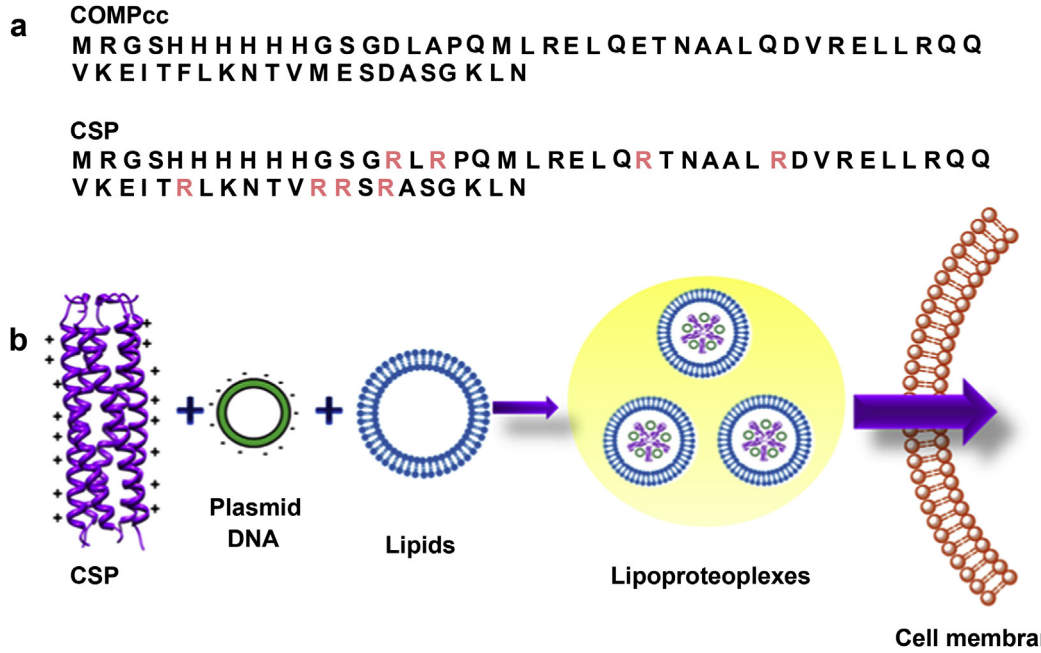

Fig. 1. a) Aligned sequences of COMPcc and CSP with mutated arginine residue positions shown in red. b) Schematic of CSP complexation with plasmid DNA and a ternary complex with cationic lipids to form lipoproteoplexes for gene delivery. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)