Lipid Nanoparticles Deliver Nucleic Acids, Including CRISPR Components, in Primary Neurons

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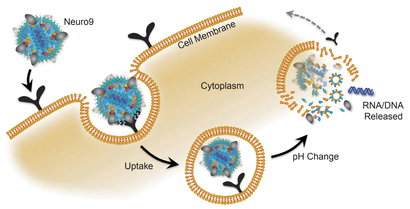
Introduction

Advances in the gene editing arena, specifically with CRISPR-Cas9, has pushed the demand for efficiently delivering payloads even further. Of the tools available, developments in the field of lipid nanoparticles (LNPs) has allowed for the reliable and efficient delivery of CRISPR components, both in research and clinical settings. Here, we bridge that gap by describing the development of an LNP delivery system for CRISPR components, robustly manufactured with clinical-grade materials using microfluidic technology at scales for in vitro applications, with the potential to move into animal models. We describe the use of lipid-based nanoparticles for highly efficient encapsulation and delivery of payloads, such as siRNA, mRNA , and plasmid. We show that representative small RNAs, mRNAs and plasmids can successfully be delivered to primary neurons. LNPs manufactured to encapsulate various nucleic acids can do so with high efficiency, encapsulating more than 95% of the payload, minimizing payload loss. Transfection efficiency of the LNPs is variable based on payload. The biological endpoint assays used to determine the accessibility of the payloads delivered varies for siRNA, mRNA, and plasmid. Using doses of 1 µg per mL of media, we achieved >90% knockdown with siRNA delivery, >90% of the primary neurons are GFP+ with GFP mRNA delivery and 60% of the primary neurons are GFP+ with GFP plasmid delivery. The LNPs are well tolerated, such that 5X the required dose have no observable cytoxicity. We demonstrated delivery of gRNA to Cas9 expressing cells to achieve up to 80% editing. We demonstrated co-transfection of Cas9 mRNA and gRNAs in separate nanoparticles to achieve editing in wild-type cells. We show that the LNPs can also be used to deliver payloads into various regions of the animal brain. These validation studies provide suitable insights in establishing strategies for efficiently delivering CRISPR components into primary cultures.

Gene Delivery via Lipid Nanoparticles

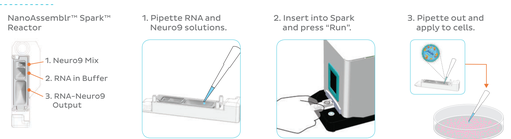
Lipid Nanoparticles (LNPs) are a platform that can be used to deliver nucleic acids to cells. LNPs mimic low density lipoproteins (LDLs), which are taken up by an endogenous pathway. LNPs are pH sensitive, and designed to release their payload into the cytoplasm.



The Microfluidic Platform

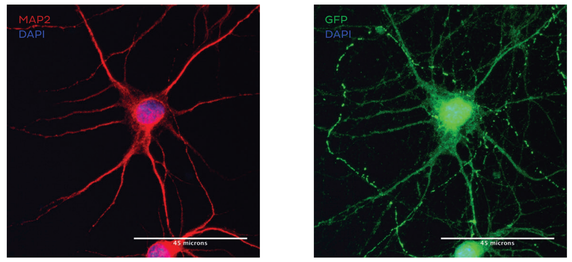
The NanoAssemblr™ platform uses microfluidics to precisely control the manufacturing of nanoparticles from µL to mL scales.

in vitro scale



in vivo scale

Proof of Concept: mRNA Delivery in Rat Primary Neurons



MAP2 positive neurons (red) show expression of GFP (green) after treatment with Neuro9 GFP mRNA-LNP in the presence of 5 µg/mL ApoE. Cells were stained with anti-MAP2 primary antibody/alexa fluor-594 conjugated secondary and anti-GFP primary antibody/alexa fluor-488 conjugated secondary antibody. Nuclei are stained with DAPI (blue)

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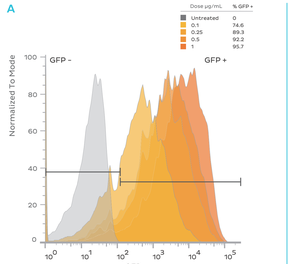

The percentage of GFP+ neurons was dose dependent, with 1 µg/mL having the highest percentage.

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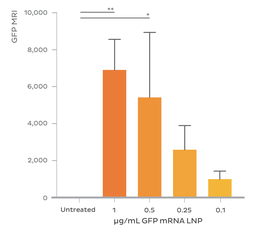
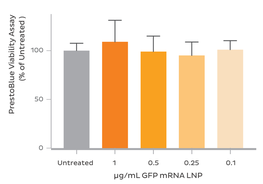


The mean fluorescent intensity (MFI) was highest for the GFP mRNA dose of 1 µg/mL, n=3 for each treatment, one-way ANOVA with Dunnet's multiple comparisons test, ** p<0.005, * p<0.05

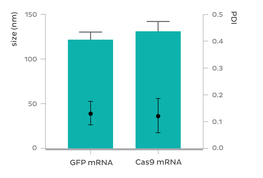
Flow cytometry analysis after 48 h treatment with Neuro9 GFP mRNA-LNP in the presence of 5 µg/mL ApoE in rat primary neurons, DIV 7, show >95% of neurons had uptaken the LNP (calculated using the fluorescent probe DiD encorporated within the nanoparticle) even when treated at different doses of GFP mRNA LNP (Data not shown).

C



Cell viability was assayed by the PrestoBlue® Cell Viability Reagent. No toxicity was observed at any dose employed, n=6 for each treatment, one-way ANOVA with Dunnett's multiple comparison test.

D



The size and polydispersity index (PDI) of the nanoparticles encapsulating different mRNAs are comparable. GFP mRNA is 996 nucleotides long, while Cas9 mRNA is 4479 nucleotides in length. The particle size and PDI is not significantly different despite using mRNAs of different lengths, student's t-test.

Methods

Primary Rat Cortical Neuron Culture
E18 rat cortical tissue was purchased from BrainBits, LLC. The cortices were removed from the shipping media and trypsinized with 0.25% trypsin-EDTA (ThermoFisher). The trypsin-EDA was then inactivated by washing the tissue with 10% FBS in DMEM (ThermoFisher), followed by DMEM alone. The tissue was triturated in Neuronal Media (NeuroCult™ Neuronal Basal Medium (StemCell) with NeuroCult™ SM1 Neuronal supplement (StemCell) and L-Glutamine (StemCell)) supplemented with L-Glutamic Acid (Sigma), and then passed through a 40µm cell strainer to form a single cell suspension. This suspension was then counted and plated on PDL coated culture plates or glass cover slips at a density of 4.8 x 104 cells/cm2. Half of the media was changed with Neuronal media without L-Glutamic Acid every 3-4 days.

Treatment of Primary Rat Cortical Neurons with LNPs
After 7 days in culture (DIV7) the 5µg/mL of ApoE was added followed by the specified LNP at the specified dose. The neurons were then incubated for 48h after which they were harvested for endpoint assessment.

Flow Cytometry
Following treatment and incubation with the specified LNP as noted above the neurons were washed with PBS and then trypsinized with 0.25% trypsin-EDTA to detach them from the culture plate. Trypsinization was inactivated by 3% FBS in PBS and the neurons were triturated to form a single cell suspension. The neurons were then pelleted and washed with PBS before being re-suspended in suspension buffer (BD biosciences) and Propidium Iodine (BD biosciences) was added to stain for apoptotic cells. The suspended, stained neurons were then passed through a 35µm cell strainer and then assed using a BD Celesta Flow Cytometer.

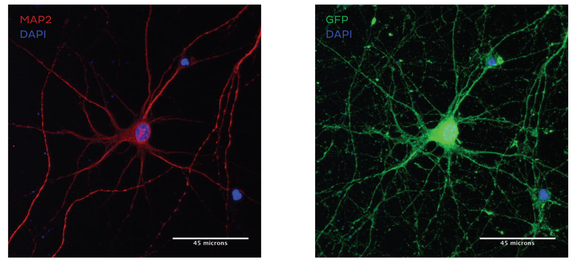
Viability Assay
The standard protocol included with the PrestoBlue® Cell Viability Reagent (ThermoFisher), following the manufacture's instructions. Values were corrected to wells containing media without neurons.

Immunocytochemistry
For this assessment the neurons were plated on PDL coated coverslips, following cultivation, treatment and incubation, the neurons were washed with PBS and fixed with 4% PFA. The neurons were permeabilized using 0.1% triton-X and then blocked with NDS followed by incubation with a MAP2 antibody (Sigma M4403) for 1 hour. The neurons were then blocked in NDS followed by incubation with a GFP antibody (AbCam ab13970) or Cas9 antibody (Diagenode C15200229) in NDS overnight. Secondary antibodies were then added, AlexaFluor-594 for MAP2 and Cas9 and AlexaFluor-488 for GFP. The coverslips were mounted on glass slides using ProLong® Diamond Antifade Mountant (ThermoFisher) and imaged on a confocal microscope. Confirmation of the selective binding of the primary and secondary antibodies was provided through no primary controls (data not shown).

In Vivo Plasmid Transfection
YAC128xNSG mice at 5 months of age were intercranially injected with LNP-encapsulating plasmid encoding a protein with an eGFP reporter. Mice were anesthetized with 1% isofluorane. The heads were prepped and a single craniotomy was made +0.5mm anterior and -1.75mm lateral to bregma. A Hamilton syringe was loaded with 5 µl of plasmid-LNP and lowered ~2.5mm from the dura. 5 µl of plasmid-LNP was injected at a rate of 0.5 µl/minute. Needle was left in place for 5 min before being retracted over course of 3 minutes. Skull was sealed with bone wax and sutured. Mice were observed for recovery until ambulatory. 48 hrs following injection mice were intercardially perfused with PBS and formalin. Brains were extracted and cryopreserved in 30% sucrose before flash frozen in isopropanol. Brains were sectioned using a cryostat at 30 µm thick serial sections with every 6th section being collected for analysis. Brains were mounted and cross-labeled with Hoescht (1:1000) for 15 min. Slices were imaged (Keyence BZ-9000) at 4x and stitched together using Keyence Viewing Software.

T7EI Cleavage Assay
Following treatment and incubation of the Cas9 HEK293 cells or primary rat neurons, genomic DNA extraction and the subsequent T7EI Cleavage assay was preformed using the Alt-R® Genome Editing Detection Kit (IDT), following the manufacture's instrutions.

Proof of Concept: Plasmid Delivery in Rat Primary Neurons



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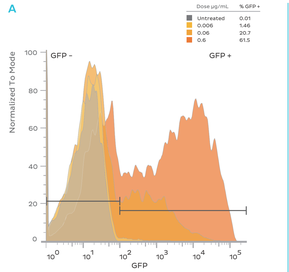

The percentage of GFP + neruons was dose dependent, with 0.6ug/mL having the higest percentage.

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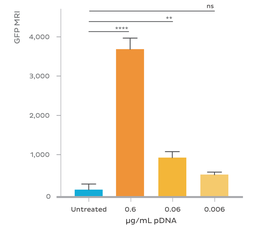

This dose reponse pattern is also observed in the GFP MFI, n=3 for each treatment one-way ANOVA with Dunnett's multiple comparisons test, **** p<0.0001, ** p<0.005

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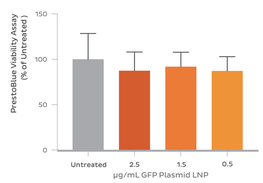

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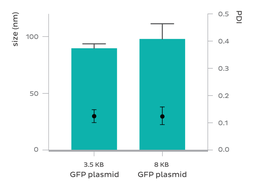
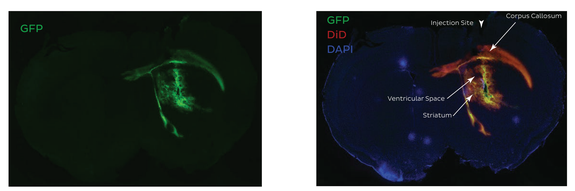


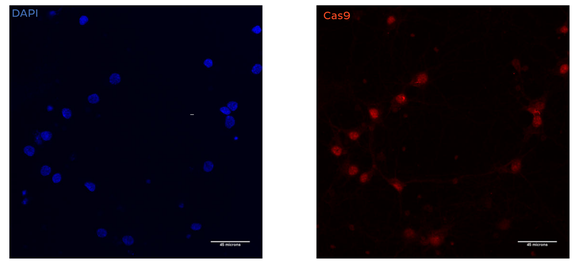
The size and PDI of the nanoparticles encapsulating different plasmids are comparable. The particle size and PDI is not significantly different despite using plasmids of different size, student's t-test.

Plasmid Delivery *in Vivo*



GFP expression (green) 48H following striatal injection of GFP plasmid-LNP in 10 month old, FVB mice. Injection of 5µl of a 3mg/mL GFP plasmid-LNP Formulation. Co-localization of nuclear staining with DAPI (blue), LNP uptake using DiD (red), and GFP expression (green) in the same image. Both LNP uptake and GFP expression co localize both within the area of injection, the striatum, and have spread to additional surrounding regions including parts of the ventricular system and the corpus callosum.

Delivery of Cas9 mRNA in Rat Primary Neurons



Primary rat neurons show Cas9 expression (Red) after treatment with Cas9 mRNA LNPs in the presence of 5ug/mL of ApoE. 48H after treatment the cells were stained for DAPI (blue) to identify nuclei, as well as Cas9 primary antibody/alexa fluor-594 conjugated secondary. At this time point the majority of the Cas9 protein has translocated to the nucleus indicating that delivery of guide RNA for the Cas9 protein must occur before this time point to ensure complexation and co-translocation to the nucleus.

Cas9 Mediated Editing in Cas9-HEK293 & Rat Primary Neurons

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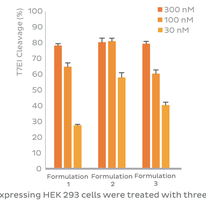
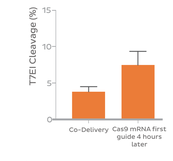


Cas9 expressing HEK 293 cells were treated with three different formulations of HPRT guide RNA LNPs. 48H following treatment the cells were harvested and effiency of editng was determined using the T7EI cleavage assay.

B



Rat primary neurons were treated with 1µg/mL of Cas9 mRNA LNPs and 3µg/mL of guide LNPs in the presence of 1µg/mL of ApoE. 48H after treatment cells were harvested and efficiency of editing was determined using hte T7EI cleavage assay.

Addition of guide RNA to Cas9 expressing cells produces robust editing in HEK293 cells. In wild-type neuronal cells further titration is necessary to ensure the correct timing for optimal editing

Conclusion

The NanoAssemblr™ platform is capable of creating highly reproducible LNPs carrying siRNA, mRNA or plasmid payloads.

These LNPs have high transfection efficiencies (>95%) in primary, rat, cortical neurons and allow for efficient delivery of their associated payloads.

The LNPs were not toxic to these primary neurons even at high dosages (50x)

LNP-mediated CRISPR-Cas9 Delivery

LNPs are versatile, and can be used to deliver Cas9 via mRNA, plasmid, or a pre-formed ribonucleoprotein complex. One or more guide strands can be delivered as well.

Our results validate that these lipid nanoparticles can be used as an effective delivery system of CRISPR-Cas9 components to mediated gene editing.

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