The Endosomal Escape Vehicle Platform Enhances Lipid Nanoparticle–Mediated Delivery of Genomic Medicines

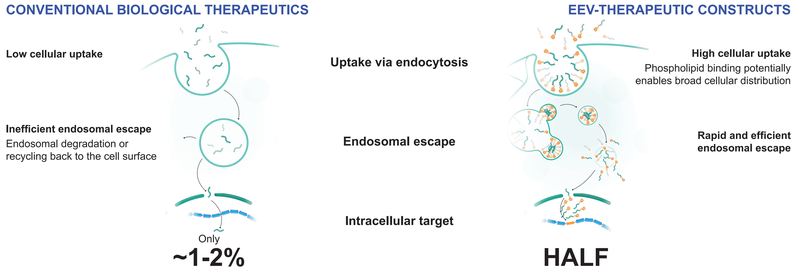
Nan-Cher Flo Yeo, Mahboubeh Kheirabadi, John Buzzo, Pinakin Sukthankar, Lucas Bongarzone, Anushree Pathak, Zekun Li, Xiang Li, **Ziqing Leo Qian**

Entrada Therapeutics, Boston, MA

INTRODUCTION

- Lipid nanoparticle (LNP) technology has emerged as a promising delivery method for nucleic acids therapeutics, including messenger RNA (mRNA) vaccines, small interfering RNA, and gene-editing modalities.[1]
- Despite tremendous success, LNP delivery platforms still face major challenges such as poor delivery efficiency due to limited cellular uptake and low endosomal escape.[2]
- To address these concerns, we have applied our Endosomal Escape Vehicle (EEV™) technology (**Figure 1**) to LNP engineering (**Figure 2A**), with the goal of improving the delivery of genomic medicines.
- The EEV platform consists of a family of proprietary cell–penetrating peptides, which has been shown previously to improve intracellular delivery of various therapeutic modalities to a wide range of tissues and cell types.[3]

Figure 1. EEV Platform Construct Mechanism of Action.



EEV, Endosomal Escape Vehicle.

OBJECTIVES

- To assess the ability of EEV-incorporated LNP (EEV-LNP) to efficiently deliver mRNA and gene-editing cargo in human cell models.
- To assess EEV-LNP–mediated delivery and effect on cell viability compared to electroporation.

CONCLUSIONS

- EEV-LNP significantly enhanced the efficiency of mRNA delivery and gene-editing efficacy compared with LNP alone and MessengerMAX in human cancer and primary immune cells.
- Additionally, EEV-LNP treatment demonstrated greater mRNA delivery and cell viability compared with electroporation.
- Our results indicate the potential of the EEV-LNP platform for improved functional delivery of genomic medicines in cell models that are traditionally difficult to transfect, indicating potential application across a range of cell types.

ACKNOWLEDGMENTS

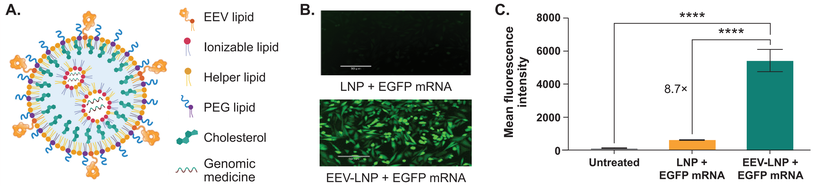
This research was funded by Entrada Therapeutics, Inc (Boston, MA). The authors would like to thank Aji Nair for assistance with poster development and Cindy Wang and Carlos Oviedo for technical support (Entrada Therapeutics, Inc). Editorial and studio support for this poster was provided by Ashfield MedComms (US), an Inizio company, and was funded by Entrada Therapeutics, Inc. References: 1. Rohner E, et al. *Nat Biotechnol*. 2022. 2. Wang X, et al. *Nat Protoc*. 2023. 3. Li X, et al. *Mol Ther Nucleic Acids*. 2023. 4. Kilchrist KV, et al. *ACS Nano*. 2019. 5. Data on File. Entrada Therapeutics. 6. Li X, et al. *J Biol Chem*. 1998.

RESULTS

Delivery of EGFP mRNA by EEV-LNP

- Greater enhanced green fluorescent protein (EGFP) mRNA delivery was observed with EEV-LNP (~9-fold) compared with LNP alone in Hela cells (**Figures 2B**, **2C**).

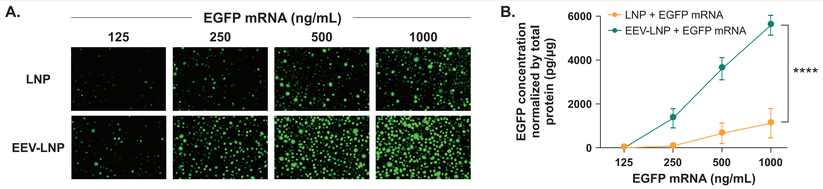
Figure 2. Enhanced EGFP mRNA Delivery by EEV-LNP.



(A) Structure of EEV-incorporated LNP. **(B, C)** Hela cells were treated with 250 ng/mL EEV-LNP or LNP, both containing EGFP mRNA. Cells were harvested after 24 hours and analyzed for GFP expression by fluorescence microscopy and FACS analysis. Only significant statistical comparisons are annotated in the graph. One-way ANOVA was used for statistical comparison; ****p<0.0001. ANOVA, analysis of covariance; EEV, Endosomal Escape Vehicle; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; LNP, lipid nanoparticle; mRNA, messenger RNA; PEG, pegylated.

- EEV-LNP demonstrated dose-dependent and significantly higher EGFP transfection efficacy in primary human macrophages ex vivo compared with LNP control (**Figure 3**).

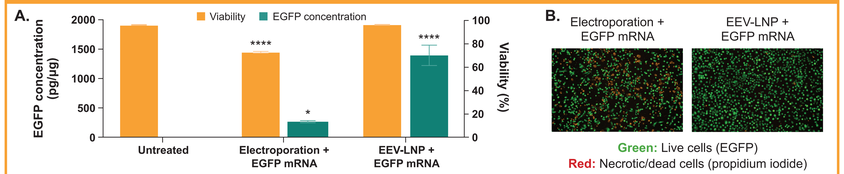
Figure 3. Uptake of EGFP mRNA With EEV-LNP in Primary Human Macrophages.



Primary CD14+ human monocytes were treated with granulocyte-macrophage colony-stimulating factor for 7 days to drive differentiation into macrophages. Cells were treated with EEV-LNP or LNP (both containing EGFP mRNA) and assessed EGFP expression by **(A)** fluorescence microscopy or **(B)** ELISA 24 hours post-treatment. Two-way ANOVA was used for statistical comparison; ****p<0.0001. ANOVA, analysis of covariance; EEV, Endosomal Escape Vehicle; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; LNP, lipid nanoparticle; mRNA, messenger RNA.

- EEV-LNP demonstrated 5-fold improvement in mRNA delivery in human primary macrophages compared with electroporation, with reduced impact on cell viability (**Figure 4**).

Figure 4. Comparison of Productive Delivery of EGFP mRNA by EEV-LNP Versus Electroporation.

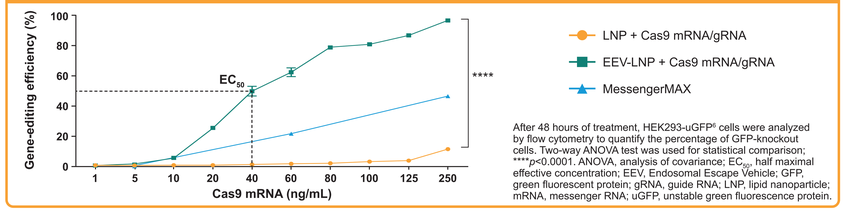


Human primary macrophages were differentiated from monocytes isolated from PBMCs. Macrophages were treated with EEV-LNP or electroporated (EGFP mRNA dose 2500 ng/10^6 cells) using Lonza nucleofection method. **(A)** At 24 hours post-treatment, EGFP protein expression was measured by ELISA. **(B)** Cell viability was quantified by LIVE/DEAD™ Cell Imaging Kit. EEV-LNP showed 5-fold improvement in EGFP mRNA delivery over electroporation, with reduced cytotoxicity. One-way ANOVA was used for statistical comparison; EGFP concentration: *p<0.05 vs untreated, ****p<0.0001 vs untreated and electroporation; Viability: ****p<0.0001 vs untreated and EEV-LNP. ANOVA, analysis of covariance; EEV, Endosomal Escape Vehicle; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; LNP, lipid nanoparticle; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell.

Delivery of Cas9 mRNA by EEV-LNP

- EEV-LNP demonstrated dose-dependent gene editing, with editing efficiency consistently higher than LNP alone and MessengerMAX across a wide concentration range from 5 to 250 ng/mL Cas9 mRNA (**Figure 5**).
  - Dose-dependent delivery of gene editing observed with EEV-LNP, demonstrating a half maximal effective concentration (EC_{50}) of approximately 40 ng/mL.
  - An approximate 33-fold enhancement was observed at a dose as low as 40 ng/mL of Cas9 mRNA compared to LNP alone.

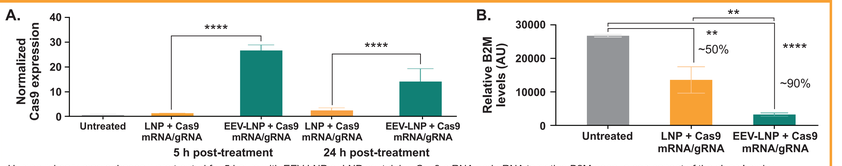
Figure 5. EEV-LNP–Mediated Co-delivery of Cas9 mRNA and gRNA in HEK293-uGFP Cells.



After 48 hours of treatment, HEK293-uGFP® cells were analyzed by flow cytometry to quantify the percentage of GFP-knockout cells. Two-way ANOVA test was used for statistical comparison; ****p<0.0001. ANOVA, analysis of covariance; EC_{50}, half maximal effective concentration; EEV, Endosomal Escape Vehicle; GFP, green fluorescent protein; gRNA, guide RNA; LNP, lipid nanoparticle; mRNA, messenger RNA; uGFP, unstable green fluorescence protein.

- EEV-LNP showed 26-times higher Cas9 expression at 5 hours post-transfection compared with LNP alone (**Figure 6A**). At 24 hours post-transfection, Cas9 expression was 7-times greater with EEV-LNP compared with LNP alone. At 7 days post-transfection, endogenous β2-microglobulin (B2M) knockdown was 40% greater with EEV-LNP compared with LNP alone (**Figure 6B**).

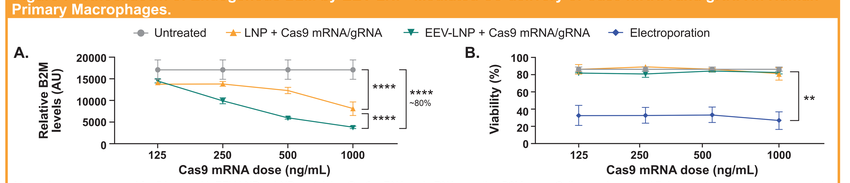
Figure 6. EEV-LNP–Mediated Co-delivery of Cas9 mRNA and gRNA in Human Primary Macrophages.



Human primary macrophages were treated for 5 hours with EEV-LNP or LNP containing Cas9 mRNA and gRNA targeting B2M gene, a component of the class I major histocompatibility complex involved in the presentation of peptide antigens to the immune system. **(A)** Cas9 protein expression was quantified by Western blot at 5 and 24 hours post-treatment. **(B)** B2M knockdown was assessed at 7 days post-treatment by FACS analysis. One-way ANOVA was used for statistical comparison; ****p<0.0001, **p<0.01. ANOVA, analysis of covariance; AU, arbitrary unit; B2M, β2-microglobulin; EEV, Endosomal Escape Vehicle; FACS, fluorescence-activated cell sorting; gRNA, guide RNA; LNP, lipid nanoparticle; mRNA, messenger RNA.

- EEV-LNP resulted in robust and dose-dependent gene editing in primary human macrophages, achieving 80% knockdown in endogenous B2M, a component of major histocompatibility complex class I that is highly expressed in macrophages (**Figure 7A**). Additionally, EEV-LNP treatment had a reduced impact on cell viability compared to electroporation (**Figure 7B**).

Figure 7 . Knockdown of Endogenous B2M by EEV-LNP–Mediated Co-delivery of Cas9 mRNA and gRNA in Human Primary Macrophages.



Macrophages were treated for 5 hours with EEV-LNP or LNP containing Cas9 mRNA and gRNA targeting B2M gene. Cells were harvested 7 days post-treatment and assessed for **(A)** knockdown of B2M protein quantified by FACS analysis. **(B)** Cell viability was assayed using LIVE/DEAD™ Cell Imaging Kit and compared to electroporation. Two-way ANOVA was used for statistical comparison; ****p<0.0001, **p<0.01. ANOVA, analysis of covariance; AU, arbitrary unit; B2M, β2-microglobulin; EEV, Endosomal Escape Vehicle; FACS, fluorescence-activated cell sorting; gRNA, guide RNA; LNP, lipid nanoparticle; mRNA, messenger RNA.