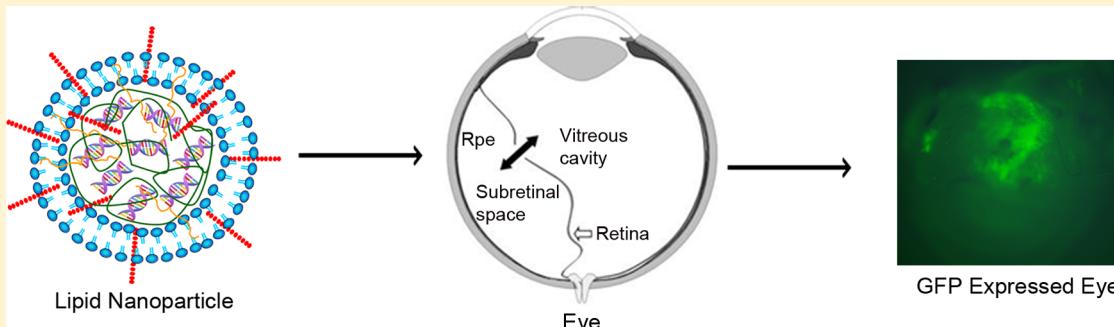


Nanoparticle-Assisted Targeted Delivery of Eye-Specific Genes to Eyes Significantly Improves the Vision of Blind Mice In Vivo

Ammaji Rajala,^{†,||,‡} Yuhong Wang,^{†,||,‡} Ye Zhu,[‡] Michelle Ranjo-Bishop,^{†,||} Jian-Xing Ma,^{‡,§} Chuanbin Mao,^{*,‡} and Raju V. S. Rajala^{*,†,‡,§,||}

[†]Department of Ophthalmology, [‡]Department of Physiology, [§]Department of Cell Biology, and ^{||}Dean A. McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, United States

^{*}Department of Chemistry and Biochemistry, University of Oklahoma, Stephenson Life Sciences Research Center, Norman, Oklahoma 73019, United States



ABSTRACT: Application of viruses as a carrier, though not safe, to deliver genes to eye tissue was successful. However, a safer, nonviral, biocompatible lipid-based nanoparticle has never been tested to treat blinding eye diseases. We created an artificial virus using a nanoparticle, liposome-protamine-DNA complex (LPD), modified with a cell permeable peptide and a nuclear localization signaling (NLS) peptide, to deliver a functional gene for eye disease treatment. In the eye, a photochemical, 11-cis-retinal, allows the visual pigment rhodopsin to absorb light in the visible range. Without the photochemical, we lose the ability to see light. Retinal pigment epithelium protein 65 (Rpe65) is the key enzyme in regulating the availability of photochemical; deficiency of this gene results in a blinding eye disease. Here we show for the first time that LPD promotes efficient delivery in a cell specific-manner, and a long-term expression of Rpe65 gene to mice lacking Rpe65 gene, leading to *in vivo* correction of blindness. Thus, LPD nanoparticles could provide a promising, efficient, nonviral method of gene delivery with clinical applications in eye disease treatment.

KEYWORDS: Lipid nanoparticles, eyes, gene therapy, nonviral vector, retina, retinal pigment epithelium

The success of gene therapy relies on the development of efficient, nontoxic gene carriers that can encapsulate and deliver foreign genetic materials into specific cell types.¹ Gene therapy carriers can be classified into two groups, viral and nonviral gene delivery systems. Although viral vectors such as adeno-associated virus (AAV) have attractive features, particularly their high gene transduction capability, they face biosafety issues, especially innate and immune barriers,² toxicity,³ and potential recombination of or complementation⁴ to vector delivery. The size of viral vectors, which restricts the insertion of genes to <5 kb, is another limitation.⁵ Despite rapid advances in gene therapy during the last two decades, major obstacles to clinical applications for human diseases still exist. These impediments include immune response, toxicity of vectors, and the lack of sustained therapeutic gene expression. Therefore, new strategies are needed to achieve safe and effective gene therapy. The ideal vector should have low antigenic potential, high capacity to accommodate genetic material, high transduction efficiency, controlled and targeted

transgene expression, and reasonable expense and safety for both the patients and the environment. These desired features led researchers to focus on nonviral vectors as an alternative to viral vectors. The nonviral vectors include polymers like polyethylenimine (PEI),⁶ and poly L-lysine (PLL),⁷ peptides, liposomes (tiny fatlike particles),⁸ and liposomes-protamine-DNA (LPD) complexes.^{9,10} However, current nonviral vectors could not achieve tissue-specific or cell-specific sustained gene expression for better treatment and eliminate the unwanted and harmful effects in non-target cells. Compacted DNA nanoparticles formulated with polyethylene glycol-substituted polylysine have been used for eye gene therapy in mouse models of eye diseases.^{11–14} We have successfully used nonviral vectors for the delivery of the vascular endothelial growth factor gene into mesenchymal stem cells.¹⁵ Our recent work shows

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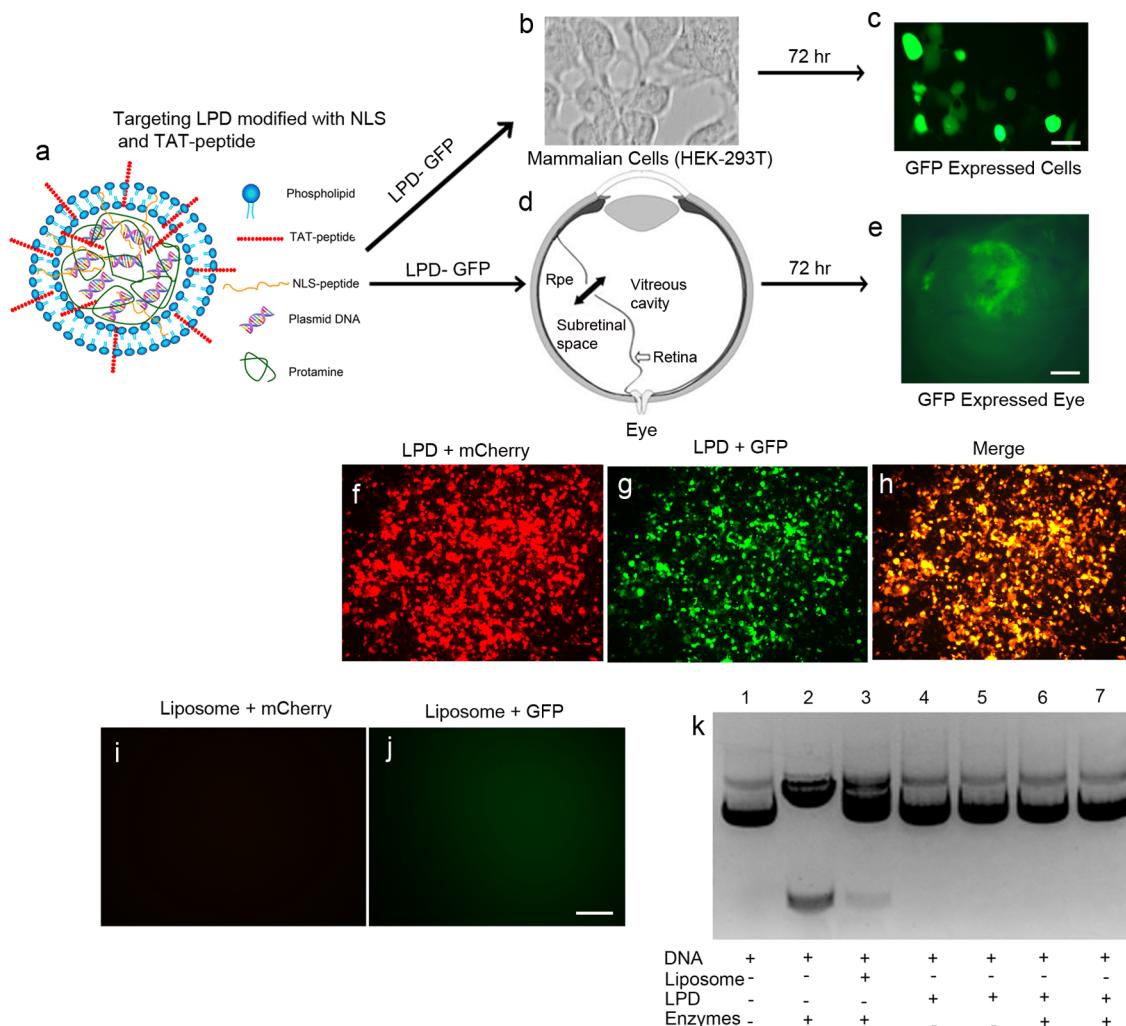


Figure 1. LPD-mediated gene delivery into mammalian and retinal cells. Schematic illustration of targeting LPD modified with NLS and TAT-peptide (a) complexed with GFP to HEK-293T mammalian cells (b,c) or by subretinal injection into eye (d,e). The successful transfection of enhanced green fluorescent protein (GFP) in mammalian cells and mouse eye is visualized by green fluorescence from GFP. mCherry (f) and GFP (g) coexpression after transfection by LPD and liposome (i,j) into mammalian cells was examined by fluorescence microscopy. Cells were examined for fluorescence 72 h after transfection. Panel h represents the merged image of mCherry and GFP. LPD protects the digestion of plasmid DNA by restriction enzymes in vitro (k). We used a 1.2 kb cDNA containing a 5' *Hind* III and a 3' *Xho* I site cloned into pcDNA3 vector. Plasmid DNA was complexed with either LPD or liposome and was incubated with restriction enzymes (*Hind* III and *Xho* I) for 60 min at 37 °C. At the end of incubation, SDS was added to a final concentration of 1%. The samples were analyzed on a 0.8% agarose gel using enzyme untreated or treated plasmid DNA as control. Lane 1, enzyme-untreated plasmid DNA; Lane 2, enzyme-treated plasmid DNA; Lane 3, enzyme-treated liposome-plasmid DNA; Lanes 4 and 5, enzyme-untreated LPD-plasmid DNA; Lanes 6 and 7, enzyme-treated LPD-plasmid DNA. Scale bar: b and c, 200 μm; e, 10 μm; f–j, 100 μm.

that cell-targeting or penetrating peptides can be integrated into LPD and other nanoparticles to promote gene transfer into mammalian cells.^{16,17} Inspired from these successes, in this work we further applied peptide-modified LPD nanoparticles to deliver functional eye-related gene for treating blinding diseases by achieving efficient, lasting, cell-specific gene expression otherwise seen in viral vectors.

The use of lipid nanoparticles as part of a system delivering drugs and genes to the retina has been suggested.¹⁸ However, an *in vivo* application has not been attempted. In this study, we developed an artificial virus, an LPD nanoparticle in combination with a nuclear localization signaling (NLS)¹⁶ peptide and a cell-penetrating transactivator of transcription (TAT) peptide,¹⁹ to produce efficient gene delivery in a cell specific manner to eye tissues with sustained expression. The key to our success arises from three unique designs of our

nanoparticles: (1) the use of biocompatible lipid molecules to pack DNA along with the biocompatible protamine molecules in the nanoparticles; (2) the integration of cell penetrating and nuclei targeting peptides into the nanoparticles to improve the efficiency of gene transfer to eye cells and the consequent lasting gene expression; and (3) the use of a DNA that carries target gene and also bears a unique promoter to achieve cell-specific gene expression.

In this Letter, the LPD was prepared according to the method reported previously¹⁶ with some modification. First, the liposomes consisting of DOTAP (1, 2-dioleoyl-3-trimethylammonium-propane), DOPE (1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine), and cholesterol (1:1:1 molar ratio) (Avanti Polar Lipids, Inc. U.S.A.) were prepared by thin film hydration. Second, protamine (1.25 mg/mL) (Sigma-Aldrich Co. LLC, U.S.A.), various peptides (NLS and TAT)

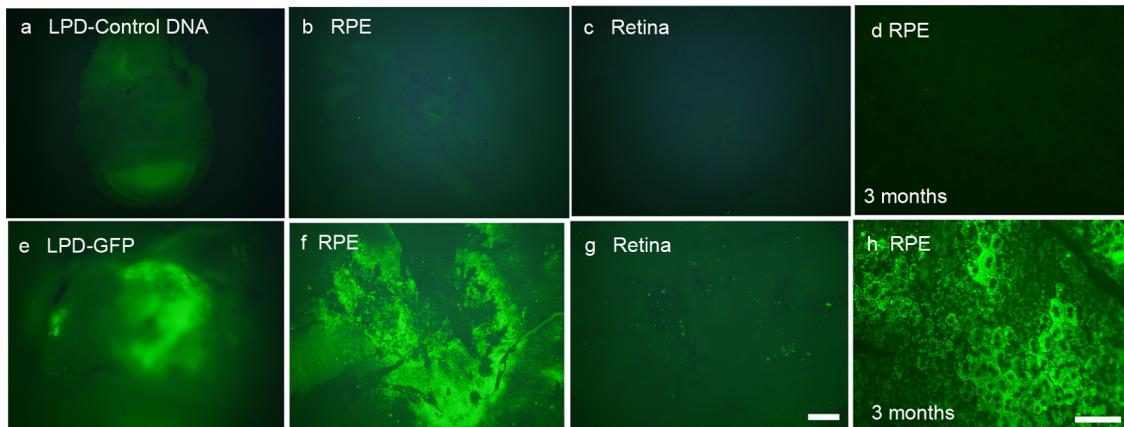


Figure 2. In vivo transfection by LPD. Plasmid DNAs of either control (pcDNA3 vector) or GFP were complexed with LPD and injected subretinally into BALB/c mice. Seventy-two hours later, eyes were removed and examined for GFP expression (a,e) under inverted fluorescence microscopy. Whole RPE (b,f) and retinal (c,g) flat mounts were prepared and examined for GFP expression under inverted fluorescence microscopy. To determine long-term expression, whole RPE (d,h) flat mounts were examined for GFP expression three months later. Scale bar: a–c and e–g, 10 μ m; d and h, 50 μ m.

(0.5 mg/mL), and the plasmid DNA (pDNA) were mixed at various weight ratios. Using a 1:20 DNA to liposome ratio resulted in the best gene expression among all examined DNA/liposome ratios tested.

We first used LPD- and liposome-mediated delivery of plasmid DNAs into cells. We prepared LPD nanoparticles¹⁶ (Figure 1a) and liposome with plasmid DNAs of green fluorescent protein (GFP) and a second generation monomeric red fluorescent protein (mCherry) for coexpression in mammalian cells. Seventy-two hours later, inverted microscopy was used to observe the coexpression of GFP and mCherry in cells. The results indicated a strong expression of both GFP and mCherry in cells that were subjected to LPD delivery but not in the transfections carried out with liposome alone (Figure 1f–j). These experiments clearly suggest that LPD nanoparticles can deliver multiple genes into cells more efficiently than liposome *in vitro*, indicating the role of protamine, NLS, and TAT peptides in LPD.

To further confirm the role of protamine in protecting DNA from attack by degrading enzymes *in vivo*, an *in vitro* restriction enzyme protection assay was performed by incubating DNA complexed with LPD or liposome in the presence of DNA-digesting enzymes. DNA was completely protected from digestion when formulated in LPD, but not when formulated in liposome in the absence of protamine (Figure 1k). This experiment suggests that protamine may protect the encapsulated DNA from attack by DNA-degrading enzymes *in vivo*.

To determine the delivery of LPD *in vivo*, mice were injected subretinally²⁰ with control plasmid DNA (LPD-control) or GFP plasmid DNA (LPD-GFP) under the control of a commonly used promoter (a region of DNA that turns on the expression of a gene to make protein) from cytomegalovirus (CMV) complexed with LPD. All animal work was performed in strict accordance with the Association for Research in Vision and Ophthalmology's statement on the "Use of Animals in Ophthalmic and Vision Research." All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma Health Sciences Center. Seventy-two hours later, eyes were harvested. Retina and retinal pigment epithelium (RPE) flat mounts (flattened tissue firmly affixed to the slide for good preservation of morphology and surface topography)²¹ were

prepared to examine the expression of GFP fluorescence. Examinations of the eyes under the inverted fluorescent microscope revealed strong fluorescence in LPD-GFP injected animals but not in the LPD-controls (Figure 2a,e). The retina is a highly organized structure made up of seven layers of cells. There are also seven different kinds of neural cells that make up the retina: two kinds of photoreceptor cells (rods and cones), retinal pigment epithelial cells (RPE), bipolar cells, amacrine cells, horizontal cells, and ganglion cells. The RPE is a partner of the neural retina and is indispensable for vision. Flat mount data showed that almost all GFP-fluorescence was exclusively associated with RPE (Figure 2b,f), and that very few cells in the retina (all other neural cell except RPE) showed GFP-fluorescence (Figure 2c,g). To determine the sustainability of gene expression, we examined GFP expression for up to 3 months; our results indicated a strong expression of GFP, even 3 months after the date of LPD injection (Figure 2d,h). These experiments suggest that LPD efficiently delivers genes *in vivo* with long-term transgene expression. Our experiments also suggest that a general promoter like CMV-promoter, commonly used for protein expression in mammalian cells may be used for RPE cell specific expression, and the reasons for its RPE cell specificity is currently unknown. To determine the cell specific delivery of LPD, we used a 225-basepair mouse rhodopsin promoter (express specifically in the retina but not RPE) to drive the expression of the GFP in the retinal rod photoreceptors and we found the expression of GFP in the flat mounts of retina (Figure 3). Addition of NLS (D K K K R K V D K K K R K V D K K K R K V) and TAT (Y G R K K R Q R R R) -peptides with LPD enhanced gene expression. The TAT-peptide belongs to arginine-rich family of peptides, which is an abundant source of membrane-permeable peptides that have potential as carriers for intracellular protein delivery.¹⁹ Even with the omission of TAT-peptide, LPD nanoparticles were able to mediate gene delivery (data not shown). Although induction of interleukin 6 is commonly observed in cases of immune system activation, no significant increase in message levels was detected between the control and LPD-injected groups (data not shown). This fact indicates that our LPD is a safer gene carrier than traditional viral carriers.

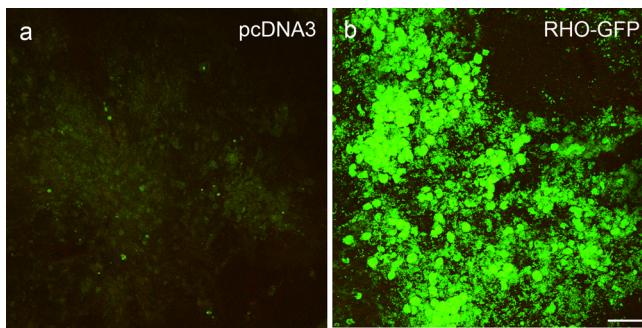


Figure 3. LPD-mediated retinal specific expression by mouse rhodopsin promoter. Plasmid DNAs of either control (pcDNA3 vector) (a) or Rhodopsin-GFP (RHO-GFP) (b) were complexed with LPD and injected subretinally into BALB/c mice. One week later, retinal flat mounts were prepared and examined for GFP expression under confocal microscopy. Scale bar: 50 μ m.

Our study suggests that LPD nanotechnology is ideal for eye gene delivery via a subretinal route. In a previous study, intravenous administration of LPD prepared in combination with DOPE decreased the efficiency of gene delivery, compared to LPD prepared with either DOTAP or DOTAP/cholesterol.¹⁰ The low efficiency of gene delivery with LPD containing DOPE has been shown to be due to its interaction with serum proteins, especially albumin.¹⁰ In light of its association with serum protein, the LPD-mediated intravenous gene delivery

had been suggested as a major factor limiting the in vivo transfection by LPD. In the current study, we delivered the LPD via subretinal route, and the interference of serum proteins was minimal. This may be one of the reasons for the LPD-mediated higher levels of transgene expression in eye tissues.

Given the sustained long-term expression profile of LPD-GFP in RPE, we elected to use LPD nanoparticles to improve vision in vivo in an RPE disease model. A mouse model lacking the Rpe65 gene has been commonly used for gene therapy studies.^{11,22–24} In the eye, a photochemical, 11-cis-retinal, allows the visual pigment rhodopsin to absorb light in the visible range. When the photochemical absorbs the light energy from light rays, it converts the light energy into nerve impulses that travel to the visual cortex of the brain and allow us to see objects. Without the photochemical, we lose the ability to see light. Retinal pigment epithelium protein 65 (Rpe65) is the key enzyme in regulating the availability of photochemical; deficiency of this gene results in a blinding eye disease. Five days after birth, we subretinally²⁰ injected Rpe65 knockout mice with LPD-GFP or control DNA or chicken Rpe65 complementary DNA²⁵ complexed with LPD. Five weeks later, fundoscopy²⁰ was performed to examine the expression of GFP. Fundoscopy, or fundus photography, is the creation of a photograph of the interior surface of the eye without harming the animal. Fundoscopy is used by eye doctors and trained

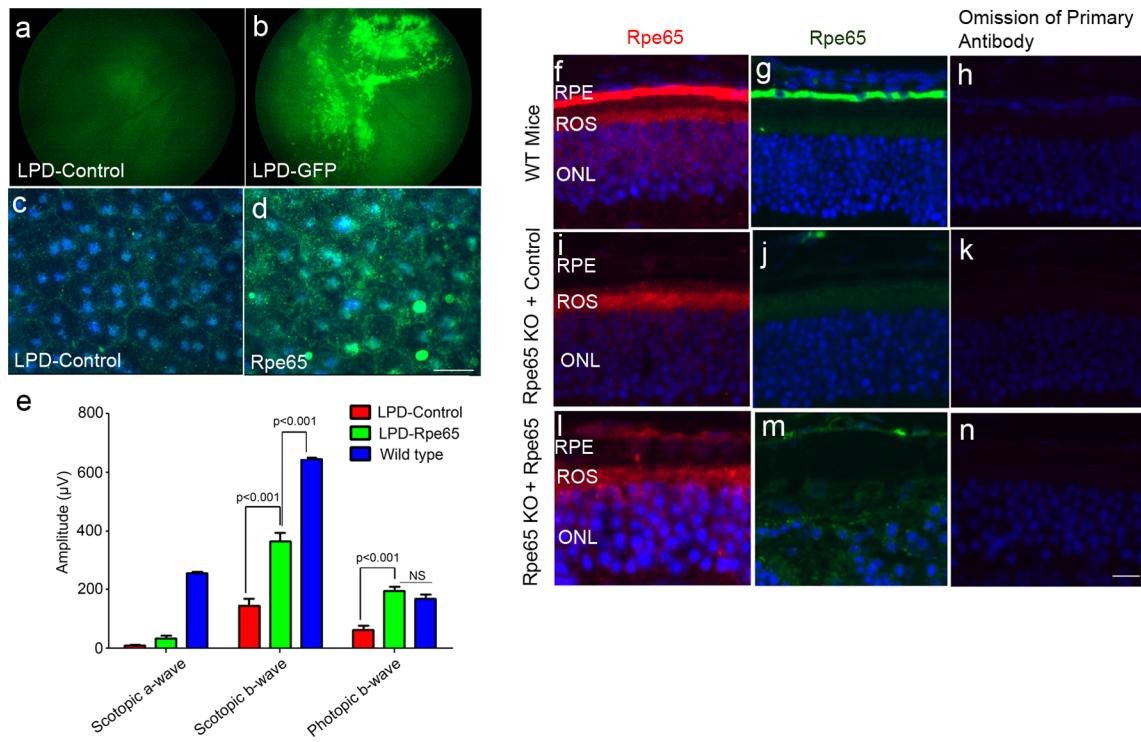


Figure 4. Rpe65-complexed LPD nanoparticles improve the vision in Rpe65 knockout (Rpe65 KO) mice. Fluorescent fundoscopy images of eyes injected with LPD-Control DNA (pcDNA3) or LPD-GFP (a,b). RPE flat mounts were prepared from LPD-control DNA and LPD-chicken Rpe65 injected eyes and costained with Rpe65 antibody (green) and DAPI (blue) to visualize nuclei (c,d). Scotopic a- and b-wave and photopic b-wave electroretinographic (ERG) analysis of wild type and Rpe65 knockout mice injected with LPD-control DNA (pcDNA3) and LPD-chicken Rpe65 (e). Values are mean \pm SEM, $n = 6$, significance between groups $p < 0.001$, NS, not significant. Immunohistochemical analysis of LPD-Rpe65 injected Rpe65 knockout mice. Prefer-fixed sections of uninjected wild type (f,g) and LPD-control (i,j), and LPD-chicken-Rpe65 (l,m) injected eyes of Rpe65 knockout mouse sections were subjected to immunohistochemical analysis with Rpe65 antibody (DALEED)³⁶ using secondary antibodies linked to 594 (red) or 488 (green) Alexa fluorophores. Panels h, k, and n represent the omission of primary antibody. Nuclei were stained with DAPI. RPE, retinal pigment epithelium; ROS, rod outer segments; ONL, outer nuclear layer. Scale bar: c and d, 50 μ m; f–n, 20 μ m.

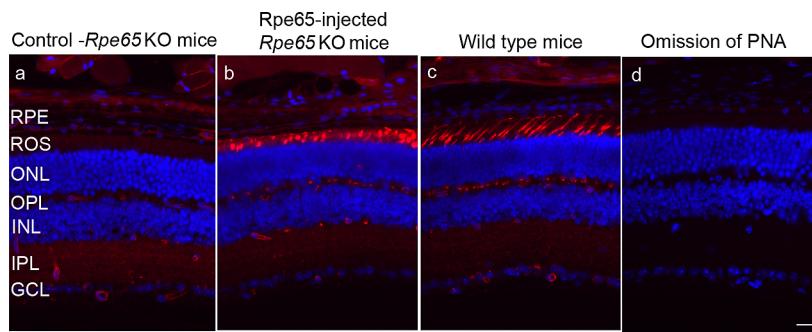


Figure 5. LPD-mediated Rpe65 gene delivery to *Rpe65* knockout mice rescues cone cell death. Retinal sections prepared from uninjected (a) and *Rpe65*-injected *Rpe65* knockout (b) and wild type mice (c) were subjected to lectin cytochemical analysis using peanut agglutinin (PNA). Panel d represents omission of PNA staining. RPE, retinal pigment epithelium; ROS, rod outer segments; ONL, outer nuclear layer, OPL, outer plexiform layer, INL, inner nuclear layer, IPL, inner plexiform layer, GCL, ganglion cell layer. Scale bar: 100 μ m.

medical professionals to monitor progression of a disease or to diagnose eye diseases.

Our results showed the expression of GFP in *Rpe65* knockout mice injected with GFP but not in those injected with the control DNA (Figure 4a,b). To determine the expression of Rpe65 in *Rpe65* knockout mice, RPE flat mounts from both groups were prepared and immunolabeled with Rpe65 antibody. Rpe65 was expressed in the *Rpe65*-injected group but not in the control DNA injected group (Figure 4c,d).

Human beings are highly dependent on vision. Our eyes respond to light rays and convert energy in the light waves to biological nerve impulses. These nerve impulses are carried by the fibers of the nerve cells in the eyes to the visual association area of the brain specialized for the perception of visual images and called the visual cortex. Rod photoreceptors provide sensitivity in dim light (night vision), while cone photoreceptors allow for color vision in bright light (day light vision). To determine the vision improvements *in vivo*, retinal function was evaluated by electroretinography (ERG)²¹ in *Rpe65* knockout mice injected with LPD-control or LPD-Rpe65. The ERG is composed of electrical potentials contributed by different cell types within the retina, and the stimulus conditions (flash or pattern stimulus, whether a background light is present, and the colors of the stimulus and background) can elicit a stronger response from certain components. Functionally, *Rpe65* knockout mice exhibit minimal or no scotopic a- and b-wave amplitudes (rod photoreceptor function) and reduced photopic b-wave (cone photoreceptor function) amplitudes.^{11,26,27} Although no significant improvement was observed in the scotopic a-wave amplitudes, significant improvements in scotopic b-wave and photopic b-wave amplitudes were observed in the LPD-Rpe65-injected group compared to the control groups (Figure 4e). Rpe65 gene delivered to *Rpe65* knockout mice exhibited ERG amplitudes of more than 55% of scotopic b-wave and almost 100% of photopic b-wave amplitudes compared to wild-type mice (Figure 4e). Consistent with the vision improvement, the immunohistochemistry results showed that expression of Rpe65 was restricted to the RPE in wild type (Figure 4f,g) and Rpe65-injected *Rpe65* knockout mice (Figure 4 l,m). Rpe65 expression was absent in *Rpe65* knockout mice injected with LPD-control DNA (Figure 4i,j). Note that the weak staining pattern observed in the *Rpe65*-injected group may be due to the reactivity of the human Rpe65 antibody toward chicken Rpe65. Collectively, these data show that LPD can generate significant vision improvements in *Rpe65* knockout

mice. The vision improvement by LPD in this study is comparable to AAV²³ and lentiviral²⁸ gene transfer of Rpe65 to *Rpe65* knockout mice.

In humans, daylight vision is primarily mediated by cone photoreceptors; chicken is a cone-dominant species.²⁹ Cone chromophore regeneration is several folds faster than rod chromophore regeneration under light conditions.³⁰ Rpe65 is located in the retinal pigment epithelium, and it has an isomeroxydrolase activity (processes all-trans retinyl esters into 11-cis retinol).^{31,32} In vitro, chicken RPE shows 11.7-fold higher isomeroxydrolase activity than bovine RPE.²⁵ We showed earlier that recombinant chicken Rpe65 has an isomeroxydrolase activity 7.7-fold higher than that in human Rpe65, suggesting that recombinant chicken Rpe65 is a more efficient enzyme than human Rpe65.²⁵ The fairly robust improvement in ERG responses is due to the high catalytic activity of chicken Rpe65, because the chromophores in cones regenerate faster.

In *Rpe65* knockout mice, the cone degeneration starts at 2 weeks of age with massive cell loss occurring in large areas of the central retina by 4 weeks of age.³³ To further confirm the functional improvement due to structural improvements we stained the retinal sections prepared from wild type, uninjected and *Rpe65*-injected *Rpe65* knockout mice with lectin cytochemical analysis using peanut agglutinin which labels cone plasma membrane around the cone inner and outer segments.³⁴ Our results indicate a complete loss of cones in *Rpe65* knockout mice injected with control DNA and preservation of cones in LPD-Rpe65 gene delivered *Rpe65* knockout mice (Figure 5) comparable to wild type control. Collectively these experiments suggest that LPD-mediated delivery of Rpe65 rescues both structural and functional phenotype of *Rpe65* knockout mice.

To demonstrate the transfection efficiency of LPD-mediated gene delivery, we delivered a cDNA encoding Src-interacting protein, Sin³⁵ fused to the N-terminal end of GFP under the control of CMV promoter into Balb/C mice. One week later, retinal sections were prepared. We examined the GFP fluorescence and colabeled the retinal sections with Rpe65 antibody. Results indicated the expression of GFP in the RPE layer of mice injected with GFP construct colabeled with Rpe65. In control mice, Rpe65 was expressed but GFP was not (Figure 6). In our experience, we generally achieve >50% of transfection efficiency with LPD (data not shown).

In summary, we formulated a liposome-protamine-DNA (LPD) complex, which was modified with cell-penetrating peptide and NLS peptide and carried a DNA capable of cell-

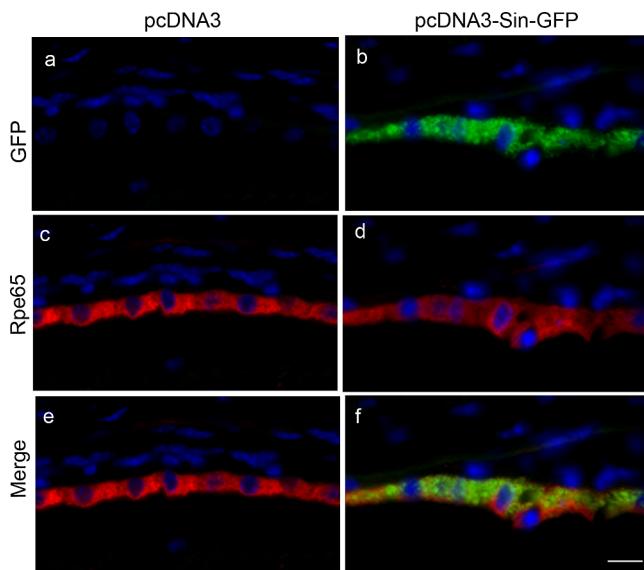


Figure 6. In vivo Rpe-specific delivery of GFP by LPD. Plasmid DNAs of either control (pcDNA3 vector) or Sin-GFP were complexed with LPD and injected subretinally into BALB/c mice. One week later, retinal sections were prepared and examined for GFP expression (a,b) under inverted fluorescence microscopy or by costaining the sections with Rpe65 antibody (c,d). Panels e and f represent the merged images of GFP and Rpe65 (e,f). Nuclei were stained with DAPI (blue). Scale bar: 20 μ m.

specific gene expression. We also found that LPD promoted efficient and lasting gene expression in vivo. Further, our vision improvement experiments in Rpe65-associated blinding eye disease mouse model suggest that LPD nanotechnology could be applied to other retinal diseases, such as retinitis pigmentosa and macular degenerations. The LPD system could be a promising nonviral gene delivery vector yielding long-term expression and durative gene transfer efficiency, making it a favorable gene carrier for future applications for eye cell-based therapies. The advantage is that this system allows us to simultaneously introduce multiple biomolecules to turn on the defective signaling pathway in vivo.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: (R.V.S.R.) raju-rajala@ouhsc.edu.

*E-mail: (C.B.M.) cbmao@ou.edu.

Author Contributions

#A.R and Y.W. equally contributed to this work.

Notes

The authors declare no competing financial interest.

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