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Combinatorial library of chalcogen-containing lipidoids for intracellular delivery of genome-editing proteins

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ABSTRACT

Protein based therapeutics with high specificities and low off-target effects are used for transient and accurate manipulation of cell functions. However, developing safe and efficient carriers for intracellular delivery of active therapeutic proteins is a long-standing challenge. Here we report a combinatorial library of chalcogen (O, S, Se) containing lipidoid nanoparticles (LNPs) as efficient nanocarriers for intracellular delivery of negatively supercharged Cre recombinase ((-30)GFP-Cre) and anionic Cas9:single-guide RNA (Cas9:sgRNA) ribonucleoprotein (RNP) for genome editing. The structure-activity relationship between the lipidoids and intracellular protein delivery efficiencies was explored and it was demonstrated that the newly developed LNPs are effective for gene recombination *in vivo*.

1. Introduction

Proteins as the workhorse biomacromolecules play important roles in the cell and life. Alteration, deficiency or malfunction of proteins within the cell often results in serious conditions [1]. Protein-based therapeutics with high tolerances and specificities, as well as low off-target effects have attracted tremendous attention during the last three decades, as either replacement therapy for protein deficiency or to elicit a therapeutic effect [2–4]. One example is the recently developed CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat associated protein 9) platform with high flexibility and specificity for genome editing through the gene deletion, insertion, activation, repression and even epigenetic modification, which could facilitate disease modeling and new treatments for various genetic disorders and infectious diseases [5–8]. However, like many other protein therapeutics, the development of safe and effective intracellular delivery systems for CRISPR/Cas9 and other genome-editing platforms is still a long-standing challenge, due to their relatively large hydrodynamic sizes, varying surface physicochemical properties, and vulnerable higher order structures. [9,10].

So far, mechanical and physical delivery methods including microinjection, electroporation and hydrodynamic injection have been employed for CRISPR/Cas9 and other genome editing protein delivery [8,11]. These methods are straightforward and usually highly efficient, but are also invasive and suffer from many practical issues which limit *in vivo* application [12,13]. Biochemical modification of CRISPR protein with functional targeting ligands like nuclear localization signal (NLS) peptide [14] and supramolecular delivery systems such as lipid and lipid-like (lipidoid) nanoparticles (LNPs) [15–17], polymeric assemblies [18–20], as well as inorganic nanoparticles-based carriers [21–23] have been explored recently. Among these, LNPs are a category of promising nanocarrier candidates which have been demonstrated by us [16], [24–27] and others [28,29] for successful gene and protein delivery applications both *in vitro* and *in vivo*.

Combinatorial library strategy has been extensively used to synthesize biomaterials for drug delivery applications [24,30]. Libraries of LNPs were synthesized through Michael addition reaction and ring opening reaction of epoxide with amines by combination of different amines head, linker compound and aliphatic chain tail groups [24,28,31–33]. In this study, we report a new library of cationic chalcogen-containing lipidoid nanoparticles

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for intracellular delivery of genome editing proteins. Due to the structure similarity of those molecules which only differ in one atom, we also elucidated the structure-activity relationship between lipidoids and intracellular protein delivery efficiencies. The chalcogen-containing lipidoids (R-O17X, Fig. 1) were synthesized by reacting lipophilic tails containing O, S and Se ethers (O17O, O17S and O17Se) with commercial available amines (10, 17, 63, etc.). Lipidoid nanoparticles were then fabricated and negatively supercharged Cre recombinase ((-30)GFP-Cre) [15] and anionic Cas9:single-guide RNA (Cas9:sgRNA) ribonucleoprotein (RNP) [16] were loaded through the supramolecular interactions, mainly electrostatic interactions. HeLa-DsRed and GFP-HEK cells were utilized for evaluation of the intracellular protein delivery and genome editing efficiencies. The physicochemical properties of LNPs were examined and the structure-activity relationship was further explored by comparing the apparent pKa and lipid membrane disruption ability. Through the *in vivo* study using transgenic Cre reporter mice, we showed that these chalcogen-containing lipidoids have the potential for *in vivo* gene recombination applications.

2. Materials and methods

2.1. General

All chemicals used for lipidoids synthesis were purchased from Sigma-Aldrich without further purification unless otherwise noted. (−30)GFP-Cre recombinase, *S. pyogenes* Cas9 (*sp*Cas9) and sgRNA were generated according to our previously reported procedures [16]. HeLa-DsRed and GFP-HEK cells were cultured in Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich) with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco). All ^{1}H NMR spectra were recorded on a Bruker AVIII 500 MHz NMR spectrometer operated in the Fourier transform mode. Hydrodynamic size and polydispersity index of nanoparticles were measured by Zeta-PALS particle size analyzer (Brookhaven Instruments). The apparent pKa values of lipidoids were determined as previously reported using 2-(*p*-toluidiny-naphthalene-6-sulphonic acid) (TNS, Sigma-Aldrich) as fluorescent probe [34]. TEM measurements were performed on a FEI Technai Transmission Electron Microscope. Fluorescence images of tissue slices were obtained using BZ-X Analyzer fluorescence microscope.

2.2. Synthesis of O17O (Fig. S1)

Sodium hydride (0.72 g, 30 mmol) was added to the solution of ethylene glycol (5.6 g, 90 mmol) in anhydrous DMF (30 mL) and stirred for 10 min at 0 °C. 1-Bromotetradecane (6.0 g, 20 mmol) and KI (3.3 g, 20 mmol) were then added and the reaction mixture was kept at 95 °C for another 4 h. After cooling to room temperature, the mixture was diluted with cold water, extracted with ethyl acetate, and dried over anhydrous sodium sulfate [35]. Compound **1** (3.3 g, yield ~ 65%) was obtained after column chromatography purification on silica gel using n-hexane/ethyl acetate as mobile phase. Then, compound **1** (3.3 g, 12.8 mmol) and triethylamine (TEA, 1.9 g, 19.2 mmol) were dissolved in anhydrous DCM (80 mL). Acryloyl chloride (1.4 g, 15.4 mmol) was added dropwise at 0 °C, and the reaction mixture was stirred overnight. After column chromatography purification, O17O was obtained as colorless oil (3.2 g, yield ~ 82%).

2.3. Synthesis of O17S (Fig. S2)

To a solution of 2-mercaptoethanol (1.1 g, 14 mmol) in acetonitrile (20 mL) was added 1-bromotetradecane (5.0 g, 18 mmol) and potassium carbonate (3.6 g, 26 mmol). The reaction solution was stirred overnight at 40 °C, filtered and concentrated [36]. Compound **2** (1.8 g, yield ~ 48%) was obtained after column chromatography purification on silica gel using n-hexane/ethyl acetate as mobile phase. In a manner similar to that for the preparation of O17O, O17S was synthesized and purified as oil-like liquid (3.5 g, yield ~ 75%).

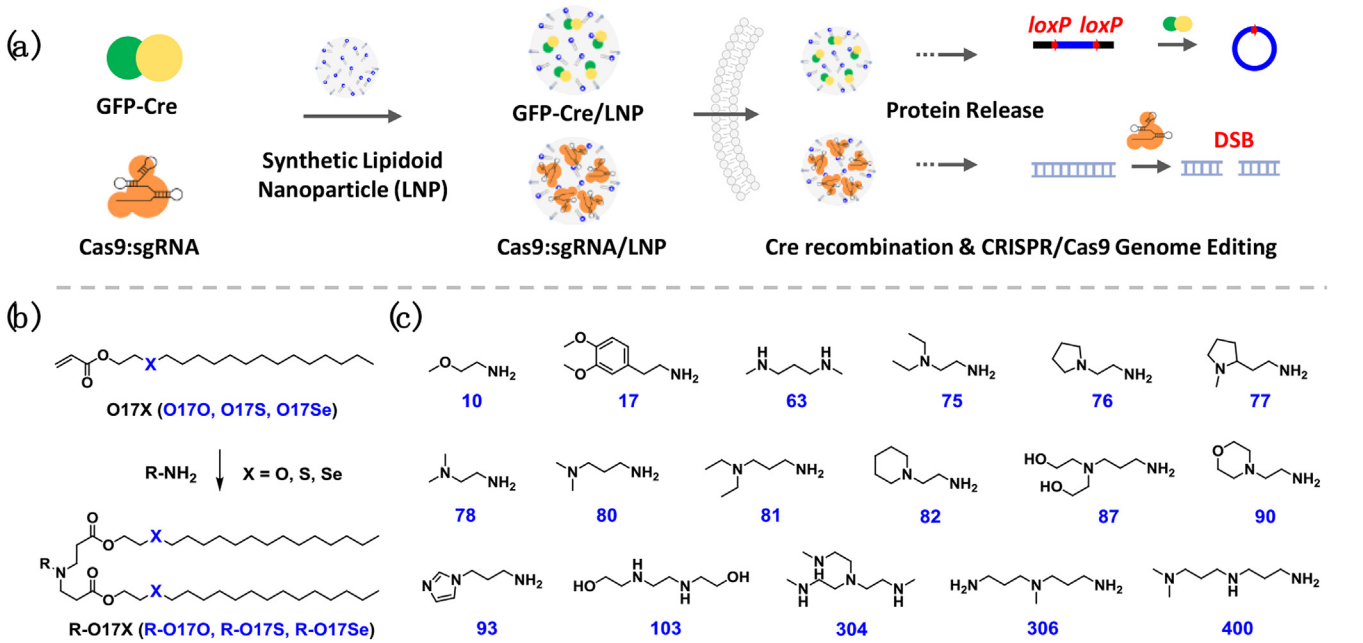

Fig. 1. (a) Encapsulation of negatively charged GFP-Cre and Cas9:sgRNA into synthetic cationic lipidoid nanoparticles (LNPs) for intracellular protein delivery and genome editing. (b) Synthetic route and lipidoids nomenclature. (c) Chemical structures of amine heads for lipidoids synthesis.

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