A Versatile Nonviral Delivery System for Multiplex Gene-Editing in the Liver

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Recent advances in CRISPR present attractive genome-editing toolsets for therapeutic strategies at the genetic level. Here, a liposome-coated mesoporous silica nanoparticle (lipoMSN) is reported as an effective CRISPR delivery system for multiplex gene-editing in the liver. The MSN provides efficient loading of Cas9 plasmid as well as Cas9 protein/guide RNA ribonucleoprotein complex (RNP), while liposome-coating offers improved serum stability and enhanced cell uptake. Hypothesizing that loss-of-function mutation in the lipid-metabolism-related genes pck9, apoC3, and angptl3 would improve cardiovascular health by lowering blood cholesterol and triglycerides, the lipoMSN is used to deliver a combination of RNPs targeting these genes. When targeting a single gene, the lipoMSN achieved a 54% gene-editing efficiency, besting the state-of-art Lipofectamine CRISPRMax. For multiplexing, lipoMSN maintained significant gene-editing at each gene target despite reduced dosage of target-specific RNP. By delivering combinations of targeting RNPs in the same nanoparticle, synergistic effects on lipid metabolism are observed in vitro and vivo. These effects, such as a 50% decrease in serum cholesterol after 4 weeks of post-treatment with lipoMSN carrying both pck9 and angptl3-targeted RNPs, could not be reached with a single gene-editing approach. Taken together, this lipoMSN represents a versatile platform for the development of efficient, combinatorial gene-editing therapeutics.

Cardiovascular disease remains a leading cause of death globally, with high plasma low-density-lipoprotein cholesterol (LDL-C) level, or hypercholesterolemia, and high plasma triglyceride level, or hyperlipidemia, as the major determinants of risk. \cite{1,2} Reduction of cholesterol is an attractive therapeutic objective, with 30–40% reduction in LDL-C correlating with paralleled reduction in cardiovascular disease risk. \cite{3} Statins, the current standard-of-care, neglect 10–20% of the high-risk patient-population due to intolerance and adverse effects with increased dosage, which motivates a genetic approach to find alternatives. \cite{3,4} The first gene target for cardioprotection was discovered when a gain-of-function mutation in proprotein convertase subtilisin/kexin type 9 (PCSK9) was identified as the cause of autosomal dominant hypercholesterolemia, driving patients into high levels of LDL-C and early coronary heart disease (CHD). \cite{5} Loss-of-function sequence variations of PCSK9 lead to significant (40%) reduction in the LDL-C level and 88% reduction in CHD. \cite{5,6} PCSK9 is an LDL receptor (LDLr) antagonist expressed in the liver, such that overexpression leads to less LDL receptors and a decrease in LDL-C removal from the plasma. \cite{7} Monoclonal antibodies targeting PCSK9 were considered the potential solution for the significant unmet need unfilled by statin drugs. \cite{8} However, PCSK9 antibodies such as alirocumab showed adverse effects including injection site reactions, neurocognitive events, ophthalmologic events, and antidrug antibody production in clinical trials. \cite{9} Small interfering RNAs (siRNAs), e.g., inclisiran, have been developed to provide a similar cardioprotective effect as the antibody therapies. \cite{10} While these siRNAs enable significant down-regulation of PCSK9, high off-target effects associated with this modality of gene manipulation remain a concern. CRISPR/Cas9-mediated gene disruption offers an alternative for higher-precision, lower frequency treatment. \cite{11}

Derived from the prokaryotic immune system, Cas9 endonuclease allows for precisely controllable gene targeting in mammalian cells when complexed with a specific guide RNA (gRNA), thereby generating a specifically localized double-stranded break at the target site. \cite{12} During the DNA repair process, the dominant pathway, nonhomologous end-joining...
of the break, often leads to frameshift errors and results in knockout of the gene.[13] This provides a simple mechanism to explore cause-and-effect in the context of lipid metabolism pathways, as specific genetic perturbations can be made and blood lipid profile changes can be measured. Similar to PCSK9, naturally occurring heterozygous mutations in ANGPTL3 and APOC3 yield cardioprotective effects through their impact in lipid metabolism in the liver.[6,14] Leveraging the CRISPR/Cas9 system to explore these three gene targets separately and in various combinations could provide valuable information for the development of cardioprotective therapeutics.

Delivery mechanisms for CRISPR/Cas9 rely heavily on viral machinery, most popularly adeno-associated virus (AAV).[15] While AAV have lower immunogenicity compared with lentivirus or adenovirus, AAV has the lowest packaging capacity of ≈5 kb.[16] This makes transduction of both the Cas9 and gRNA difficult and increasingly so for multiplexing. Further, cloning is required for each gene target, which contributes to slower workflow when screening potential gRNA designs.[17] Nanoparticle delivery of CRISPR/Cas9 elements has become a viable alternative, providing transient delivery of various forms of the CRISPR/Cas9 cassette, ranging from plasmid encoding the Cas9 endonuclease and gRNA, Cas9 mRNA with gRNA, to the Cas9/gRNA ribonucleoprotein (RNP) complex.[18] Liposomes and lipid-based nanoparticles have been investigated extensively as nonviral carriers, enabling effective drug/gene delivery with limited risk of immunogenicity and allowing for tunable surface properties via lipid composition.[19] However, many commercially available lipid-based carriers have limitations in vivo, and often rely on electrostatic self-assembly with cargo, providing relatively low loading efficiency of low solubility or charge density cargos.[20] Hypothesizing that integrating liposome with a core capable of loading diverse therapeutic cargo may resolve the aforementioned limitation and preserve liposome's favorable cell entry, we designed a mesoporous silica nanoparticle (MSN) core to provide a liposome-coated MSN (lipoMSN) system for delivery of CRISPR/Cas9 elements.[21] MSN provides high surface area for the electrostatic loading of lower charge density Cas9/gRNA RNP cargo and additionally shelters its gRNA component susceptible to the degradative extracellular and endosomal environments.[22]

While lipoMSNs have proven their efficacy in the delivery of a variety of cargos, from small molecule drugs, peptides, to nucleic acids (siRNA and plasmid), they have not yet been successfully employed in the context of multiplex gene editing using multiple Cas9/gRNA RNPs.[23] In this work, we demonstrate that our lipoMSN delivery system is viable in its ability to deliver Cas9/gRNA RNP as well as Cas9 plasmid with gRNA through electrostatic loading. Further, we apply this system to target three different cardioprotective genes simultaneously in order to study the potential synergistic effects that arise from multiple pathway manipulation. Since previous multiplex gene editing relied on in vivo transcription of the CRISPR/Cas9 components via plasmid or RNA delivery, or delivery in separate vehicles, our work provides a unique opportunity to study the effects of multiple Cas9/gRNA RNPs coloaded into a singular delivery vehicle.[24] Chadwick et al., used separated adenoviruses to deliver CRISPR base editors targeting angptl3 and pck9 but could not detect synergistic effect.[4] This could be due to the un-synchronized editing of any given cell. With our proposed system, we are able to reduce the potential compensation mechanisms of these nonredundent pathways of lipid metabolism, which can provide insight of potential synergistic effects.[25]

We first tested whether our lipoMSN system could deliver various CRISPR/Cas9 editing elements, including low charge density Cas9 protein (≈160 kDa), short gRNA (≈100 nucleotides), and large Cas9 plasmid (≈10 kb).[26] While gRNAs and plasmids have been previously loaded into various nanoparticle platforms,[27] loading of nonuniform, weakly-charged Cas9/gRNA RNP (≈1.4 mV) presents a challenge, compared with the loading of uniformly negative-charged Cas9 plasmid (≈16 mV) or gRNA (≈14 mV; Figure 1A).[28] Screening of two different MSN cores—functionalized with carboxyl (−COOH) or amino (−NH2) group—showed that while all cores were comparable (Figures S1 and S2, Supporting Information), amine-function- alization on MSN, yielding a positive charge between 30 and 40 mV, resulted in efficient loading of Cas9/gRNA RNP as well as the Cas9 plasmid and gRNA at an MSN-to-cargo ratio of 20 to 1 (w/w) (Figure S3, Supporting Information).

Because liver was the initial therapeutic target, we then optimized the formulations in the primary mouse hepatic cell line (AML-12). Despite the loading capabilities, MSN alone provided low uptake and poor serum stability (Figures S1A and S4, Supporting Information). In contrast, coating with liposome—confirmed via transmission electron microscopy (TEM) (Figure 1B; Figure S5, Supporting Information)—improved both cellular uptake and transfection efficiency of MSN (Figure S1A,B, Supporting Information). Under the optimized liposome/MSN/cargo ratio (20/20/1), gRNA-loaded and Cas9-T2A-EGFP plasmid (px458)-lipoMSNs showed improved uptake (98% at 4 h) and transfection (25% at 24 h), respectively. Seeing positive results given by liposome-coating, an iterative optimization process on lipid composition of the liposome was subsequently carried out (Figure S6, Supporting Information), yielding the best composition with 65% DOTAP, 30% cholesterol, 3.75% DOPE, and 1.25% DSPE-PEG (Figure S7, Supporting Information).

With the optimized liposome composition and liposome/ MSN/cargo ratio, the lipoMSN showed relatively uniform physical characteristics in size and surface charge despite the varied cargos (Figure 1C). To date, there is no single platform allowing direct comparison of gene editing efficacy between different formats of CRISPR/Cas9 elements, although some attempts have been made.[29] As the first delivery system capable of delivering CRISPR elements in three different formats (Cas9 plasmid + gRNA, all-in-one plasmid encoding both Cas9 and gRNA, Cas9/gRNA RNP), we carried out a head-to-head comparison between these formats. When delivered by the lipoMSN, the Cas9/gRNA RNP gave the highest gene editing (Figure 1D,E), similar to reported results obtained through electroporation.[30] Notably, our lipoMSN outperformed the current gold standard for Cas9/gRNA RNP delivery, lipofectamine CRISPRMax. Surveyor assays showed Cas9/gRNA-loaded lipoMSN produced a gene disruption efficiency of 54%, which was superior to Lipofectamine CRISPRMax with Cas9/gRNA RNP (30%) as well as Lipofectamine 3000 with the all-in-one Cas9/gRNA plasmid (33%). The use of Cas9/gRNA RNP has increased in popularity in the field because of its high editing fidelity,[29,30] This in conjunction
with our maximized editing efficiency led us to continue our work using our lipoMSN system with Cas9/gRNA RNP.

After seeing effective gene editing with Cas9/gRNA RNP-loaded lipoMSN, we next explored multiplex gene editing with our system to disrupt the three genes (pcsk9, apoc3, and angptl3) in disparate pathways involved in LDL metabolism. As shown in Figure 2A, Pcsk9 inhibits LDLR recycling, and Apoc3 inhibits lipoprotein lipase activity, while Angptl3 inhibits the expression of LDLR as well as lipoprotein lipase. Simultaneous disruption of these three genes may show synergy on lowering LDL-C level, thereby boosting cardioprotection efficacy; yet, multiplex gene editing provided the next challenge of maintaining significant gene editing for these three different gene targets while keeping the total Cas9/gRNA RNP dose constant. Editing efficiency at the pcsk9 target site remains consistent despite dosage of pcsk9-targeting Cas9/gRNA RNP being a half or a third of that in the single-targeting group. Similar results were obtained at the apoc3 and angptl3 loci as well (Figure 2B–E). Our results imply that the limiting factor for effective gene editing lies with the delivery of CRISPR/Cas9 elements into the cell, not the quantity delivered per cell, which is also supported by previous reports with Cas9 plasmid and mRNA. It may be that the number of RNP only needs to meet a threshold to provide targeted gene editing, such that when the combination of three different RNP are delivered into one cell, they provide similar amounts of gene editing to all three gene targets as a monogenic Cas9/gRNA RNP.

To validate that our gene editing resulted in reduced expression of the three targets, pcsk9, angptl3, and apoc3, we first performed reverse-transcription quantitative PCR (RT-qPCR) on the treated AML-12 cells. Interestingly, in addition to the expected results of reduced expression of the gene target with treatment by the respective Cas9/gRNA RNP, we found collateral effects of the gene editing. For example, pcsk9 expression was significantly upregulated by 50% and 25% with editing of apoc3 and angptl3, respectively. In contrast, when treated with a combination of all three Cas9/gRNA RNPs, the expression levels of pcsk9, apoc3, and angptl3 were most significantly reduced by 50%, 80%, and 85%, respectively (Figure 3A–C). This suggests the potential for Pcsk9, Angptl3, or Apoc3 to compensate for each other through uncharacterized feedback loops as they all show effects on lipid uptake and metabolism in the liver. Our singular lipoMSN delivery approach targeting all three genes may take advantage of the overlap of these pathways by removal of these compensation mechanisms. We looked at increased ldlr expression as a result of the different RNP treatments in order to predict most effective synergistic gene editing combinations for further exploration. Disruption
of all three target genes led to up-regulation of ldlr expression by fivefold at 24 h post-treatment (Figure 3D), whereas the ldlr level was significantly increased at 48 h post-treatment in the single- and dual- (pcsk9 + angptl3) disruption groups (Figure 3E). Enzyme-linked immunosorbent assay (ELISA) also confirmed the Ldlr upregulation after Cas9-mediated pcsk9 disruption using our lipoMSN (Figure 3F).

To determine the lipoMSN delivery system’s efficacy at RNP multiplex gene editing in vivo, six groups of 5-week-old C57BL/6J female mice were treated in various combinations (Figure 4A). These combinations (pcsk9, angptl3, pcsk9 + angptl3, pcsk9 + apoc3 + angptl3, Cas9 protein alone, phosphate buffered saline (PBS) control) were designed to validate the synergistic effects between pcsk9- and angptl3-targeting. LipoMSN was given twice through intravenous administration, for a total dose of 10 mg per kg of Cas9/gRNA RNP. Blood of each mouse was drawn weekly beginning one week before treatment in order to determine treatment effects on triglycerides and cholesterol. We also monitored the changes in weight, blood high-density-lipoprotein cholesterol (HDL-C) and alanine transaminase (ALT) levels to determine potential toxicity of our lipoMSN. Serum triglycerides showed a significant lasting

Figure 2. Surveyor Assay confirmation of multiplex gene targeting using lipoMSN. A) Schematic illustrating the roles of Pcsk9, Apoc3, and Angptl3 on lipid metabolism. B–D) Surveyor assays showing in vitro editing of pcsk9 (B), apoc3 (C), and angptl3 (D) was durable despite lowered doses of target gRNA. E) Gene editing efficiency quantification using Image J. Results are presented as average ± SEM (n = 4). Positive control of Lipofectamine CRISPRMax delivering targeted RNP noted by “+”, while negative control of Cas9 protein only noted with “−”. Control and singular target (1) RNP was delivered at 2 µg mL⁻¹, while target-specific RNP for dual-targeting (2) was at 1 µg mL⁻¹ each (2 µg mL⁻¹ total) and triple-targeting (3) was at 0.67 µg mL⁻¹ (2 µg mL⁻¹ total).

Figure 3. qPCR measurement of gene regulation after CRISPR/Cas9 disruption of singular or multiple genes. A–C) RT-qPCR validation on the pcsk9 (A), apoc3 (B), and angptl3 (C) expression levels in the AML-12 cells at 48 h post-treatment of the lipoMSN with Cas9/gRNA RNP relative to the untreated control after normalization to GAPDH expression. D,E) RT-qPCR calculated expression of ldlr at 24 h (D) and 48 h (E). F) Ldlr protein amount is quantified at 48 h post-treatment measured by ELISA. Results are presented as average ± SEM (n = 4). A–E) qPCR data is relative to the untreated control after normalization to GAPDH expression. Significance was determined using one-way ANOVA with Tukey’s posthoc test, and represented as *p < 0.05 and **p < 0.01.
effect in the single angptl3-targeted group, with a 25% decrease observed even at week 4 post-treatment (Figure 4B). The unexpected finding in the PBS control group, which showed an observable drop in serum triglycerides from week 1 to week 2, may have been due to variations in time of blood collection, which has previously been shown to have an impact on blood lipids. Serum cholesterol measurements showed a significant effect for all the treated groups. At week 4 post-treatment, single gene disruption lowered the cholesterol level by \(\approx 30\%\) (31.7% and 28.2% for the \(pcsk9\)- and \(angptl3\)-targeted groups, respectively), while dual- \((pcsk9 + angptl3)\) and triple- \((pcsk9 + angptl3 + apoc3)\) gene disruption gave more substantial reduction (56.5% and 43.18%, respectively; Figure 4C). The results of mouse weight, HDL-C and ALT measurements indicated that our lipoMSN did not cause any significant adverse effects, as no significant difference was observed in each indicator between groups (Figure 4D–F). Further, collection and H&E staining of the heart, liver, lung, kidney, and spleen yielded no observable damage in any treatment groups (Figure S11, Supporting Information). Dual disruption of both \(pcsk9\) and \(angptl3\) was more effective on lowering serum cholesterol than any singular disruption. This finding was different from that by the Musunuru and group in their adenovirus-based gene editing where no synergy between the two targets was observed. Serum cholesterol measurements showed a significant effect for all the treated groups. At week 4 post-treatment, single gene disruption lowered the cholesterol level by \(\approx 30\%\) (31.7% and 28.2% for the \(pcsk9\)- and \(angptl3\)-targeted groups, respectively), while dual- \((pcsk9 + angptl3)\) and triple- \((pcsk9 + angptl3 + apoc3)\) gene disruption gave more substantial reduction (56.5% and 43.18%, respectively; Figure 4C). The results of mouse weight, HDL-C and ALT measurements indicated that our lipoMSN did not cause any significant adverse effects, as no significant difference was observed in each indicator between groups (Figure 4D–F). Further, collection and H&E staining of the heart, liver, lung, kidney, and spleen yielded no observable damage in any treatment groups (Figure S11, Supporting Information). Dual disruption of both \(pcsk9\) and \(angptl3\) was more effective on lowering serum cholesterol than any singular disruption. This finding was different from that by the Musunuru and group in their adenovirus-based gene editing where no synergy between the two targets was observed. The main reason could be due to the difference in delivery approach; the previous approach, where two individual adenoviruses were applied to target \(pcsk9\) and \(angptl3\), did not ensure a high probability that any given cell would receive both viral vectors. Plausibly, as the editing kinetics of Cas9/gRNA RNP is faster, similar effect might also take a longer period to show when using viral machinery for gene targeting. Nevertheless, to put our lipoMSN efficacy into context, alirocumab, an anti-PCSK9 drug in clinical trials, provides human patients with \(\approx 61\%\) reduction in LDL-C with a biweekly dosing, which in preclinical studies have shown an approximately 50% reduction in total cholesterol in mice. To validate that the blood lipid profile was a result of the lipoMSN-RNP treatment, ELISA assays were used to measure the decrease in \(Pcsk9\) and \(Angptl3\) after the treatment. Reduced circulating \(Pcsk9\) was observed in the \(pcsk9\)-targeting group’s serum samples at both weeks 1 and 4 post-treatment (Figure S8B,C, Supporting Information). Circulating \(Angptl3\) showed no significant differences, but this could be due to the decreased editing efficiency at \(angptl3\), which was supported by our sequencing results. Further, clinical studies measuring circulating \(Angptl3\) in patients with homozygous and heterozygous loss-of-function mutations show that heterozygous mutations do not provide a statistically significant decrease in \(Angptl3\) compared to a healthy control, implying that significant gene disruption may be required to provide measurable decreases in \(Angptl3\). At our end-point (week 4 post-administration), we were able to detect an indel rate of 24.8% at the target \(pcsk9\) locus, but only 7.2% at the \(angptl3\) site (Figure S10, Supporting Information). Similar disparities in gene editing were observed in our in vitro validation as well (Figure 2). The gene disruption efficiency of \(angptl3\) was lower than that of \(pcsk9\), which could be due to differences in gRNA’s targeting capability or Cas9 affinity, and a further optimization on gRNA design may resolve this issue.

In conclusion, we designed and tested this lipoMSN platform for effective Cas9/gRNA delivery for multiplex gene editing, which enabled exploration of three cardioprotective gene targets in the liver. This easy-to-assemble delivery system leverages on the MSN core to load variable cargos from small gRNA, plasmid, to large protein, while the liposome coating
provides consistent and predictable physical characteristics despite the cargo. Gene editing efficiency when delivering a single gRNA reached 54% in vitro and 24.8% in vivo at week 4 post-treatment. The efficiency was not significantly compromised with codelivery of three different Cas9/gRNA RNP, which allowed synergistic effects to be detected when a singular vehicle was used to disrupt the three cardioprotective genes, pcsk9, apoc3, and angpl1. The in vivo gene disruption of these genes provides encouraging evidence that the multiplex lipoMSN platform offers significant improvement over single-target therapy. Collectively, this study suggests an effective approach of discovering synergistic therapeutic targets using multiplexed nonviral gene editing.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of interest
The authors declare no conflict of interest.

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