Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles

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A central challenge to the development of protein-based therapeutics is the inefficiency of delivery of protein cargo across the mammalian cell membrane, including escape from endosomes. Here we report that combining bioreducible lipid nanoparticles with negatively supercharged Cre recombinase or anionic Cas9:single-guide (sg)RNA complexes drives the electrostatic assembly of nanoparticles that mediate potent protein delivery and genome editing. These bioreducible lipids efficiently deliver protein cargo into cells, facilitate the escape of protein from endosomes in response to the reductive intracellular environment, and direct protein to its intracellular target sites. The delivery of supercharged Cre protein and Cas9:sgRNA complexed with bioreducible lipids into cultured human cells enables gene recombination and genome editing with efficiencies greater than 70%. In addition, we demonstrate that these lipids are effective for functional protein delivery into mouse brain for gene recombination in vivo. Therefore, the integration of this bioreducible lipid platform with protein engineering has the potential to advance the therapeutic relevance of protein-based genome editing.

Significance

The therapeutic potential of protein-based genome editing is dependent on the delivery of proteins to appropriate intracellular targets. Here we report that combining bioreducible lipid nanoparticles and negatively supercharged Cre recombinase or anionic Cas9: single-guide (sg)RNA complexes drives the self-assembly of nanoparticles for potent protein delivery and genome editing. The design of bioreducible lipids facilitates the degradation of nanoparticles inside cells in response to the reductive intracellular environment, enhancing the endosomal escape of protein. In addition, modulation of protein charge through either genetic fusion of supercharged protein or complexation of Cas9 with its inherently anionic sgRNA allows highly efficient protein delivery and effective genome editing in mammalian cells and functional recombination delivery in the rodent brain.


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knockout with efficiencies higher than 70% in cultured human cells. Finally, we demonstrate that these lipid nanoparticles can deliver genome-editing protein into the mouse brain for effective DNA recombination in vivo.

Results and Discussion

Lipid Synthesis and Nanoparticle Formulation. The bioreducible lipids were synthesized by heating an amine and acrylate in Teflon-lined glass screw-top vials for 48 h, and the crude products were purified using flash chromatography. The lipids were named with amine number (Fig. 2) and O14B; the latter indicates the carbon number of the hydrophobic tail and the bioreducible nature of lipids. All lipids were formulated with cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and C16-PEG₂₀₀₀-ceramide (SI Materials and Methods) for the following studies.

Screening Lipids for Protein Delivery. To demonstrate the necessity of the negative charge on the protein for assembling a nanoparticle with cationic lipids, we first fused a negatively supercharged GFP variant, (-27)GFP, to Cre recombinase and generated (-27)GFP-Cre. The fusion of (-27)GFP with Cre allows the study of protein uptake by analysis of intracellular GFP fluorescence, as well as a functional readout of Cre activity. In this study, we used HeLa-DsRed cells that express red fluorescent DsRed on Cre-mediated gene recombination to evaluate (-27)GFP-Cre delivery efficiency using different lipids. After delivery of protein-containing nanoparticles, the cellular uptake of different (-27)GFP-Cre/lipid complexes was quantified by counting GFP-positive cells. As shown in Fig. 3A, treatment of (-27)GFP-Cre (25 nM protein) without lipids showed a very low ratio of GFP-positive cells, indicating that the protein is not able to enter cells without cationic lipid. Interestingly, the proportion of GFP-positive cells after treatment was dependent on the nature of the lipid headgroup used in the delivery. Seven of the 12 lipids delivered (-27)GFP-Cre with a higher efficiency than that of a commercial lipid reagent, Lipofectamine 2000 (LFP2K). Complexation of (-27)GFP-Cre with lipids 8-O14B, 7-O14B, and 8-O14B resulted in more than 50% GFP-positive cells. In particular, 8-O14B (chemical structure shown in Figs. 1 and 2) delivered (-27)GFP-Cre in the highest efficiency among the 12 lipids, with more than 80% GFP-positive cells observed after treatment.

Lipid and (-27)GFP-Cre Binding Study. The complexation of 8-O14B and (-27)GFP-Cre to form nanoparticles was examined using dynamic light scattering (DLS; Table S1) analysis and transmission electronic microscopy (TEM) imaging (Fig. S1). The size of 8-O14B nanoparticles increased from 100 to 240 nm with the addition of (-27)GFP-Cre (Table S1), indicating formation of a nanoparticle between the lipid and (-27)GFP-Cre. ζ Potential measurement indicated the surface charge decrease of 8-O14B from 7 to -18 mV, confirming that the electrostatic binding of (-27)GFP-Cre and 8-O14B neutralized the positive charge of the lipid nanoparticles. In contrast, the addition of the same concentration of Cre protein (without the supernegative (-27)GFP fusion) had a negligible effect on the size and surface charge of the 8-O14B nanoparticles (Table S1). The DLS study indicates the necessity of electrostatic attraction between protein and lipid for nanoparticle loading.

Circular dichroism (CD) spectra showed negligible change in the secondary structure of (-27)GFP-Cre in the presence of 8-O14B (Fig. S2A). Moreover, no shift in the wavelength for GFP fluorescence from (-27)GFP-Cre was observed, further confirming the retention of the native structure of (-27)GFP-Cre after complexation with bioreducible lipids (Fig. S2B).

Endocytosis and Endosome Escape of Lipid/Protein Nanoparticles. We previously reported that the lipid-like nanoparticles deliver protein mainly through an endocytosis-dependent pathway (11). To probe the detailed mechanism of bioreducible lipid to deliver protein, we treated HeLa-DsRed cells with 8-O14B/(-27)GFP-Cre...
complexes in the presence of endocytosis inhibitors. As shown in Fig. S3, both sucrose, a clathrin-mediated endocytosis inhibitor, and the cholesterol-depleting agent methyl-β-cyclodextrin (M-β-CD), strongly suppressed cellular uptake of 8-O14B/(-27)GFP-Cre nanoparticles. In addition, treatment of the dynamin II inhibitor dynasore significantly reduced the delivery of (-27)GFP-Cre. In contrast, an inhibitor of caveolin-mediated endocytosis, nystatin, had minimal effect on the delivery of (-27)GFP-Cre. These data indicate that 8-O14B/(-27)GFP-Cre nanoparticles enter cells mainly through clathrin-mediated endocytosis, in which plasma cholesterol and dynamin also play roles in the uptake of the lipid/protein nanocomplex.

The intracellular trafficking of 8-O14B/(-27)GFP-Cre nanoparticles after entering cells was studied by visualizing subcellular (-27)GFP-Cre accumulation and protein localization using confocal laser scanning microscopy (CLSM) imaging. As shown in Fig. 4, treatment of HeLa-DsRed cells with (-27)GFP-Cre alone (12.5 nM protein) showed no (-27)GFP delivery, which was consistent with the cellular uptake study (Fig. 2A). Treatment with 8-O14B/(-27)GFP-Cre nanoparticles (12.5 nM protein) showed significant accumulation of GFP fluorescence in the cytosol and nucleus, with a low level of colocalization with endosome/lysosome, indicating the efficient endosome escape of 8-O14B/(-27)GFP-Cre nanoparticles. The inherent nuclear localization signal presented on the Cre recombinase enables the accumulation of (-27)GFP-Cre in nuclei for effective gene recombination (18).

Cre Protein Delivery-Mediated Gene Recombination. The resulting DsRed expression from successful Cre-mediated recombination in (-27)GFP-Cre lipid nanoparticle-treated HeLa-DsRed cells was analyzed 24 h after protein delivery. As shown in Fig. 3B, (-27)GFP-Cre alone is incapable of inducing DsRed expression. Treatment of cells with nanoparticles formulated from (-27)GFP-Cre and the seven lipids that efficiently delivered (-27)GFP-Cre in the cellular uptake study (Fig. 3A) showed comparable or higher percentages of DsRed-positive cells than that of LPF2K. The best lipid for protein delivery identified in the cellular uptake analysis, 8-O14B, delivered (-27)GFP-Cre to a high efficiency, resulting in 80% DsRed-positive cells. In addition, 8-O14B/(-27)GFP-Cre delivery resulted in significantly enhanced recombination efficiency compared with treatment with Lipofectamine RNAiMax (4), indicating that efficient escape of 8-O14B/(-27)GFP-Cre from the endosome facilitates improved Cre-mediated gene recombination.

Delivery of (-27)GFP-Cre/8-O14B induced DsRed expression in a protein concentration-dependent manner. When the concentration of (-27)GFP-Cre delivered to cells was increased from 6.25 to 25 nM, the DsRed-positive cells increased from 10% to 80% (Fig. 5A), and no Ds-Red positive cells were observed when treatment was performed with uncomplexed protein, demonstrating the necessity of lipid for an effective Cre-mediated gene recombination. Furthermore, HeLa-DsRed cells treated with 8-O14B/(-27)GFP-Cre nanoparticles retained viability above 85% at all protein concentrations studied (0–50 nM) as measured by a MTT assay (Fig. S4), indicating that the 8-O14B lipid is highly biocompatible and displays low cytotoxicity.

We next studied the chemical structure–activity relationship of the bioreducible lipids for protein delivery, and this study was enabled by our facile, combinatorial lipid synthesis approach. For this purpose, lipids conjugated from amine 8 and acrylates featuring tails with 12, 14, 16, and 18 carbon atoms, were synthesized for (-27)GFP-Cre delivery. All four lipids showed comparable protein encapsulation efficiency, with more than 90% (-27)GFP-Cre encapsulated under the optimized delivery condition using 8-O14B (Fig. S5A). However, these lipids showed quite different capability for protein delivery. Lipids with tails containing
GFP-Cre was encapsulated with nanoparticles. More than 90% of cells when 25 nM (−27)GFP-Cre nanoparticles was exposed to HeLa-DsRed cells (Fig. S5B). The percentage of DsRed expressed cells was as high as 80% following these treatments (Fig. 5B). 8-O12B, on the other hand, had the lowest protein delivery efficiency, with less than 20% GFP-positive and DsRed-recombined cells under the same conditions (Fig. S5B and Fig. 5B). These findings demonstrate that lipid nanoparticles with the shorter 12-carbon hydrophobic tail had lower protein delivery efficiency, consistent with our previous observations (11). Taken together, these findings highlight the advantages of using a combinatorial strategy to identify effective protein delivery vehicles.

**Protein Charge Determines Gene Recombination Efficiency.** To further demonstrate the essential role of the electrostatic binding between lipids and negatively supercharged proteins for an effective protein delivery, Cre recombinase with and without different supernegative GFP fusions were designed and evaluated for their ability to mediate gene recombination. To this end, we fused Cre recombinase to four GFP variants with negative charges of −7, −20, −27, or −30. The negative charge density of negative GFP-fused proteins determines their encapsulation efficiency by 8-O14B nanoparticles. More than 90% of (−27) and (−30)GFP-Cre was encapsulated into 8-O14B (Fig. S6A), whereas less than 30% of (−7) and (−20)GFP-Cre was encapsulated with 8-O14B nanoparticles under the same conditions.

The higher encapsulation of (−27)GFP-Cre and (−30)GFP-Cre into 8-O14B nanoparticles enhanced protein delivery and gene recombination efficiency of these proteins relative to the (−7) and (−20)GFP-Cre constructs. Analysis of the cellular uptake of different protein/lipid complexes indicated that (−27)GFP-Cre and (−30)GFP-Cre nanoparticle treatment showed higher GFP fluorescence intensity than cells treated with (−7)GFP-Cre and (−20)GFP-Cre (Fig. S6B). In addition, the treatment of HeLa-DsRed cells with the 8-O14B complex of (−27)GFP-Cre or (−30)GFP-Cre both resulted in 80% DsRed-positive cells (Fig. 6), demonstrating a significantly higher gene recombination efficiency than that of (−7)GFP-Cre, (−20)GFP-Cre, or Cre without supernegative GFP fusion delivery (Fig. 6).

**Anionic Cas9:sgRNA Delivery for Genome Modification.** Having demonstrated the success and high efficiency of bioreducible lipids to deliver Cre recombinase for gene recombination, we next investigated whether these lipids are able to deliver the genome-editing protein Cas9 and facilitate Cas9-mediated genetic modification of mammalian cells. CRISPR-associated protein 9 (Cas9) can bind to and cleave a target DNA sequence that is complementary to the first 20 nucleotides of an sgRNA (19). Cas9-induced double-strand breaks could be repaired by nonhomologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, enabling targeted genome editing and cell engineering for the treatment of genetic diseases (3, 20, 21). Two limitations for the use of the Cas9:sgRNA complex for genome editing are delivery into target cells and modification at off-target DNA sites (22–24). We and others have shown that delivery of the Cas9:sgRNA ribonucleoprotein complex results in comparable efficacy and reduced off-target cleavage events compared with traditional plasmid-based delivery methods (4, 25). The ribonucleoprotein complex is anionic, facilitating complexation with cationic nanoparticles without the need for fusion with a supernegatively charged protein. The ability of bioreducible lipids to deliver anionic Cas9:sgRNA complex for genome editing was demonstrated by targeting genomic EGFP reporter gene in HEK cells. The efficient delivery of Cas9:sgRNA and on-target Cas9 cleavage of GFP-expressing HEK cells induce NHEJ in the EGFP reporter gene and result in the loss of cell fluorescence. For this purpose, we treated HEK cells with 25 nM Cas9 and 25 nM EGFP-targeting sgRNA with and without lipid complexation, and the GFP expression profile was analyzed and summarized in Fig. 7. Five of the 12 lipids delivered the Cas9:sgRNA complex and induced the loss of EGFP-positive cells with an efficiency higher than 50%. In particular,
In vivo delivery of Cre recombinase to mouse brain. Rosa26<sub>−</sub> mouse was microinjected with 0.1 µL 50 µM (−27)GFP-Cre alone or the same amount of protein complexed with lipid 8-O14B. After 6 d, the tdTomato expression indicative of Cre-mediated recombination in dorsomedial hypothalamic nucleus (DM; X = +0.20, Y = −1.6, Z = −5.0), mediodorsal thalamic nucleus (MD; X = +0.25, Y = −1.4, Z = −2.2), and bed nucleus of the stria terminalis (BNST; X = +0.9, Y = +0.4, Z = −4.0) was visualized using fluorescent microscopy. (Scale bar, 100 µm.)
commercial Lipofectamine are restricted by its toxicity and inflammatory side effects (26, 27), our combinatorial strategy to develop synthetic lipids has the potential to discover lipids that overcome these barriers. We (11) and others (28) have previously shown that lipids designed in a combinatorial fashion have low immunogenicity and toxicity.

The efficient and localized delivery of genome-editing proteins to the mouse brain demonstrated here may eventually lead to a protein-based approach for correcting genetic diseases and neurological disorders. For example, the single injection of nanoparticles containing a Cas9/sgrRNA complex into brain regions rich in dopaminergic neurons could enhance dopamine signaling and potentially alleviate some symptoms of Parkinson’s disease. One current treatment for Parkinson’s disease is deep brain stimulation. Genome editing offers several potential advantages including being less invasive and avoiding the risk of electrode-induced inflammation, because genome editing can affect a permanent genomic change following a single injection. We showed here how injection into the brain effects highly spatially localized delivery of our nanoparticles, potentially enabling control over the subpopulation of cells to which our agent is delivered and minimizing the risk of unintended effects in other cells. We predict that one of the major challenges for this approach will be to deliver the genome editing protein to enough cells to effect a significant change in phenotype. More experiments must be done to characterize and optimize the pharmacokinetics, efficacy, and safety of this strategy in animal models.

Materials and Methods

Details describing synthesis, formulation, and characterization of lipid nanoparticles; protein expression procedure; protein delivery in vitro and in vivo; and cellular uptake mechanism studies can be found in SI Materials and Methods. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees (#AN-6598) at Baylor College of Medicine.

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