Nanoparticles That Deliver RNA to Bone Marrow Identified by in Vivo Directed Evolution

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Supporting Information

ABSTRACT: Bone marrow endothelial cells (BMECs) regulate their microenvironment, which includes hematopoietic stem cells. This makes BMECs an important target cell type for siRNA or gene editing (e.g., CRISPR) therapies. However, siRNA and sgRNA have not been delivered to BMECs using systemically administered nanoparticles. Given that in vitro nanoparticle screens have not identified nanoparticles with BMEC tropism, we developed a system to quantify how >100 different nanoparticles deliver siRNA in a single mouse. This is the first barcoding system capable of quantifying functional cytosolic siRNA delivery (where the siRNA drug is active), distinguishing it from in vivo screens that quantify biodistribution (where the siRNA drug went). Combining this approach with bioinformatics, we performed in vivo directed evolution, and identified BM1, a lipid nanoparticle (LNP) that delivers siRNA and sgRNA to BMECs. Interestingly, chemical analysis revealed BMEC tropism was not related to LNP size; tropism changed with the structure of poly(ethylene glycol), as well as the presence of cholesterol. These results suggest that significant changes to vascular targeting can be imparted to a LNP by making simple changes to its chemical composition, rather than using active targeting ligands. BM1 is the first nanoparticle to efficiently deliver siRNA and sgRNA to BMECs in vivo, demonstrating that this functional in vivo screen can identify nanoparticles with novel tropism in vivo. More generally, in vivo screening may help reveal the complex relationship between nanoparticle structure and tropism, thereby helping scientists understand how simple chemical changes control nanoparticle targeting.

INTRODUCTION

siRNAs can elucidate how genes cause disease. In a typical example, a lipid nanoparticle (LNP) delivers siRNA that inhibits a target gene in vivo; this circumvents the need to breed inducible genetic knockout mice, a process which can take over a year. Nanoparticles that efficiently deliver siRNA to hepatocytes, lung and heart endothelial cells, and immune cells have been used in this way. For example, a LNP with tropism to hepatocytes delivered siRNAs targeting endosomal genes; this uncovered how Rab5 influenced endocytosis. Similar approaches have been applied to hypertensive heart disease, extracellular matrix signaling, cancer, glucose homeostasis, and other phenotypes. In addition to its utility as an in vivo scientific reagent, siRNA has treated tissue-speciﬁc “angiocrine” signaling is implicated in many diseases but is poorly understood. BMECs are diﬃcult to study in large part because manipulating gene expression in vivo is challenging.

A method to directly evolve LNPs with novel tropisms in vivo would facilitate in vivo studies and RNA therapies. However, most LNPs display an affinity for the liver; this is thought to be driven by physiological advantages including slow blood flow and discontinuous vasculature in hepatic sinusoids. As a result, systemically administered RNA delivery to nonliver organs remains challenging. One unexplored contributor to LNP liver tropism is the process by which LNPs are selected. Like all nanoparticles, LNPs are initially tested in vitro, before a few lead LNPs are tested in vivo. However, in vivo LNP delivery is in

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uncovered how cholesterol structure in others have designed in vivo nanoparticle screens with DNA in vitro nanoparticle delivery can predict hepatocyte delivery.34 The course of 2 experiments, using bioinformatics to deliver siRNA to cells in vivo. We studied 160 LNPs over simultaneously quantifying how >100 LNPs functionally tumor delivery,37 compared in vitro and in vivo delivery,33 and fi barcodes. Barcoding studies have identi nanoparticles.32 We compared in vitro and in vivo nanoparticle relationship in endothelial cells or macrophages.33 However, biodistribution mediated by >300 LNPs and found no nanoparticles that systemically delivered siRNA to endothelial cells in different tissues. We found multiple nanoparticles that have been reported to deliver siRNA to endothelial cells in the lung, heart, kidney, liver, lymph nodes, spleen, brain, and pancreas. An ionizable LNP named 7C1 we reported4 was the most efficient siRNA delivery vehicle for lung, heart, and kidney endothelial cells in our search, silencing target genes by 50% after systemic siRNA doses as low as 0.02 mg/kg (Figure 1B). No LNPs in our search targeted BMECs after systemic administration. On this basis, we synthesized the 7C1 lipid as we described,3 and investigated whether the “original” 80:20 formulation silenced BMECs in vivo. We formulated LNPs by combining the 7C1 ionizable lipid (Figure S1B) with C12PEG2000 at a molar ratio of 80:20; the mass ratio of 7C1 and PEG to siRNA was 5:1 as reported. We injected mice with siRNA targeting Luciferase (siLuc, the control group) or ICAM-2 (siICAM-2). Both validated4,11,12 siRNAs were chemically modified to minimize off-target gene silencing and reduce immunostimulation (Figure S1C). We injected mice intravenously with a 1.0 mg/kg dose of siRNA, waited 3 days, isolated endothelial cells (CD31+CD45−) from bone marrow, lung, and heart using fluorescence activated cell sorting (FACS), and quantified ICAM-2 protein expression using mean fluorescent intensity (MFI) (Figure S1D). As expected, ICAM-2 protein expression was reduced in lung and heart endothelial cells isolated from mice treated with siICAM-2, relative to mice treated with the siLuc control. ICAM-2 protein expression did not change in BMECs (Figure 1C), demonstrating the 80:20 7C1 formulation did not target BMECs.

We hypothesized BMEC tropism could be impacted by the (i) size of the 7C1 LNP or the (ii) chemical composition of the PEG and “helper lipids” added into the LNP. The chemical composition hypothesis was substantiated by evidence that PEG structure influences pharmacokinetics of liver-targeting LNPs in vivo.41 Given that in vitro nanoparticle delivery to endothelial cells does not predict in vivo nanoparticle delivery to endothelial cells,33 we tested our hypothesis in vivo. We reasoned that—like AAV delivery systems—in vivo directed evolution could identify LNPs efficiently by refining the “chemical space” which we were investigating (Figure 1D). This approach has been an important advance in AAVs; directed evolution has identified viruses that deliver genes to

**RESULTS**

We performed a literature search (Figure S1A) to identify nanoparticles that systemically delivered siRNA to endothelial cells in different tissues. We found multiple nanoparticles that have been reported to deliver siRNA to endothelial cells in the lung, heart, kidney, liver, lymph nodes, spleen, brain, and pancreas. An ionizable LNP named 7C1 we reported4 was the most efficient siRNA delivery vehicle for lung, heart, and kidney endothelial cells in our search, silencing target genes by 50% after systemic siRNA doses as low as 0.02 mg/kg (Figure 1B). No LNPs in our search targeted BMECs after systemic administration. On this basis, we synthesized the 7C1 lipid as we described,3 and investigated whether the “original” 80:20 formulation silenced BMECs in vivo. We formulated LNPs by combining the 7C1 ionizable lipid (Figure S1B) with C12PEG2000 at a molar ratio of 80:20; the mass ratio of 7C1 and PEG to siRNA was 5:1 as reported. We injected mice with siRNA targeting Luciferase (siLuc, the control group) or ICAM-2 (siICAM-2). Both validated4,11,12 siRNAs were chemically modified to minimize off-target gene silencing and reduce immunostimulation (Figure S1C). We injected mice intravenously with a 1.0 mg/kg dose of siRNA, waited 3 days, isolated endothelial cells (CD31+CD45−) from bone marrow, lung, and heart using fluorescence activated cell sorting (FACS), and quantified ICAM-2 protein expression using mean fluorescent intensity (MFI) (Figure S1D). As expected, ICAM-2 protein expression was reduced in lung and heart endothelial cells isolated from mice treated with siICAM-2, relative to mice treated with the siLuc control. ICAM-2 protein expression did not change in BMECs (Figure 1C), demonstrating the 80:20 7C1 formulation did not target BMECs.

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the brain, eye, liver, and other tissues. Refining the LNP chemical space is important; between 100 million and 200 billion chemically distinct LNPs could be formulated with validated chemistries.

We designed and validated a high throughput barcoding system to simultaneously quantify how >100 LNPs functionally delivered siRNA in vivo. This screening system is distinct from previous barcoding approaches, which quantify biodistribution (Figure 2A). We used a validated high throughput microfluidics platform to formulate LNP-1, with chemical structure 1, so it carried siICAM-2 and barcode 1. We repeated this process N times, formulating LNP-N, with chemical structure N, to carry siICAM-2 and barcode N. The size and stability of all N LNPs was tested individually using dynamic light scattering (DLS); stable LNPs with hydrodynamic diameters between 20 and 200 nm were pooled together (other LNPs were discarded). The mass ratio of the siRNA:DNA barcode was 10:1. To identify LNPs that functionally delivered siICAM-2 into the cytoplasm, we pooled the stable LNPs together, administered them to mice intravenously, waited 3 days, and isolated ICAM-2Low endothelial cells (i.e., cells with low ICAM-2 MFI) from bone marrow using FACS. We isolated and amplified the barcodes using universal primers and performed deep sequencing to identify barcode sequences that were enriched in the ICAM-2Low cells (Figure 2A). We quantified “normalized delivery” of each barcode; normalized delivery is a calculation of the times each individual barcode is found in a sample, divided by the sum of all barcode counts in that sample (Figure S1E). Normalized delivery is analogous to counts per million in RNA-seq experiments and can be used to quantify LNP biodistribution. Specifically, normalized delivery quantifies how efficiently a barcode was delivered, relative to all other nanoparticles tested. For example, if barcode 10 is twice as abundant as barcode 11, then we hypothesize nanoparticle 10 delivered the barcode twice as efficiently as barcode 11.

We rationally designed the DNA barcodes (Figure S1F). Specifically, we designed DNA barcodes with minimal 2° structure and G-quadruplex formation by separating our previously reported randomized 7 nucleotide region into semirandomized NWNH and NWH site. This increased DNA polymerase access during barcode amplification. We also included universal primer sites so all the barcodes were amplified with 1 set of primers (Figure S1G). The “barcode region” of the DNA barcode was 8 nucleotides and located in the middle of the sequence. We designed the barcodes with a base distance of 3; each barcode was distinct from all other barcodes at 3 of the 8 positions. Using a QC score of 30, this reduced the odds of a “false call” by the Illumina Sequencing machines to less than 1/10⁹. Of the 65 536 (i.e., 4⁸) potential barcode combinations, we selected 156 which would work together on Illumina sequencers. We also flanked the primer sites with 3 additional phosphorothioate-modified nucleotides to reduce exonuclease degradation.

We performed control experiments to evaluate whether coformulating the barcode and a siRNA into the LNP would affect delivery. First, we formulated LNPs with siRNA or siRNA + barcode, and measured size with DLS; there was no difference (Figure S1H). As a second control experiment, we formulated the “80:20” 7C1 formulation with barcodes and a control siRNA (siLuc) or barcodes and siCAM-2. Control mice were injected with 1.5 mg/kg total nucleic acid, while experimental mice were injected with a total nucleic acid dose of either 1.5, 0.5, or 0.16 mg/kg. 72 h after injection, lung delivery is a calculation of the times each individual barcode is found in a sample, divided by the sum of all barcode counts in that sample (Figure S1E). Normalized delivery is analogous to counts per million in RNA-seq experiments and can be used to quantify LNP biodistribution. Specifically, normalized delivery quantifies how efficiently a barcode was delivered, relative to all other nanoparticles tested. For example, if barcode 10 is twice as abundant as barcode 11, then we hypothesize nanoparticle 10 delivered the barcode twice as efficiently as barcode 11.

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endothelial cells were isolated and ICAM-2 protein expression was quantified as MFI using flow cytometry. As expected, we observed a dose-dependent increase in ICAM-2 protein silencing as the siICAM-2 dose increased (Figure 2B,C). We also observed an increase in the number of endothelial cells that were ICAM-2Low (Figure 2D). These data suggested that LNP-mediated delivery of siICAM-2 and barcode silenced target genes as expected in vivo.

To test the hypothesis that BMEC tropism was impacted by altering LNP size or LNP chemical composition of the PEG and “helper lipids”, we designed a library of LNPs consisting of 7C1 lipomer and cholesterol, and 5 different lipid-PEGs (Figure 3A, Figure S2A). We investigated how PEG molecular weight (2000 and 3000 Da) affected delivery; PEG molecular weight can change PEG conformation at the LNP surface, which can alter interactions between nanoparticles and the body.47 We also varied the lipid tails in the PEG (fully saturated with 14, 16, or 18 carbons). This can change “on/off kinetics” of PEG by altering the stability with which the hydrophilic PEG is “anchored” into the LNP membrane.47 Each PEG type was formulated into 24 distinct formulations with 7C1 and cholesterol (Figure 3C). Of the 120 LNPs formulated to carry siRNA and barcode, 115 were stable, with diameters between 20 and 200 nm. These 115 LNPs were pooled together and intravenously injected at a total nucleic acid dose of 1.5 mg/kg. As a control, we compared the diameter of the pooled LNPs (53 nm) to the individual LNPs making up the pool, and found they were similar (Figure 3D). We included 2 additional negative controls, which were 2 naked barcodes. As expected, the normalized delivery of both naked barcode controls in ICAM2Low BMECs was lower than the normalized delivery for all barcodes delivered by LNPs (Figure 3E).

We tested the hypothesis that LNP size affected BMEC tropism. We plotted normalized delivery against the diameter for all 115 LNPs and observed no correlation ($R^2 = 0.06$) (Figure 3F). To exclude the possibility that these results were an artifact of testing many LNPs, we plotted normalized delivery against diameter for the top and bottom 10%; once again, we found no relationship (Figure 3G). Finally, we plotted the size of the top and bottom 10% and found no statistical difference (Figure 3H). Taken together, this evidence did not support our size-based hypothesis. We then
tested the hypothesis that LNP chemical composition affected BMEC tropism. We analyzed the material characteristics of top performing LNPs. Specifically, we looked for material properties that were enriched in the top 10% LNPs. An example calculation (which does not include real data) for enrichment is shown in Figure S2B. In top-performing LNPs, we observed an enrichment of nanoparticles with either low (1–2.5%) or high (15–20%) PEG mole percent (Figure 4A). Additionally, we observed that nanoparticles containing either C16PEG2000 or C18PEG2000 were enriched (Figure 4B). To further confirm that PEG structure influenced delivery, we performed a paired analysis, comparing normalized delivery of LNPs that had identical ratios of 7C1, cholesterol, and PEG, but had either C18PEG2000 or C14PEG3000. C14PEG2000 performed significantly better compared to C18PEG3000 (Figure 4C). Taken together, this provided preliminary evidence to support the hypothesis that LNP composition affected BMEC targeting more than size. To confirm that our screening methodology could be used to identify LNPs that functionally deliver siRNA to BMECs, we formulated the top performing LNP to carry both siLuc and siICAM-2 (Figure S2C). We intravenously injected mice with 1.0 mg/kg siRNA and measured ICAM-2 MFI on BMECs by flow cytometry. We observed a 16% reduction in ICAM-2 MFI with the winner from screen 1 (Figure S2D), which was 2.2-fold more potent (Figure S2E) than the “original” 80:20 formulation (Figure 1B).

To further identify LNPs with improved potency in BMECs, we designed a second LNP library that was evolved from the first; the second library was informed by our PEG enrichment data. More specifically, LNPs for library 2 were made with 7C1, cholesterol, and either C14PEG2000 or C18PEG2000. In some formulations, we also included DSPC (Figure 5A, Figure S2F), since DSPC may improve the encapsulation of nucleic acids,48 and may alter how nanoparticles interact with serum proteins in the “protein corona”.49 We formulated LNPs with 20 distinct molar ratios (Figure 5B). Of the 40 formulated, 31 formed stable nanoparticles with diameters between 20 and 200 nm. These stable LNPs were pooled together; as a control, we compared the diameter of the pooled LNPs (43 nm) to the individual LNPs, and found they were similar (Figure S2G). Pooled LNPs were administered to mice at a total nucleic acid dose of 1.5 mg/kg. Three days later, we isolated ICAM-2Low BMECs using FACS, and sequenced the barcodes. We made several observations that gave us confidence in the results. The first observation was that ICAM-2 silencing in the second library was ~1.5× more robust than the first library (Figure S5C). This suggested our second library contained more potent LNPs than the first. We noted that the experimental variance in potency of the second library was larger than the variance in the first; we analyzed mouse weight, sex, and age (Figure S2H), but were unable to come up with a specific hypothesis for this observation except for normal experimental variance in vivo; furthermore, the delivery of individual LNPs to ICAM-2Low BMECs was consistent between technical replicates in both library 1 and 2 by paired One-way ANOVA (Figure S2I,J). Both library 1 and 2 were well tolerated (Figure S2K,L).

The second observation was that both negative controls (naked barcodes) once again had lower normalized delivery than all barcodes carried by LNPs (Figure 5D). Third, we observed size and chemical composition results that were consistent with library 1. Specifically, we analyzed the relationship between LNP size and delivery, and observed no correlation between the size of all 31 LNPs and delivery (Figure 5E). We did not observe any relationship between the normalized delivery and size for the top and bottom 10% (Figure 5F), and there was no statistical difference in size between the top and bottom 10% (Figure 5G). We next analyzed which chemical characteristics were enriched in the top 10% of LNPs. When we analyzed LNP chemical composition, we found LNPs with high PEG percentages (15 to 20%) were enriched (Figure 5H), as were LNPs with C14PEG3000 (Figure 5I). Additionally, LNPs formulated with 80 mol % 7C1 (Figure 5J), 0% DSPC (Figure 5K), and 0.1–10% cholesterol were enriched (Figure 5L). These enrichment data suggested that a 7C1-based nanoparticle with formulation molar ratio of 80% 7C1:10–20% cholesterol:15–20% C14PEG2000 would be highly active in BMECs. We then tested the top 3 LNPs found in screen 2 (Figure S3A). Notably, all 3 reduced ICAM-2 expression in BMECs more than (i) 7C1 and (ii) the top performing LNP from screen 1 (Figure S3B).

Interestingly, we noticed that the chemical composition of the top performing individual LNP (BM1) exactly matched the enriched chemical characteristics from Figure 5 (Figure 6A). We then selected the top performing LNP from library 2, and compared its (i) chemical composition, (ii) physical traits, (iii) in vitro uptake mechanism, and most importantly, (iv) ability to functionally deliver siRNA/sgRNA that manipulate BMEC gene expression in vivo (Figure 6) to that of “original” 80:20 7C1. Both 7C1 and BM1 formed stable LNPs with diameters between 45 and 50 nm and had a narrow polydispersity index (PDI) (Figure 6B). BM1 was also stable for over 10 days when stored at 4 °C (Figure S3C). Additionally, the pK₅ of each
Figure 5. Analysis of LNP size and chemical traits from a second library further suggests BMEC targeting is influenced by LNP chemical composition. (A) LNPs from library 2 were made with 7C1 lipomer, cholesterol, and DSPC. (B) Two different PEG types were used in library 2: C16PEG2000 and C18PEG2000. These structures were selected based on data from LNP library 1. (C) 20 different formulation ratios were used for each of the two PEG types in library 2. (D) ICAM-2 protein silencing in BMECs 3 days after mice were injected with the library of LNPs at a total dose of 1.5 mg/kg. Notably, ICAM-2 silencing was more potent in BMECs than library 1. (D) Normalized DNA delivery in BMECs for 31 LNPs and 2 naked barcodes; as expected, the naked barcodes performed poorly. (E) Correlation between LNP diameter (nm) and normalized DNA delivery in BMECs for all 31 LNPs in Library 2. (F) Correlation between LNP diameter (nm) and normalized DNA delivery in the top and bottom 10% LNPs based on performance from library 2. (G) Diameter (nm) of top and bottom 10% LNPs. Taken together, (E–G) further suggest BMEC targeting is not influenced by LNP size between 20 and 200 nm. (H) Enrichment of LNPs containing PEG mol % between 15 and 20% in BMECs in vivo. (I) Enrichment of LNPs containing C18PEG2000 in BMECs in vivo. (J) Enrichment of LNPs containing 80 mol % 7C1 in BMECs in vivo. (K) Enrichment of LNPs containing 0 mol % DSPC in BMECs in vivo. (L) Enrichment of LNPs containing 0.1–10 mol % cholesterol in BMECs in vivo.
LNP was between 6.45 and 6.55, indicating that each has a net neutral charge in blood (pH = 7.4) (Figure 6B, Figure S3D,E), but could become cationic in early endosomes. To measure how "original" 7C1 and BM1 are endocytosed in vitro, we formulated both "original" 80:20 7C1 and BM1 to carry siGFP tagged with AlexaFluor647 and applied each LNP at a dose of 20 nM siRNA to Immortalized Mouse Aortic Endothelial Cells (IMAECs). iMAECs are endothelial cells that are freshly isolated from mice; they recapitulate important endothelial phenotypes.49 After 1 h, cells were washed and siRNA uptake was measured by flow cytometry. BM1 endocytosis was 40% less than 7C1 (Figure 6C). When cells were pretreated by genistein (caveolin-inhibitor) and chlorpromazine (clathrin-inhibitor), endocytosis of 7C1 decreased by at least 40%; however, BM1 endocytosis only decreased in the presence of chlorpromazine, relative to cells not treated with inhibitors (Figure 6D, Figure S3F,G). We next tested the potency of BM1 at delivering siRNA to BMECs in vivo. We intravenously injected BM1 at a dose of 1 mg/kg siRNA; after 3 days, we isolated bone marrow and measured ICAM-2 MFI using flow cytometry. Compared to BMECs from mice treated with BM1 carrying siLuc, mice treated with BM1 carrying siICAM-2 showed 37% protein silencing (Figure 6E,F). This represents a 4.8X increase in potency compared to original 7C1.

Given that we specifically evolved BM1 to target BMECs, we compared its potency to original 7C1 in lung and heart endothelial cells. We observed no difference in potency between 7C1 and BM1 in these tissues (Figure 6G). We then quantified biodistribution of 7C1 and BM1 using QUANT, a highly sensitive ddPCR-based method.50 Specifically, we quantified DNA barcode biodistribution in lung, heart, and bone marrow ECs, as well as CD34+ hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (Figure S3I). Biodistribution to BMECs increased 4.7X in mice treated with BM1, relative to original 7C1 formulation (Figure S3J). We did not observe significant differences in biodistribution in the other analyzed cell types. These biodistribution data were similar to the relative siRNA-mediated gene silencing we observed.

Confident that BM1 could potently deliver siRNA to BMECs, we next tested if it could also deliver sgRNA. We formulated BM1 at a dose of 1 mg/kg carrying an sgRNA targeting ICAM-2 and intravenously injected into mice constitutively expressing SpCas9.51 This sgRNA was chemically modified with three phosphorothioates on each termini and 2′-O-methyl ribose modifications at select positions.
(Figure S3K),52 Five days after injection, we isolated BMECs and CD34+ HSPCs and measured indels at ICAM-2 via Tracking Indels by Decomposition (TIDE). BM1 led to a 15% indel (insertions and deletions) rate in BMECs (Figure 6H) and undetectable levels of indels in CD34+ HSPCs (Figure S3L). BM1 was well tolerated in mice with both siRNA and sgRNA (Figure S3M,N).

**DISCUSSION**

The first systemically administered siRNA therapy was approved in August 2018.20 In this system, siRNAs are delivered to hepatocytes using an ionizable LNP.1 This illustrates the clinical potential of RNA therapeutics and highlights the need for “nonliver” RNA delivery vehicles. The nanomedicine field is well positioned to make advances in nonliver delivery; thanks to important advances in nanoparticle synthesis, between 100 million and 200 billion chemically distinct nanoparticles can be formulated using available materials. However, nanoparticles must still be tested laboriously 1 by 1 in vivo. And as a result, most nanoparticles are only tested in vitro, which leaves many potential therapeutic molecules undiscovered.

Here we report that coformulating a DNA barcode and siRNA into the same LNP can facilitate high throughput screens that quantify functional cytoplasmic siRNA delivery. This approach can help scientists in several ways. First, over the course of several experiments, it is possible to study thousands of nanoparticles delivering siRNA to any combination of cells. This could accelerate the discovery of new nanomedicines. Notably, we predict that it will eventually be feasible to study how up to 500 LNPs deliver siRNA in a single mouse. Second, we envision studies designed to systematically identify the traits that alter nanoparticle targeting directly in vivo. In this example, we tested two hypotheses: LNP (1) size or (2) chemical properties affect targeting. Over the course of our experiments, we consistently found no evidence to support hypothesis 1 and multiple lines of evidence to support hypothesis 2. Interestingly, our data suggested that making seemingly small changes to the LNP formulation—in our case, changing the lipid tail of the PEG and adding cholesterol—altered nanoparticle tropism. Notably, minor changes to PEG composition have altered the pharmacokinetics and function of liver-targeting LNPs.53 However, the mechanisms mediating this effect remain unclear. In future studies, we hope to test two hypotheses. First, that PEG on/off rates in serum are altered by changing the lipid tail of the PEG. Second, that the inclusion of cholesterol alters the serum lipoproteins to which the LNP binds. It is also possible that both hypotheses are incorrect, and instead, that a yet to be discovered, multivariate effect is causing these effects. Broadly, these data suggest that LNP targeting can be altered by making small changes to the chemical composition, which may offer a simple alternative to traditional approaches, which rely on active targeting ligands.53—55 These data need to be substantiated by other recent reports,41,56 but need to be validated in other laboratories. If vascular tropism can be altered by simple changes to the LNPs, then these data will be helpful by informing the number of physical and chemical variables that need to be considered when formulating chemically diverse nanoparticle libraries.

It is important to note several limitations with the current study. First, this screening system will not work with toxic or unstable nanoparticles. Second, like all DNA-based screens, it is important to include all the controls we have described herein. Third, we only used two iterative libraries; we believe future iterative libraries will be able to identify LNPs with even greater BMEC tropism. Finally, given the size of these data sets, it will be important to collaborate with “big data” scientists, to understand which new, cutting edge bioinformatic approaches can be applied to these in vivo delivery data sets. Even with these nuances, we believe this methodology offers a solution to many technical/practical issues that impede the translation of new nanoparticles into the clinic.

**MATERIALS AND METHODS**

**Nanoparticle Formulation.** Nanoparticles were formulated using a microfluidic device as previously described.26 Briefly, nucleic acids (siRNA and DNA barcodes) were diluted in citrate buffer while lipid-amine compounds, alkyl tailed PEG, cholesterol, and DSPC were diluted in ethanol. PEG, cholesterol, and DSPC was purchased from Avanti Lipids. Citrate and ethanol phases were combined in a microfluidic device by syringe pumps.

**DNA Barcoding.** Each chemically distinct LNP was formulated to carry its own unique DNA barcode and siRNA. For example, LNP1 carried DNA barcode 1 and siCAM2, while the chemically distinct LNP2 carried DNA barcode 2 and siCAM2. Single stranded DNA sequences were purchased from Integrated DNA Technologies (IDT). To ensure equal amplification of each sequence, we included universal forward and reverse primer regions. Each barcode was distinguished using a unique 8 nucleotide sequence. An 8 nucleotide sequence can generate 65 536 distinct barcodes. We used 156 distinct sequences designed to prevent sequence “bleaching” on the Illumina MiniSeq sequencing machine.

**Nanoparticle Characterization.** LNP hydrodynamic diameter was measured using a plate reader formatted dynamic light scattering machine (Wyatt). LNPs were diluted in sterile 1X PBS to a concentration of ~0.06 μg/mL and analyzed. LNPs were only included if they formed monodisperse populations with diameter between 20 and 200 nm. Particles that met these criteria were dialyzed with 1X phosphate buffered saline (PBS, Invitrogen), and were sterile filtered with a 0.22 μm filter.

**Animal Experiments.** All animal experiments were performed in accordance with the Georgia Institute of Technology’s IACUC. C57BL/6j (#000664) and constitutive SpCas9 (#026179) mice were purchased from The Jackson Laboratory and used between 5 and 12 weeks of age. In all experiments, we used N = 3–5 mice/group. Mice were injected intravenously through the lateral tail vein. The nanoparticle concentration was determined using NanoDrop (Thermo Scientific).

**Cell Isolation and Staining.** Cells were isolated 72 h (for screens) or 120 h (for in vivo gene editing) hours after injection with LNPs unless otherwise noted. Mice were perfused with 20 mL of 1× PBS through the right atrium. As we previously described4,33 tissues were sterile filtered with a 0.22 μm filter.

**PCR Amplification for Illumina Sequencing.** All samples were amplified and prepared for sequencing using nested PCR (Figure S1G). Two μL of primers were added to 5 μL of Kapa HiFi 2X master mix, and 3 μL template DNA/water. The second PCR, added Nextera XT chemistry, indices and i5/i7 adapter regions. Dual-indexed samples were run on a 2% agarose gel to ensure that PCR reaction occurred before being pooled and gel purified.

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Deep Sequencing. Illumina sequencing was conducted in Georgia Institute of Technology’s Molecular Evolution core. Runs were performed on an Illumina Miniseq Primers were designed on the basis of Nextera XT adapter sequences.

Barcode Sequencing Normalization. Counts for each particle, per cell type, were normalized to the barcode LNP mixture applied to cells or injected into the mouse.

TNS Assay. The pKₐ of 7C1 and BM1 was measured as previously described. Briefly, a stock solution of 10 mM HEPES (Sigma), 10 mM MES (Sigma), 10 mM sodium acetate (Sigma), and 140 mM sodium chloride (Sigma) was prepared and pH adjusted with hydrogen chloride and sodium hydroxide to a range of pH between 4 and 10. Using 4 replicates for each nanoparticle at each pH, 140 μL pH-adjusted buffer was added to a 96-well plate, followed by the addition of 5 μL of 2-(p-toluidino)-6-naphthalene sulfonic acid (60 μg/mL). Sul of each nanoparticle was added to each well. After 5 min of incubation under gentle shaking, fluorescence absorbance was measured using excitation wavelengths of 325 nm and emission wavelength of 435 nm.

In Vitro Endocytosis. Immortalized mouse aortic endothelial cells (IMAECs) were seeded in a 24 well plate at 40,000 cells per well and allowed to culture overnight. 7C1 and BM1 were formulated to carry Alexa647-tagged siRNA using microfluidics. After formulation, both LNPs were dialyzed for 2 h in 1X PBS. One hour prior to incubation with each LNP, inhibitors of clathrin-mediated endocytosis (chlorpromazine, 100 mM, Alfa Aesar), caveolae-mediated endocytosis (genistein, 100 mM, TCI America), and macropinocytosis (5-(N-ethyl-N-isopropyl) Amiloride, EIPA, 50 mM, Toronto Research Chemicals) were added to IMAECs at a dose of 20 nM siRNA/well. LNPs were left on the cells for 1 h before the cells were washed 2× with PBS, trypsinized and prepared for flow cytometry using a BD Accuri C6.

RNA Interference. siRNAs were chemically modified at the 2’ position to increase stability and negate immunostimulation. 72 h after injection, tissues were isolated and protein expression was determined via flow cytometry. ICAM2 mean fluorescent intensity in siLuc-treated mice was normalized to 100%.

QUANT Biodistribution. 7C1 and BM1 LNPs were formulated to carry the DNA barcodes utilized in this study. Mice were injected at a dose of 0.5 mg/kg. After 4 h, tissues were isolated and endothelial cells from the lung, heart, and bone marrow, as well as CD34+ HSPCs were isolated by FACS. DNA barcodes were isolated using QuickExtract (Epicenter). Biodistribution was measured as previously described. Briefly, the QX200 Droplet Digital PCR System (Bio-Rad) was used to prep and analyze all ddPCR results. All PCR samples were prepared with 10 μL ddPCR with ddPCR Supermix for Probes (Bio-Rad), 1 μM of Reverse/Forward Primers, 1 mM of template, and 8 μL water. 20 μL of each reaction and 70 μL of Droplet Generation Oil for Probes (Bio-Rad) were loaded into DG8 Cartridges and covered with DG8 Gaskets. Cartridges were placed in the QX200 Droplet Generator to create water—oil emulsion droplets. Cycle conditions for PCR were as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 1 cycle of 95 °C for 10 min. Plates were stored at 4 °C until ran on the QX200 Droplet Digital PCR System. For each biological rep, 2 technical repetitions were completed. In all cases, technical reps were averaged.

In Vivo Cas9 Editing. Mice constitutively expressing SpCas9 were injected with BM1 carrying 1 mg/kg of sgICAM2. sgICAM2 was modified with 2′ O-methyl ribose at select positions and 3 phosphorothioates at both the 5′ and 3′ termini. Five days after injection, cells were isolated via FACS. Indels were measured by TIDES.

Data Analysis and Statistics. Sequencing results were processed using a custom R script to extract raw barcode counts for each tissue. These raw counts were then normalized with an R script prior for further analysis. Statistical analysis was done using GraphPad Prism 7; more specifically paired 2-tail t-test or one-way ANOVAs were used where appropriate. Data is plotted as mean ± standard error mean unless otherwise stated.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b08976.

Literature Review of Delivery to Endothelial Cells, Structure of 7C1, siRNA Sequences, Representative FACS Gating, Representative Normalization, Barcode Sequences, Diagram of PCR Scheme, Dynamic Light Scattering, LNP Library Chemical Composition, Enrichment Diagram, Winning LNP Confirmation, Library Diameter, Consistency of Technical Replicates, Mouse Weights, LNP Stability, LNP Ionizability, LNP Endocytosis, LNP Biodistribution, sgRNA Sequence, HSPC Indels (PDF)

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Notes

The authors declare the following competing financial interest(s): C.D.S., M.P.L., and J.E.D. have filed intellectual property related to this work. All data, analyses, and scripts used in the paper are available upon requests made to dahlanlab.org.

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REFERENCES


