A method for gene knockdown in the retina using a lipid-based carrier

Joshua A. Chu-Tan,1,2 Nilisha Fernando,1 Riemke Aggio-Bruce,1 Adrian V. Cioanca,1 Krisztina Valter,1,2 Nektaria Andronikou,1,3 Xavier deMollerat du Jeu,1,3 Matt Rutar,2 Jan Provis,1,2 Riccardo Natoli1,2

1The John Curtin School of Medical Research, The Australian National University, Acton, ACT, Australia; 2The Australian National University Medical School, Acton, Australia; 3Thermo Fisher Scientific, Carlsbad, CA; 4School of Biomedical Sciences, The University of Melbourne, Kenneth Myer Building, Melbourne, Australia

Purpose: The use of small non-coding nucleic acids, such as siRNA and miRNA, has allowed for a deeper understanding of gene functions, as well as for development of gene therapies for complex neurodegenerative diseases, including retinal degeneration. For effective delivery into the eye and transfection of the retina, suitable transfection methods are required. We investigated the use of a lipid-based transfection agent, Invivofectamine® 3.0 (Thermo Fisher Scientific), as a potential method for delivery of nucleic acids to the retina.

Methods: Rodents were injected intravitreally with formulations of Invivofectamine 3.0 containing scrambled, Gapdh, IL-1β, and C3 siRNAs, or sterile PBS (control) using a modified protocol for encapsulation of nucleic acids. TdT-mediated dUTP nick-end labeling (TUNEL) and IBA1 immunohistochemistry was used to determine histological cell death and inflammation. qPCR were used to determine the stress and inflammatory profile of the retina. Electoretinography (ERG) and optical coherence tomography (OCT) were employed as clinical indicators of retinal health.

Results: We showed that macrophage recruitment, retinal stress, and photoreceptor cell death in animals receiving Invivofectamine 3.0 were comparable to those in negative controls. Following delivery of Invivofectamine 3.0 alone, no statistically significant changes in expression were found in a suite of inflammatory and stress genes, and ERG and OCT analyses revealed no changes in retinal function or morphology. Injections with siRNAs for proinflammatory genes (C3 and IL-1β) and Gapdh, in combination with Invivofectamine 3.0, resulted in statistically significant targeted gene knockdown in the retina for up to 4 days following injection. Using a fluorescent Block-It siRNA, transfection was visualized throughout the neural retina with evidence of transfection observed in cells of the ganglion cell layer, inner nuclear layer, and outer nuclear layer.

Conclusions: This work supports the use of Invivofectamine 3.0 as a transfection agent for effective delivery of nucleic acids to the retina for gene function studies and as potential therapeutics.

Delivery of small nucleic acids as a gene therapy has been intensely investigated for the treatment of neurodegenerative diseases, including retinal degenerations [1-8]. The eye is the ideal structure for developing and testing translational gene therapies for neurodegeneration due to the small size and enclosed structure, immune privilege, and easy accessibility of the eye and the availability of animal models for retinal diseases [9,10].

However, delivery of drugs to the retina located in the posterior part of the eye has historically been challenging [11]. Intravitreal delivery of drugs is a commonly used technique that requires injection into the vitreous body of the eye. However, frequent administration of intravitreal injections can lead to several complications, including retinal detachment, endophthalmitis, and increased intraocular pressure [11,12], an issue that may be bypassed with the development of new topical ocular formulations with the ability to reach the retina [13]. In addition to the complexities of retinal physiology that deem delivery to the tissue challenging, the stability and actual transfection efficiency of small nucleic acids injected into the eye have to be considered [14].

For a gene therapy to be effective, an efficient vector or carrier is required. Several key aspects are crucial for effective gene therapy vectors: enhancing gene transfer efficiency, cell specificity, safety, and long-term expression [15,16]. In the past decade, there have been significant efforts in improving the delivery of small nucleic acids directed toward finding safe and efficient gene therapy reagents and vehicles. They can be split into two major groups: viral vectors and non-viral vectors.

Viral vectors include lentiviruses and retroviruses, but the one with the most success in terms of intraocular delivery is the use of adenoassociated viruses (AAVs) [17–19]. However, there are limitations regarding the consistent use...
of AAVs within the eye, specifically their tendency to cause inflammatory responses at high doses, limiting their ability to be repeatedly injected [20-25].

Lipid-based transfection agents have been explored as a potentially safer alternative to viral vectors due to the ease of their production and low toxicity (reviewed in [10]). Many efforts in the past decade have gone into improving transfection efficiency of lipid-based formulations with limited toxicity [26]. Lipid-based nanosystems, including solid lipid nanoparticles and liposomes, have been shown to be efficient carriers of siRNA to the retina to manage retinal diseases [14]. Invivofectamine 3.0 (Thermo Fisher Scientific, Waltham, MA) was developed as a lipid-based carrier for in vivo work to enhance delivery to the target tissue while minimizing unwanted cytotoxicity within the tissue. Previous studies have demonstrated the use of Invivofectamine as a mode of delivery to the retina [27,28], where the difficulties of reduced transfection efficiency due to the inner limiting membrane (ILM) were overcome [29,30].

In this study, we demonstrate the efficacy of Invivofectamine 3.0 as a carrier for siRNA into the retina. The results indicate that the use of Invivofectamine 3.0 lipid-based transfection is a quick, low-toxicity, and efficient method for retinal transfection of small nucleic acids with minimal to no toxicity or negative effects on retinal function, inflammation, or cell death. This is a simple method for modulating and controlling dysregulated gene expression using siRNA in rodent animal models.

METHODS

Animals: All animal procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the Australian National University (ANU) Animal Experimentation Ethics Committee (Protocol Number: A2014/56, A2017/41). Adult albino Sprague-Dawley (SD) rats age between 90 and 120 days and C57BL/6J mice age between 60 and 90 days were used for all experiments. The animals were born and raised in a 12 h:12 h light-dark cycle in dim light conditions (5 lux). Atropine sulfate 1% w/v eye drops (Bausch and Lomb, Sydney, Australia) were used to dilate pupils during photosensitization damage in mice and before intravitreal injections. Ketamine (100 mg/kg; Troy Laboratories, Glendenning, Australia) and xylazil (12 mg/kg; Troy Laboratories) were used to anesthetize animals before the intravitreal injections, electroretinography (ERG), and optical coherence tomography (OCT) were performed.

Preparation of encapsulated small nucleic acids with Invivofectamine 3.0: The procedure for the preparation of siRNAs is outlined in Figure 1. SiRNAs were encapsulated using Invivofectamine 3.0, a cationic liposome-based formulation according to the manufacturer’s instructions (Thermo Fisher Scientific) with some modifications. The SiRNAs used in this study are listed in Table 1. The following equates to the protocol used for siRNA with a molecular weight of 13,000 g/mol:

- Equal volumes of siRNA (diluted to 2.4 μg/μl in nuclease-free water) and complexation buffer were mixed. The siRNA:complexation buffer solution was then mixed in a 1:1 ratio with the Invivofectamine 3.0 formulation warmed to room temperature. The mixture was then incubated for 30 min at 50 °C. Following the incubation step, the mixture was diluted 15-fold in 0.1 M sterile, endotoxin-free UltraPure PBS (Thermo Fisher Scientific, Waltham, MA). Diaphragm was conducted using an Amicon Ultra-4 Centrifugal Filter Unit (Merck Millipore, Burlington, MA) to purify the siRNA:Invivofectamine 3.0 complexes. The siRNA:Invivofectamine 3.0 complex was added to the filter column and spun at 4,000 ×g for approximately 1 h to purify and concentrate the solution to 2 μg/μl. The concentrated complex was then diluted with sterile 0.1 M PBS, so that each siRNA complex was encapsulated at a final concentration of 0.33 μg/μl in sterile PBS unless stated otherwise. The concentrations of siRNA used were based on previously published studies [27,28].

- Preparation of encapsulated small nucleic acids with in vivo-jetPEI: Silencer™ FAM-labeled GAPDH siRNA (Thermo Fisher Scientific) was reconstituted in nuclease-free water at a stock concentration of 4 μg/μl and diluted in 10% glucose solution in nuclease-free water. The in vivo-jetPEI® (Polyplus transfection, Illkirch, France) transfection reagent was prepared in 10% glucose solution in nuclease-free water and used at an nitrogen/nucleic acid phosphate (N/P) ratio of 7 (0.14 μl of transfection reagent per 1 μg of GAPDH siRNA). Equal volumes of the siRNA solution and the in vivo-jetPEI® solution were mixed and incubated at room temperature for 15 min to achieve complexation. The maximum concentration of nucleic acid that could be encapsulated was 0.5 μg of Gapdh siRNA which was delivered intraventrically at a final glucose concentration of 5%.

Intravitreal injections: Intravitreal injections were performed as outlined schematically in Figure 1 and Figure 2. Animals were anesthetized using an intraperitoneal injection of ketamine (100 mg/kg) and xylazil (12 mg/kg). A pupil dilator (tropicamide 0.5% w/v eye drops; Bausch and Lomb) was administered to the ocular surface of each eye (Figure 2A).
A string loop was tied around the eye to allow for easier access to the injection site (Figure 2B). The injection site was swabbed with 5% povidone iodine (Betadine, Mundipharma, Sydney, Australia) before the injection (Figure 2C). siRNA:Invivofectamine 3.0 complexes were injected intravitreally into the rodent eyes with the aid of a stereo microscope (M125; Leica Microsystems, Wetzlar, Germany). A 30-gauge needle was first used to make a punch incision, 0.5 mm posterior to the temporal limbus. A 10 μl NanoFil syringe with an attached 34 gauge NanoFil needle (World Precision Instruments, Sarasota, FL) was then inserted through the incision, angled toward the optic nerve (Figure 2D–F). Three microliters of the complex was injected into each rat eye at a concentration of 0.33 μg/μl, and 1 μl into each mouse eye at a concentration of 1 μg/μl (Figure 2G). The cloudiness of Invivofectamine 3.0 was visible through the animal eye.

---

**A. IVF 3.0 SOLUTION**

1. **IVF 3.0 MIX**

   - **IVF 3.0 (RT)** add to **Complexation buffer + siRNA**
   - 2. **50°C 30mins**
   - 3. **Dilute in PBS**
   - 4. **Spin down in filter column**
   - 5. **Dilute in PBS to correct concentration**

---

**B. INTRAVITREAL INJECTION**

1. **Anaesthetize animal**
2. **Pupil dilate**
3. **“Bulge out” eye**
4. **Swab antiseptic**
5. **Pilot hole**
6. **Inject 3μl of IVF3.0 solution**

---

Figure 1. Schematic of Invivofectamine 3.0 preparation and intravitreal injections. **A**: Invivofectamine 3.0 was added to a mixture of complexation buffer and siRNA of choice. This solution was incubated for 30 min at 50 °C. The solution was diluted in PBS and then spun down in a filter column to the correct concentration, which then was ready for injection. **B**: The animals were anesthetized and their pupils dilated with atropine sulfate. The eye was then drawn forward with a string loop. Betadine iodine was swabbed on the injection site before insertion of the pilot hole with a 30-gauge needle. The 34-gauge NanoFil syringe was then inserted and 3 μl (for rats) or 1 μl (for mice) of the solution injected. The injection process is described in more detail in Figure 2.
The animals were singly euthanized with CO₂ gas inhalation in a sealed cage. The superior surface of the eye was marked before extraction for orientation purposes. Whole eyes were injected and immersion fixed with 4% paraformaldehyde for 4 h at 4 °C and then processed as described previously \[33,34\]. The eyes were sectioned in the parasagittal plane at a thickness of 16 μm and mounted on poly-L-lysine slides. Retinas were collected via a corneal incision for RNA extraction (further described in section “Quantitative real-time PCR”).

**Measurement of retinal function using ERG:** Full-field scotopic ERG was performed to assess the animals’ retinal function after intravitreal injections as described previously \[32\]. ERG was performed using an LED-based system (FS-250A Enhanced Ganzfeld, Photometric Solutions International, Huntingdale, Australia). Briefly, mice were dark-adapted overnight, anesthetized using an intraperitoneal injection of ketamine (100 mg/kg) and xylazil (12 mg/kg), and the pupils dilated with 1% w/v atropine sulfate (Bausch and Lomb). A single- or twin-flash paradigm over a stimulus intensity range of 6.3 log cd s m⁻² (range −4.4 to 1.9 log cd s m⁻²) was used to elicit mixed (rod and cone) or isolated cone responses, respectively. Measurements of the cone a-wave and b-wave responses were performed using Lab Chart 8 (AD Instruments, Dunedin, New Zealand).

**Optical coherence tomography:** Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) was used to determine changes in retinal morphology. OCT and fundus images of the retina were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) as previously described \[28\] at 1 and 7 days post-injection. Animals were anaesthetized and restrained on a custom-made platform attached to the OCT machine. A rodent contact lens was placed on the eye (polymethylmethacrylate [PMMA] lenses, radius of curvature of the central optic zone of 2.70 mm and diameter of 5.20 mm, Cantor + Nissel, Brackley, UK). Fundus and cross-sectional images were taken from 0 to 3 mm superior to the optic nerve. Retinal thickness and outer nuclear layer (ONL) depth were measured in two OCT transects per retina in the region of interest (1–2 mm superior to the optic nerve), which is the location of focal retinal damage in rodents in PD \[32\], with three points sampled across each image. The ONL thickness ratios were calculated as the ONL thickness relative to the distance between the outer limiting membrane (OLM) and the ILM. Fundus images of the area of interest (superior to the optic nerve) were taken to detect any retinal lesions.

**TUNEL analysis of cell death:** TdT-mediated dUTP nick-end labeling (TUNEL) was used to quantify photoreceptor cell death in retinal cryosections, using a previously published protocol \[35\] with an In Situ Cell Death Detection Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland). TUNEL positive cells were counted in the ONL along the full length of the retinal sections cut on the superoinferior plane including the optic disc. The final counts for each experimental group were

| siRNA complex                        | Company                | Catalogue number |
|--------------------------------------|------------------------|------------------|
| **Gapdh** (Glyceraldehyde 3-phosphate dehydrogenase) | Thermo Fisher Scientific | AM4650           |
| **II-1β** (Interleukin-1β)           | Thermo Fisher Scientific | s127941          |
| **C3** (Complement component C3)     | Thermo Fisher Scientific | s63165           |
| **Block-It Alexa Fluor Red**         | Thermo Fisher Scientific | 14,750,100       |
| **Scrambled negative siRNA**         | Thermo Fisher Scientific | 12,935,300       |
the average of at least four biological replicates counted at comparable locations in the retina.

**Immunohistochemistry:** Immunohistochemistry was performed on retinal cryosections according to previously described protocols, with minor modifications [36]. IBA1 (1:500, Wako, Osaka, Japan) and GFAP (1:500, Dako, Glostrup, Denmark) were used as primary antibodies, which were later conjugated to Alexa-488 or Streptavidin-594 (Thermo Fisher Scientific) as secondary antibodies. Fluorescence in sections was captured in Z-stacked form, using a laser-scanning A1+ confocal microscope. Images were captured

---

**Figure 2.** Images of intravitreal injection into the rat eye. **A:** Rat eye after administration of atropine sulfate for pupil dilation. **B:** A string loop was tied around the eye to “bulge out” the eye. **C:** Betadine iodine was swabbed on the surface of the sclera at the injection site. **D:** A pilot hole was made in the superotemporal region with a 30-gauge needle on the sclera behind the lens. **E:** The pilot hole is clearly seen on the sclera. **F:** A 34-gauge needle attached to a NanoFil syringe was used for injections and was inserted into the pilot hole at a similar angle. **G:** The cloudy Invivofectamine 3.0 solution was injected and should be visible through the lens. **H:** Chlorsig antibiotic was swabbed on the injection site with GenTeal eye gel applied to prevent dryness.
Optical densitometry was used to determine relative fluorescence intensity and transfection efficiency of fluorescent Block-It siRNA. All sections were measured in three different locations in the same retinal region (superior, 2–3 mm from the optic nerve due to the larger rat retina [31]). The measurements were taken from the whole retinal region from the ILM to the OLM. In addition, single layer measurements were taken from the ganglion cell layer (GCL), inner nuclear layer (INL), and ONL individually, in arbitrary fluorescence intensity units. All regions of interest were measured using the Nikon A1 confocal microscope.

Quantitative real-time PCR: Total RNA was extracted from the retinal samples in microscale according to the manufacturer’s protocol (RNAqueous Total Isolation Micro Kit, Thermo Fisher Scientific). The concentration and purity of the RNA samples were determined using an ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Only samples with a 260/280 ratio between 1.9 and 2.1 were considered for analysis. The RNA samples were stored at −80 °C indefinitely.

Following purification of RNA, cDNA was synthesized using a Tetro cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer’s protocol. A 20 μl reaction mixture was used in conjunction with 1 μg RNA, 500 ng oligo(dT) primer, and 200 U reverse transcriptase. Gene expression was measured via qRT-PCR using TaqMan probes, cDNA, and TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), as shown in Table 2. The TaqMan probes, cDNA, and TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) were plated in a 384-well transparent plate, and amplification of each sample was performed in technical duplicate using a QuantStudio 12 K Flex real-time polymerase chain reaction (RT-PCR) machine (Thermo Fisher Scientific). Analysis was performed using the comparative cycle threshold method (ΔΔCt). The relative fold change was expressed as a percentage change compared to negative siRNA or PBS controls and normalized to two reference genes, Actβ and Hprt1 for when Gapdh was assayed in knockdown experiments, and Gapdh and Actβ for all others.

Statistics: All graphing and statistical analysis was performed using Prism 6 (GraphPad Software, San Diego, CA). Statistically significant trends in time-course data sets were ascertained using a one-way or two-way ANOVA to determine statistical significance (p<0.05); Tukey’s or Sidak’s post hoc tests were applied where multiple statistical comparisons were required. An unpaired Student’s t test was used for single comparisons.

RESULTS

Inflammatory and cell death profile at 24 h post-Invivofectamine 3.0 injection: Two previous formulations of Invivofectamine (Invivofectamine 1MFG and Invivofectamine 2.0) displayed a change in the inflammatory profile of the retina following delivery (Appendix 1). This presented with IBA1 positive cells exhibiting an amoeboid, activated profile and moving toward the GCL and vitreous body, which is where the formulation was injected. This observation indicates recruitment of resting mononuclear phagocytes (microglia and macrophages) to an inflammatory site. Quantification of this revealed a statistically significant difference in the IBA1 cell location in Invivofectamine 2.0-injected retinas (Appendix 1, p>0.05). The inflammatory profile following Invivofectamine 3.0 delivery required further evaluation. IBA1 positive mononuclear phagocytes displaying a resting, ramified morphology were evident in the control and Invivofectamine 3.0-injected eyes (Figure 3A–D). Mononuclear phagocytes appeared to have a normal distribution within the GCL, inner plexiform layer (IPL), and INL of the retinal tissue in the untreated needle-stick and PBS controls, as well as in the Invivofectamine 3.0-injected retinas. Using a general stress marker (GFAP), no differences were shown in the expression profile between the needle-stick and PBS controls when compared with Invivofectamine 3.0 (Figure 3E–H). The TUNEL assay showed that there was no discernible difference in TUNEL positive photoreceptor cells in the ONL of the Invivofectamine 3.0-injected and control animals (Figure 3I–L). Quantification of the total number of IBA1 labeled cells in each retinal section revealed no difference between any of the control animals and the Invivofectamine 3.0-injected animals (Figure 3M, p>0.05).

A suite of retinal stress and inflammatory primers was run using quantitative real time polymerase chain reaction (qRT-PCR) to further investigate the retinal profile after Invivofectamine 3.0 injection. There were no statistically significant changes in the expression of the stress and inflammatory markers tested (Il-1β, Nos3, Fgf-2, Ccl4, Ccl17, Cxc11, Edn2, Socs1, and Stat6) after 24 h post-injection with Invivofectamine 3.0 (Figure 3O, p>0.05). Ccl2, Il-10, and Il-6 levels were undetermined (UD) as they were deemed too low for the software to effectively quantify.
Clinical safety indicators post-injection: After 24 h post-injection with Invivofectamine 3.0, OCT retinal (Figure 4A, C) and fundus images (Figure 4B, D) displayed no indication of retinal damage. There were no differences in the ONL thickness ratios taken from the superior region at 1 mm from the optic nerve (region of focal retinal damage in PD mice [32]), quantified from the OCT retinal images, compared with control animals injected with PBS (Figure 4E, p>0.05). The ERG cone a-wave and b-wave responses displayed no statistically significant differences between the Invivofectamine 3.0-injected animals and the PBS control animals (Figure 4F–H, p>0.05).

After 7 days post-injection, there was no difference in the ONL thickness ratios, again quantified from the OCT retinal images, between the Invivofectamine 3.0-injected and PBS control animals (Figure 4I, p>0.05). The same was seen with the 7-day post-injection ERG responses, with the cone a-wave and b-wave responses all showing no changes (Figure 4J–L, p>0.05), indicating no difference in ERG response between these treatment groups.

Gene knockdown efficiency: We also compared the efficacy of Invivofectamine 3.0 with another commercially available lipid-based transfection reagent, in vivo-jetPEI® (Polyplus transfection), demonstrating that Invivofectamine 3.0 had a greater knockdown capability at the earliest time point as Gapdh siRNA encapsulated at in vivo-jetPEI® displayed no statistically significant difference from the control (Figure 5A, p>0.05). However, the in vivo-jetPEI® could deliver the siRNA only at a maximum concentration of 0.5 μg/μl as opposed to the 1 μg/μl allowed for Invivofectamine 3.0. To
Figure 3. Histological indicators of cellular stress in the retina 24 h post-injection. 

A–D: IBA1 positive microglia (labeled in red) displayed a normal profile with ramified shape and distributed within the inner layers of the retina. This was true for the non-injected PBS and needle-stick controls as well as the Invivofectamine 3.0-injected animals.

E–H: The GFAP profile between the control group and the Invivofectamine 3.0 group had the same profile with labeling only in the inner limiting membrane (ILM) and beneath the inner nuclear layer (INL). Labeled Müller cell processes were not evident.

I–L: TdT-mediated dUTP nick-end labeling (TUNEL) positive labeling was little to absent in all samples.

M: Quantification of IBA1 positive cells revealed no differences between all samples.

N: Quantification of TUNEL cells revealed no differences between all samples.

O: Quantitative real-time PCR (qRT-PCR) showed no statistically significant changes in a suite of inflammatory and retinal stress genes in Invivofectamine 3.0-injected animals compared to the PBS controls. Ccl2, II-10, and II-6 expression was undetermined (UD, expression levels were too low for the software to detect; n=4, scale bars represent 100 μm).
Figure 4. ERG and OCT indicating retinal safety and health following Invivofectamine 3.0 injection. 
A–D: Representative images of retinal and fundus images taken from the PBS control animals and the Invivofectamine 3.0-injected animals showed no indicators of retinal stress at 24 h post-injection. 
E: Outer nuclear layer (ONL) ratios measured at 24 h post-injection revealed no statistically significant differences between the PBS-injected controls and the Invivofectamine 3.0-injected animals. 
F–H: Cone a-wave and b-wave responses measured from electroretinography (ERG) showed no differences between the PBS-injected controls and the Invivofectamine 3.0-injected animals at 24 h post-injection. 
I: At 7 days post-injection, no differences were still seen in the ONL ratios between the two groups. 
J–L: Cone a-wave and b-wave ERG responses again showed no differences at 7 days post-injection between the PBS-injected controls and the Invivofectamine 3.0-injected animals (*p<0.05 using unpaired Student t test (E, F, I, J) and two-way ANOVA with Sidak’s post hoc test (G, H, K, L), n=6, scale bar represents 200 μm).
test the duration of the targeted gene knockdown following the intravitreal delivery of Gapdh siRNA, we analyzed retinal gene expression at 1, 3, 5, and 7 days post-injection. A knock-down of Gapdh, when carried by Invivofectamine 3.0, was evident for up to 3 days post-injection, with 5 and 7 days post-injection showing no statistically significant knockdown compared to the PBS-injected controls (Figure 5B, p<0.05).

SiRNAs for Il-1β and C3, two major contributors to inflammation in the retina [37,38], were used to further test gene knockdown under stressed conditions induced by PD. Il-1β siRNA injection with Invivofectamine 3.0 showed a statistically significant knockdown of Il-1β by approximately 90% at 48 h post-injection when compared to the negative siRNA controls (Figure 5B, p<0.05). Injection with C3 siRNA encapsulated with Invivofectamine 3.0 also resulted in statistically significant knockdown of C3 for 4 days post-injection when compared to the animals injected with negative siRNA controls following PD (Figure 5C, p<0.05).

Retinal location of Invivofectamine 3.0 delivery: We used Invivofectamine 3.0 in combination with Block-It siRNA (Thermo Fisher Scientific) to visualize the localization of Invivofectamine 3.0 delivery of fluorescent siRNA within the retina.

Figure 5. Efficiency of gene knockdown with Invivofectamine 3.0. A: In vivo-jetPEI®: Gapdh siRNA produced no statistically significant change in gene Gapdh expression 1 day post-injection when compared to controls whereas Invivofectamine 3.0 showed a statistically significant knockdown. B: Invivofectamine 3.0: Gapdh siRNA produced statistically significant targeted gene knockdown of Gapdh at 1 and 3 days post-injection. Five and 7 days showed no statistically significant difference compared to negative siRNA controls. C: Invivofectamine 3.0 complexed with siRNA against the inflammatory chemokine Il-1β also produced statistically significant knockdown. D: Invivofectamine 3.0:C3 siRNA allowed for a statistically significant gene knockdown as well (*p<0.05 using one-way ANOVA with Tukey’s post hoc test (A) and unpaired Student t test (C, D), n=4–11).
the retina. The negative control showed low levels of fluorescence with only background autofluorescent staining visible in the outer segments (Figure 6A, B). An intravitreal injection with fluorescent Block-It led to fluorescence dispersed throughout all layers of the retina with the strongest amounts of fluorescent staining visualized in the central retina from the stitched whole retinal images (Figure 6C). Strong fluorescence staining could be seen throughout the GCL, INL, and ONL. In some instances, whole ganglion cells, bipolar cells, and photoreceptors were transfected (Figure 6D, indicated by the arrows).

Quantitative analysis of the fluorescence seen post-injection revealed a statistically significant increase in the fluorescence intensity of the Block-It siRNA when compared to negative siRNA-injected retinas when measuring all retinal layers from the OLM to the ILM (Figure 6E, p<0.05). The GCL, INL, and ONL all individually showed statistically significant increases in fluorescent staining in animals injected with Block-It siRNA when compared to negative controls (Figure 6F, p<0.05).

DISCUSSION

Delivery of nucleic acids into the retina will enable a greater understanding of the mechanisms behind normal retinal function, paving the way for novel therapeutics and treatment strategies, not only for retinal disorders but also other neurodegenerative diseases. In this study, we demonstrated that the intravitreal delivery of Invivofectamine 3.0 provides a reliable and safe transfection method of siRNA into all layers of the retina. First, we found that injection with Invivofectamine 3.0 results in no signs of increased immunogenicity, general stress, or cell death. Second, we found no statistically significant changes in retinal function or morphology after intravitreal administration of Invivofectamine 3.0 up to 1 week post-injection. Third, we found that Invivofectamine 3.0 penetrates the inner retina and the outer retina, providing up to 3–4 days of statistically significant, targeted gene knockdown from a single injection. Taken together, we demonstrated the use of Invivofectamine 3.0 is a quick and efficient method for studying the effects of nucleic acid delivery globally in the retina.

One of the primary issues with gene delivery vehicles is the ability for transfection into the relevant cell type, let alone the retina itself [39]. Non-viral and viral systems have had trouble with efficient penetration into the inner and outer retinal layers in vivo. The method of gene vector delivery into the eye plays a role in this, due to the many layers of the retina and the differing efficiencies of transfection into those layers. The two primary injection techniques for retinal transfection are subretinal and intravitreal injections. Although subretinal injections are efficient in transfecting the two cell types (photoreceptors and RPE) that contribute to many retinal degenerations, they are technically challenging and invasive by nature, with evidence of foveal thinning, macular holes, choroidal effusions, and ocular hypotension [40-42].

Intravitreal injection for gene vector administration, however, is less invasive as it allows for the broad distribution of vectors throughout the retina. Adverse effects associated with intravitreal injections are perceived to be short-term, with retinal detachment, endophthalmitis, and vitreous hemorrhaging all noted as possible complications [11,12,14]. Using the proposed method, Invivofectamine 3.0 can transfect the GCL, INL, and ONL of the retina through intravitreal injections, with all three layers showing significant transfection of siRNA when carried by Invivofectamine 3.0. There was evidence of whole cells being transfected in these three layers, with cells in all of these layers being clearly labeled. The fluorescence depicted in the figure had dissipated within 24 h, at which point visualization and imaging of the fluorescence were no longer possible. This could be due to leaching of the fluorescent tag. Increasing the nucleic acid dose is an immediate way in which the longevity may be maximized. Additionally, the size of the eye must be taken into account due to the volume of vitreous humor for intravitreal injections. Concentrations must be adjusted accordingly, and we found that successful transfection in the mouse eye requires a larger relative nucleic concentration for its size. However, we are confident with the gene expression knockdown results of up to 3–4 days, and therefore, the quick half-life of the fluorescent tag in the retina was not a major concern.

Successful gene therapies require safe and effective delivery systems, with minimal side effects. When comparing the two broad classes of delivery systems currently available (viral and non-viral), non-viral systems appear to be more easily produced, less toxic, and less immunogenic [23,24,43,44]. In this study, we showed that Invivofectamine 3.0 has low levels of immunogenicity or acute toxicity in the retina, allowing for the potential of repeated administration, which is limited using viral vectors [43]. Furthermore, using two clinical methods of analysis, we demonstrated no loss of retinal function using ERG, while OCT showed normal morphology, following Invivofectamine 3.0 transfection.

One of the reported major drawbacks of non-viral, lipid-based delivery systems is that they are reported to be less effective than their viral counterparts, rarely accomplishing transgene expression at therapeutic levels [39,45]. Various approaches have been used to improve this efficiency,
including the use of nanocarriers [11,14]. In the present study, we showed that Invivofectamine 3.0 can sustain targeted gene knockdown for up to 3–4 days using an siRNA for Gapdh, which is abundant in the retina. Additionally, with siRNAs for C3 and Il-1β, we demonstrated effective reduction of gene expression. C3 and Il-1β are key mediators of inflammation in retinal degeneration with peak C3 expression at 5–7 days of PD in mice [28,32] and peak Il-1β expression at 24 h of PD in rats [28]. In comparison with another commercially available lipid-based formulation, in vivo-jetPEI, only 0.5 μg/μl of siRNA could be encapsulated in a microinjection of 1 μl which is required for mouse eyes. This does not imply that siRNA at 1 μg/μl used with in vivo-jetPEI® would not perform in the same manner; however, with limitations in the current formulation, this concentration is not possible. This demonstrates the packaging capability of Invivofectamine 3.0, and

Figure 6. Localization of Invivofectamine 3.0 transfection with Block-It siRNA. A: Whole, stitched images of the retina show a representation of the distribution of Block-It siRNA with only autofluorescence of the outer segments in the negative control animals. B: Central images of the retina revealed no fluorescence in the negative siRNA-injected animals except autofluorescence of the outer segments. Grey lines indicate the cellular layers. C: Animals injected with Block-It display a similar pattern of autofluorescence but also have fluorescence visible in the inner and outer layers of the retina both peripherally and centrally. D: In the central retinal images, fluorescence can be seen throughout the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) of the retina. Whole cells being transfected can be seen throughout the sections (white arrowheads). E: Quantification of fluorescence intensity revealed a statistically significant increase in animals injected with Block-It siRNA when compared to those injected with negative siRNA. F: Densitometry measurements of the retinal layers revealed statistically significant increases in fluorescence in the GCL, INL, and ONL in animals injected with Block-It (*p<0.05 using unpaired Student t test, n=4).
the maximum siRNA allowed for in the Invivofectamine 3.0 preparation demonstrated statistically significant gene knockdown capabilities when compared with in vivo-jetPEI®.

The 3–4 day timeframe (Gapdh) may limit the suitability of Invivofectamine 3.0 for long-term use, but for future considerations, new strategies are needed to increase the efficacy of gene knockdown and the longevity of delivery, both key aspects of therapeutic gene delivery. To increase knockdown time, heavier modification of the nucleic acids themselves could be beneficial. For the formulation, increasing the proportion of pegylated lipids results in a longer retention time in the blood, but it has not been tested in an ocular model, and may result in a loss of efficacy. Although currently not as effective long-term as viral-based approaches, lipid-based transfection is useful for proof-of-principle studies, and has been demonstrated to be effective in models of focal retinal degeneration [28,46,47]. Additionally, with the advent of lipid-based topical vehicles that can reach the posterior part of the eye, such as myriocin [13], the ease with which we can study gene silencing effects in the retina may be even more improved in the near future.

Although we demonstrated successful transfection of the siRNA:Invivofectamine 3.0 complex through to the inner retina, an important distinction is that this approach does not provide for targeting of a particular retinal cell type leading to potential pan-retinal knockdown involving multiple cell types. Viral vectors can be adapted to target uptake by a specified cell type [48-50]. Similar approaches could be harnessed for non-viral vectors through the intrinsic design of the chemical vectors [39,51]. A previous study showed successful targeted delivery of DNA and siRNA tumor cells due to chemical modifications of the lipid-based delivery agent to contain specific antibodies that mediate this recognition [52]. To our knowledge, this has yet to be achieved in the retina.

The results of this study indicated that Invivofectamine 3.0 is an effective tool for expediting gene function studies and identifying possible therapeutics in the neural retina. We demonstrated that a single injection of this lipid-based transfection of siRNA was able to penetrate all layers of the rodent retina and allowed for up to 3–4 days of targeted gene knockdown. If the longevity of the gene knockdown can be increased further, lipid-based transfection may be considered a time-effective alternative to viral-based strategies for ocular gene expression studies.

APPENDIX 1. INFLAMMATORY PROFILE OF INVIVOFECTAMINE FORMULATIONS.

To access the data, click or select the words “Appendix 1.” (A-J) The IBA1 profile following the delivery of various Invivofectamine formulations was visualized with an increase in amoeboid, activated microglia present in Invivofectamine 1M and 2.0 formulations. (K) Quantification determined an increase in IBA1 positive microglia in Invivofectamine 2.0 in the ganglion cell layer of the retina. (L) No differences in TUNEL were observed (*p<0.05, one-way ANOVA with Tukey’s post hoc test).

ACKNOWLEDGMENTS

This study was partly supported through funding from the Thermo Fisher Scientific Applications and Claims Expansions Grant with two of the authors (NA and XM) members of the Thermo Fisher Scientific team. Other funding bodies are The National Health and Medical Research Council (APP1127705, 2017-2019), the Australian Government Research Training Program Scholarship and the Australian National University Translational Fellowship. The authors do not have any other competing interests or conflicts of interest to disclose with regards to this paper.

REFERENCES

1. Ali RR, Sarra GM, Stephens C, Alwis MD, Bainbridge JW, Munro PM, Fauser S, Reichel MB, Kinnon C, Hunt DM, Bhattacharya SS, Thrasher AJ. Restoration of photoreceptor ultrastructure and function in retinal degeneration slow mice by gene therapy. Nat Genet 2000; 25:306-10. [PMID: 10888879].
2. Besch D, Zrenner E. Prevention and therapy in hereditary retinal degenerations. Doc Ophthalmol 2003; 106:31-5. [PMID: 12675483].
3. Campochiaro PA. Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders. Gene Ther 2006; 13:559-62. [PMID: 16195702].
4. Kjellstrom S, Bush RA, Zeng Y, Takada Y, Sieving PA. Retinoschisin gene therapy and natural history in the Rs1h-KO mouse: long-term rescue from retinal degeneration. Invest Ophthalmo Vis Sci 2007; 48:3837-45. [PMID: 17652759].
5. Schlichtenbrede FC, da Cruz L, Stephens C, Smith AJ, Georgiadis A, Thrasher AJ, Bainbridge JW, Seeliger MW, Ali RR. Long-term evaluation of retinal function in Pph2rd2/Rd2 mice following AAV-mediated gene replacement therapy. J Gene Med 2003; 5:757-64. [PMID: 12950066].
6. Gulyaeva LF, Kushlinskiy NE. Regulatory mechanisms of microRNA expression. J Transl Med 2016; 14:143-[PMID: 27197967].
7. Berber P, Grassmann F, Kiel C, Weber BH. An Eye on Age-Related Macular Degeneration: The Role of MicroRNAs in Disease Pathology. Mol Diagn Ther 2017; 21:31-43. [PMID: 27658786].

8. Li Z, Rana TM. Therapeutic targeting of microRNAs: current status and future challenges. Nat Rev Drug Discov 2014; 13:622-38. [PMID: 25011539].

9. Conley SM, Cai X, Naash MI. Nonviral ocular gene therapy: assessment and future directions. Curr Opin Mol Ther 2008; 10:456-63. [PMID: 18830921].

10. Oliveira AV, Rosa da Costa AM, Silva GA. Non-viral strategies for ocular gene delivery. Mater Sci Eng C 2017; 77:1275-89. [PMID: 28532005].

11. Bucolo C, Drago F, Salomone S. Ocular drug delivery: a clue from nanotechnology. Front Pharmacol 2012; 3:188. [PMID: 23125835].

12. Wadhwa S, Paliwal R, Paliwal SR, Vyas SP. Nanocarriers in ocular drug delivery: an update review. Curr Pharm Des 2009; 15:2724-50. [PMID: 19689343].

13. Platania CBM, Dei Cas M, Cianciolo S, Fidilio A, Lazzara F, Paroni R, Pignatello R, Stretto E, Ghidoni R, Drago F, Bucolo C. Novel ophthalmic formulation of myriocin: implications in retinitis pigmentosa. Drug Deliv 2019; 26:237-43. [PMID: 30883241].

14. Amadio M, Pascale A, Cupri S, Pignatello R, Osera C, V DA, AG DA, Leggio GM, Ruozzi B, Govoni S, Drago F, Bucolo C. Nanosystems based on siRNA silencing HuR expression counteract diabetic retinopathy in rat. Pharmocol Res 2016; 111:713-20. [PMID: 27475885].

15. Miyazaki M, Obata Y, Abe K, Furuasu A, Koji T, Tabata Y, Kohno S. Gene transfer using nonviral delivery systems. Perit Dial Int 2006; 26:633-40. .

16. Nishikawa M, Hashida M. Nonviral approaches satisfying various requirements for effective in vivo gene therapy. Biol Pharm Bull 2002; 25:275-83. [PMID: 11913519].

17. Boye SE, Boye SL, Lewin AS, Hausswirth WW. A comprehensive review of retinal gene therapy. Mol Ther 2013; 21:509-19. .

18. Carvalho LS, Vandenberghhe LH. Promising and delivering gene therapies for vision loss. Vision Res 2015; 111:124-33. .

19. Trapani I, Puppo A, Auricchio A. Vector platforms for gene therapy of inherited retinopathies. Prog Retin Eye Res 2014; 43:108-28. [PMID: 25124745].

20. Navarro J, Risco R, Toschi M, Schattman G. Gene therapy and intracytoplasmatic sperm injection (ICSI) - a review. Placenta 2008; 29:193-9. .

21. Kafri T, Morgan D, Krahl T, Sarvetnick N, Sherman L, Verma I. Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy. Proc Natl Acad Sci USA 1998; 95:11377-82. [PMID: 9736744].

22. Thomas CE, Birkett D, Anozie I, Castro MG, Lowenstein PR. Acute direct adenoviral vector cytotoxicity and chronic, but not acute, inflammatory responses correlate with decreased vector-mediated transgene expression in the brain. Mol Ther 2001; 3:36-46. .

23. Bessis N, GarciaCozar FJ, Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. Gene Ther 2004; 11:Suppl 1S10-7. [PMID: 15454952].

24. Thomas CE, Ehhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 2003; 4:346-58. [PMID: 12728277].

25. Han IC, Burnight E, Ulferts MJ, Worthington KS, Russell SR, Sohn EH, Mullins RF, Stone E, Tucker BA, Wiley LA. Helper-Dependent Adenovirus Transduces the Human and Rat Retina but Elicits an Inflammatory Reaction When Delivered Subretinally in Rats. Hum Gene Ther 2019; 31:456426.

26. Koirala A, Conley SM, Naash MI. A review of therapeutic prospects of non-viral gene therapy in the retinal pigment epithelium. Biomaterials 2013; 34:7158-67. [PMID: 23796578].

27. Rutar M, Natoli R, Provis JM. Small interfering RNA-mediated suppression of Ccl2 in Muller cells attenuates microglial recruitment and photoreceptor death following retinal degeneration. J Neuroinflammation 2012; 9:221. [PMID: 22992301].

28. Natoli R, Fernando N, Jiao H, Racic T, Madigan M, Barnett NL, Chu-Tan JA, Valter K, Provis J, Rutar M. Retinal Macrophages Synthesize C3 and Activate Complement in AMD and in Models of Focal Retinal Degeneration. Invest Ophthalmol Vis Sci 2017; 58:2977-90. [PMID: 28605809].

29. Matsuda T, Cepko CL. Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci USA 2004; 101:16-22. [PMID: 14603031].

30. Pitkanen L, Pelkonen J, Ruponen M, Ronkko S, Urtti A. Neural retina limits the nonviral gene transfer to retinal pigment epithelium in an in vitro bovine eye model. AAPS J 2004; 6:e25. [PMID: 15760110].

31. Rutar M, Provis JM, Valter K. Brief exposure to damaging light causes focal recruitment of macrophages, and long-term destabilization of photoreceptors in the albino rat retina. Curr Eye Res 2010; 35:631-43. [PMID: 20597649].

32. Natoli R, Jiao H, Barnett NL, Fernando N, Valter K, Provis JM, Rutar M. A model of progressive photo-oxidative degeneration and inflammation in the pigmented C57BL/6J mouse retina. Exp Eye Res 2016; 147:114-27. [PMID: 27155143].

33. Rutar M, Natoli R, Kozulin P, Valter K, Gatenby P, Provis JM. Analysis of complement expression in light-induced retinal degeneration: synthesis and deposition of C3 by microglia/macrophages is associated with focal photoreceptor degeneration. Invest Ophthalmol Vis Sci 2011; 52:5347-58. [PMID: 21571681].

34. Fernando N, Natoli R, Valter K, Provis J, Rutar M. The broad-spectrum chemokine inhibitor NR58–3.14.3 modulates...
macrophage-mediated inflammation in the diseased retina. J Neuroinflammation 2016; 13:47-.[PMID: 26911327].

35. Natoli R, Zhu Y, Valter K, Bisti S, Eells J, Stone J. Gene and noncoding RNA regulation underlying photoreceptor protection: microarray study of dietary antioxidant saffron and photobiomodulation in rat retina. Mol Vis 2010; 16:1801-22. [PMID: 20844572].

36. Rutar M, Natoli R, Chia RX, Valter K, Provis JM. Chemokine-mediated inflammation in the degenerating retina is coordinated by Muller cells, activated microglia, and retinal pigment epithelium. J Neuroinflammation 2015; 12:8-.[PMID: 25595590].

37. Anderson DH, Radeke MJ, Gallo NB, Chapin EA, Johnson PT, Curletti CR, Hancox LS, Hu J, Ebright JN, Malek G, Hauser MA, Rickman CB, Bok D, Hageman GS, Johnson LV. The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis revisited. Prog Retin Eye Res 2010; 29:95-112. [PMID: 19961953].

38. Zhao M, Bai Y, Xie W, Shi X, Li F, Yang F, Sun Y, Huang L, Li X. Interleukin-1beta Level Is Increased in Vitreous of Patients with Neovascular Age-Related Macular Degeneration (nAMD) and Polypoidal Choroidal Vasculopathy (PCV). PLoS One 2015; 10:e0125150-[PMID: 25978536].

39. Al-Dosari MS, Gao X. Nonviral gene delivery: principle, limitations, and recent progress. AAPS J 2009; 11:671-81. [PMID: 19834816].

40. Jacobson SG, Cideciyan AV, Ratnakaram R, Heon E, Schwartz SB, Roman AJ, Peden MC, Aleman TS, Boye SL, Sumaroka A, Conlon TJ, Calcedo R, Pang JJ, Erger KE, Olivares MB, Mullins CL, Swider M, Kaushal S, Feuer WJ, Iannaccone A, Fishman GA, Stone EM, Byrne BJ, Hausswirth WW. Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. Arch Ophthalmol 2012; 130:9-24. [PMID: 21911650].

41. Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Minguozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM, Rossi S, Lyubarsky A, Arruda VR, Konkle B, Stone E, Sun J, Jacobs J, Dell’Osso L, Hertle R, Ma JX, Redmond TM, Zhu X, Hauck B, Zelenai O, Shindler KS, Maguire MG, Wright JF, Volpe NJ, McDonnell JW, Auricchio A, High KA, Bennett J. Safety and efficacy of gene transfer for Leber’s congenital amaurosis. N Engl J Med 2008; 358:2240-8. [PMID: 18441370].

42. Bloquel C, Bourchard E, Berdugo M, Ben Ezra D, Behar-Cohen F. Non-viral ocular gene therapy: potential ocular therapeutic avenues. Adv Drug Deliv Rev 2006; 58:1224-42. [PMID: 17095114].

43. Witlox MA, Lamfers ML, Wuisman PI, Curiel DT, Siegal GP. Evolving gene therapy approaches for osteosarcoma using viral vectors Bone 2007; 40:797-812. review[PMID: 17189720].

44. Halbert CL, Miller AD, McNamara S, Emerson J, Gibson RL, Ramsey B, Aitken ML. Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5, and 6 in cystic fibrosis and normal populations: Implications for gene therapy using AAV vectors. Hum Gene Ther 2006; 17:440-7. [PMID: 16610931].

45. Dang JM, Leong KW. Natural polymers for gene delivery and tissue engineering. Adv Drug Deliv Rev 2006; 58:487-99. [PMID: 16762443].

46. Chu-Tan JA, Rutar M, Saxena K, Aggio-Bruce R, Essex RW, Valter K, Jiao H, Fernando N, Woolf Y, Madigan MC, Provis J, Natoli R. MicroRNA-124 Dysregulation is Associated With Retinal Inflammation and Photoreceptor Death in the Degrading Retina. Invest Ophthalmol Vis Sci 2018; 59:4094-105. [PMID: 30098196].

47. Natoli R, Fernando N, Madigan M, Chu-Tan JA, Valter K, Provis J, Rutar M. Microglia-derived IL-1beta promotes chemokine expression by Muller cells and RPE in focal retinal degeneration. Mol Neurodegener 2017; 12:31-[PMID: 28438165].

48. Kotterman MA, Schaffer DV. Engineering adeno-associated viruses for clinical gene therapy. Nat Rev Genet 2014; 15:445-51. [PMID: 24840552].

49. Petros-Silva H, Linden R. Advances in recombinant adeno-associated viral vectors for gene delivery. Curr Gene Ther 2013; 13:335-45. [PMID: 24060313].

50. Schon C, Biel M, Michalakis S. Retinal gene delivery by adeno-associated virus (AAV) vectors: Strategies and applications. Eur J Pharm Biopharm 2015; 95:343-52.

51. Simicikova M, Prather KL, Prazeres DM, Monteiro GA. Towards effective non-viral gene delivery vector. Biotechnol Genet Eng Rev 2015; 31:82-107. [PMID: 27160661].

52. Xu L, Huang CC, Huang W, Tang WH, Rait A, Yin YZ, Cruz I, Xiang LM, Pirolo KF, Chang EH. Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. Mol Cancer Ther 2002; 1:337-46. [PMID: 12489850].
