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Scleral Permeability of a Small, Single-Stranded Oligonucleotide

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ABSTRACT

Developing more effective ocular drug delivery systems is essential to improving the treatment of posterior segment eye disease. The large target area provided by the sclera and potentially less vision threatening complications are advantages of transscleral administration compared to more traditional modalities of drug delivery to the posterior segment. We aimed to determine the permeability coefficient for the *in vitro* diffusion of a small, single-stranded, oligonucleotide across human sclera. Transscleral permeability was measured by placing 100 μL of 2.96×10^{-4} mol single-stranded, fluorescein-labeled oligonucleotide (MW = 7998.3) on the episcleral surface of sclera mounted in a perfusion chamber. Fractions of choroidal perfusate were collected hourly for 24 hours. The permeability constant or K_{trans} for the transscleral diffusion of the naked, single-stranded, fluorescein-labeled oligonucleotide was $7.67 \pm 1.8 \times 10^{-7}$ cm/s (mean \pm SEM, $N = 7$). The permeability constant or K_{trans} after intrascleral injection of the same fluorescein-labeled oligonucleotide was $1.32 \pm 0.42 \times 10^{-7}$ (mean \pm SEM, $N = 4$). This analysis demonstrates that diffusion of a naked, 24-base, single-stranded, fluorescein-labeled oligonucleotide can be accomplished by both of the described methods. The ability to deliver single-stranded oligonucleotides across the sclera may prove to be advantageous given the development of several novel therapeutic strategies that use similar molecules.

INTRODUCTION

New methods for drug delivery to the posterior segment of the eye, including the posterior vitreous, retina, retinal pigment epithelium (RPE), and choroid, have generated specific interest in the ophthalmic community (1). The treatments of uveitis, age-related macular degeneration (AMD), diabetic retinopathy, and retinitis pigmentosa are in part limited by the difficulty in achieving therapeutic concentrations of drugs to these target tissues (2). Developing more effective ocular drug delivery methods with less vision threatening complications is essential to improving the treatment of posterior segment eye disease.

Achieving adequate levels of therapeutic agents in the posterior segment has been difficult by topical application (3–5). Delivering bioactive agents to the posterior segment either across the sclera

or via intrascleral injection may avoid many complications associated with intraocular delivery as well as limit systemic toxicity. Intraocular administration, via intravitreal injections or surgical implantation of intraocular sustained-release devices, is effective in achieving therapeutic drug levels, but due to their invasive nature, these methods carry risk for significant complications. Repeated intravitreal injections are required for chronic diseases and have the potential complications of retinal detachment, endophthalmitis, vitreous hemorrhage, and cataract formation (6). Surgical implantation of intraocular sustained-release devices, such as the Vitrasert ganciclovir implant used in the treatment of cytomegalovirus, avoids repeated injections (7,8). However, periodic intraocular surgery is required to insert and replace these implants. Surgical placement and removal carry many complications similar to those associated with intravitreal injection. Further, surgical implantation requires operating room facilities resulting in substantially higher costs. Systemic administration can deliver drugs to the posterior eye, but the systemic levels necessary to penetrate the blood–retinal barrier are often associated with dose limiting side-effects and toxicity (9).

The large target area provided by the sclera and potentially less vision threatening complications are advantages of transscleral administration compared to more traditional modalities of drug delivery to the posterior segment. The sclera, by virtue of its large surface area, accessibility, and relatively high permeability, may provide a useful vector for delivering drugs into the posterior segment of the eye (4,9). Drug administration to the posterior segment via subtenons or intrascleral injection should have a lower incidence of vision threatening complications because of the less invasive nature in comparison to intravitreal injections or surgical implantation.

Periocular injection should also have less systemic toxicity than oral or parenteral administration due to a more localized drug delivery. However, for a drug to reach the posterior segment following periocular injection, it must first diffuse across the sclera. The rate and extent of intraocular penetration are primarily dependent on the permeability of the sclera.

The sclera is an elastic tissue composed of collagen fibrils and proteoglycans that contain approximately 70% water and account for 95% of the total surface area of the eye with an average surface area of 17 cm² (4,9). Diffusion through an aqueous route is the primary method of drug permeation across the sclera. A fiber matrix model of transscleral drug delivery described by Edwards and Prausnitz (10) can be used to predict the scleral permeability of solutes ranging from low molecular weight drugs to macromolecules. This model and other studies demonstrate that molecular weight greatly influences scleral permeability, with permeability decreasing exponentially with increasing molecular weight (6). However, other studies have shown that the molecular radius of a solute is actually a better predictor of scleral permeability (2). Additional studies suggest that the sclera is quite permeable to a wide range of solutes (MW 285–69,000) (11). Ahmed and Patton (12) first suggested that transscleral diffusion could be utilized to deliver drugs to the posterior segment. Using topical application of inulin and timolol in rabbits, Ahmed and Patton (12) demonstrated that noncorneal, transscleral absorption contributed significantly to drug penetration into intraocular tissues, and neither local vasculature nor resorption from the systemic circulation accounted for these intraocular levels of solute. Earlier studies have shown the sclera to be permeable to compounds up to 70 kDa, but more recent *in vitro* experiments have shown that rabbit sclera is permeable to higher molecular weight dextrans, up to 150 kDa, as well as to the proteins IgG and bovine serum albumin (6). Preliminary experiments have shown successful transscleral delivery of larger molecular weight, protein-based reagents to the retina and choroid is possible (13).

Diffusion of oligonucleotides through the sclera to the posterior segment should theoretically be possible due to several properties of the sclera and oligonucleotides. First, oligonucleotides possess a large negative charge making them very hydrophilic. Because the sclera is an aqueous milieu, transscleral diffusion is greater for hydrophilic compounds compared with hydrophobic compounds. Second, oligonucleotides have a smaller molecular radius relative to a liposome or viral vector complex, both of which have been demonstrated to reach the posterior segment. Certain kinds of cationic liposomes applied topically to the ocular surface have been demonstrated to facilitate transfer of a reporter gene to retinal ganglion cells in rats (14). It is assumed that the route of penetration is via the

sclera. These properties of small oligonucleotides and the sclera combined with evidence supporting the successful delivery of a liposome to the posterior segment, support the possibility of delivering oligonucleotides to the posterior segment via transscleral diffusion. Recent data demonstrate successful delivery of a naked RNA aptamer across the sclera by diffusion (15). Furthermore, the aptamer appeared to maintain its function after diffusing across the sclera (15).

To our knowledge, no study has determined the permeability coefficient for the transscleral diffusion of a naked, single-stranded oligonucleotide. The purpose of this study is to determine the *in vitro* permeability coefficients of a small, naked, single-stranded, fluorescein-labeled oligonucleotide after either episcleral administration or intrascleral injection.

Another aim of this study is to evaluate intrascleral injection, a novel approach to periocular drug delivery. The intrascleral injector is a drug-delivery device specifically designed for less-invasive administration of drugs to the posterior segment by micro-injection into the sclera. We hypothesized that the intrascleral injector might permit more complete absorption of a drug by creating a small, localized depot of drug that is less susceptible to periocular washout. Furthermore, intrascleral injection may reduce systemic toxicity compared to subconjunctival injection by lessening systemic absorption. We determined the permeability coefficient of the same oligonucleotide after intrascleral injection of a small bolus of the oligonucleotide.

MATERIALS AND METHODS

Oligonucleotide Design and Synthesis

The single-stranded oligonucleotide used in this study was designed to direct the repair of the genomic mutation in the tyrosinase gene of the New Zealand White rabbit using genoplasty (16). The C1118A point mutation (GenBank accession number AF210660) results in a T373K amino acid substitution in the mature tyrosinase protein in the New Zealand White rabbit (17). This mutation has been demonstrated to be the same mutation responsible for human type 1 oculocutaneous albinism (17,18). The oligonucleotide consists of 24 nucleotides. The nucleotide at position 13 aligns with the C1118A point mutation resulting in a noncomplementary base pair to direct the repair of the mutant tyrosinase gene through unknown mechanisms. Phosphorothioate linkages rather than phosphodiester linkages were utilized for the six outermost bonds on both ends of the oligonucleotide to impede degradation of the molecule. The nucleotide sequence is 5'- ACC TGG GAC ATC GTT CCA TTC ATA -3'. After synthesis, the oligonucleotide was high-performance liquid chromatography (HPLC) and ion exchange purified (Applied Biosystems; Foster City, CA) to ensure that only the full-length oligonucleotide with purinated nucleotides was used in the diffusion assays.

Determination of *In Vitro* Transscleral Permeability

Scleral tissue specimens, excised from moist-chamber stored human globes (Georgia Eye Bank, Atlanta, GA), were prepared and mounted in a perfusion chamber (5). The temperature of the water jacketed perfusion chamber was kept at 37°C by a circulating water bath and the transscleral pressure was maintained at 15 mmHg, to simulate intraocular pressure, by varying the height of the water column in the outflow tubing. Transscleral permeability was measured by placing 100 μ L of 2.96×10^{-4} mol fluorescein-labeled, oligonucleotide (MW = 7998.3) on the episcleral surface of the sclera while perfusing the choroidal side with a Balanced Salt Solution (BSS); (Alcon Laboratories, Fort Worth, TX). Fractions of the choroidal perfusate were collected hourly for 24 hours. Fluorescence of each hourly sample was measured using a spectrofluorometer. Scleral permeability was determined by calculating the permeability constant, K_{trans} (cm/second), using the following equation

$$K_{\text{trans}} = (R_{\text{total}}/(A \times t)) \times (1/D),$$

where R_{total} equals the total moles through sclera in time t (second), A equals the surface area of the sclera (cm^2), and D equals the concentration of original solution in the donor chamber (mol/mL). At completion, the scleral tissue was trephinated, dividing the exposed area from the surrounding non-exposed area. Fluorescein-labeled oligonucleotide was extracted from the exposed tissue in 1 mL of BSS and the amount of the oligonucleotide was determined by fluorescence measurements. Scleral permeability K_{trans} (cm/second) was calculated for the transscleral diffusion method using seven independent trials.

Determination of *In Vitro* Permeability After Intrasceral Injection

Human donor eye sclera was mounted in the perfusion apparatus as described previously. Rather than placing oligonucleotide on the episcleral surface, an intrasceral delivery device (Figure 1A, Insite Vision Inc., Alameda, CA) was used to inject $5 \mu\text{L}$ of 1.49×10^{-4} mol of fluorescein labeled oligonucleotide ($\text{MW} = 7998.3$) into the sclera. At the start of the experiment, $100 \mu\text{L}$ of BSS was placed on the episcleral surface. BSS was perfused to the choroidal side and fractions of the choroidal perfusate were collected every hour for 24 hours. Fluorescence was measured for each hourly sample using the spectrofluorometer. Scleral permeability K_{trans} (cm/second) was calculated for the intrasceral injection method using four independent trials.

Characterization of the Intrasceral Injection

To determine the placement of oligonucleotide by the intrasceral injector, a human eye bank scleral specimen was mounted in the perfusion apparatus and the intrasceral injector was used to inject $5 \mu\text{L}$ of India ink. The scleral specimen was fixed, paraffin embedded, and sectioned prior to hematoxylin and eosin staining. Light microscopy and digital photography were used to analyze the slides.

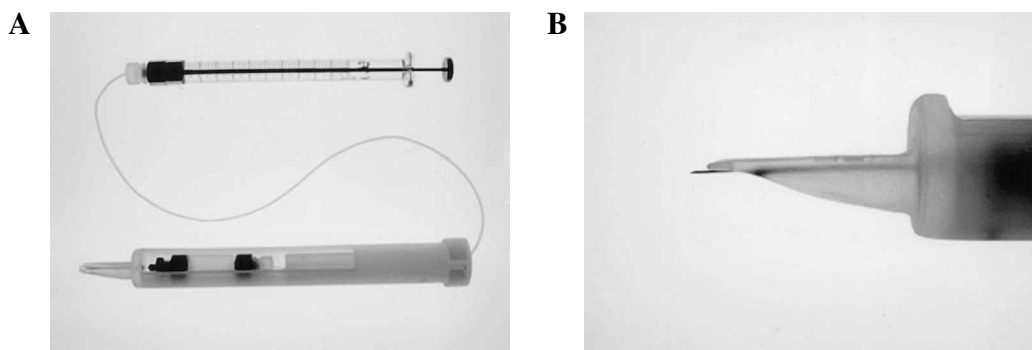


FIGURE 1. Intrasceral Injector. (A) Photograph of Intrasceral Injection Device (Insite Vision, Alameda, CA) Used to Inject Small Amount of Oligonucleotide into Sclera. This device was designed to deliver small, localized amounts of solution within the sclera and thus reduce the amount of sclera required to diffuse across to reach the posterior segment. Theoretically, this device should also limit nonocular tissue exposure, which may be advantageous with more toxic medications. The injection device consisted of a 33-gauge beveled stainless-steel needle connected to a $10 \mu\text{L}$ Hamilton gas tight syringe via a piece of HPLC grade PEEK tubing. (B) The tip of the intrasceral injector has a plastic “stop” to prevent the complete penetration of the scleral stroma.

Statistical Analysis

All average values are reported as mean \pm SEM (standard error of the mean).

RESULTS

In Vitro Transscleral Permeability

Figure 2 shows the transscleral diffusion of the naked, single-stranded, fluorescein-labeled oligonucleotide as a function of time. The permeability constant or K_{trans} for the transscleral diffusion of the naked, single-stranded, fluorescein-labeled oligonucleotide was $7.67 \pm 1.8 \times 10^{-7}$ cm/second (mean \pm SEM, N = 7). After 24 hours, $21.93 \pm 5.44\%$ of the total amount of oligonucleotide had diffused across the sclera, leaving approximately $3.35 \pm 1.18\%$ in the sclera itself and $12.94 \pm 4.31\%$ in the donor chamber (mean \pm SEM).

Characterization of the Intrasccleral Injection

Light microscopy (Fig. 3) demonstrates placement of the $5 \mu\text{L}$ of India ink using the intrasccleral injector. The device delivered the fluid bolus to a depth of approximately 80% of the scleral thickness. The India ink appeared to remain between the scleral lamellae and did not exude back toward the episcleral surface.

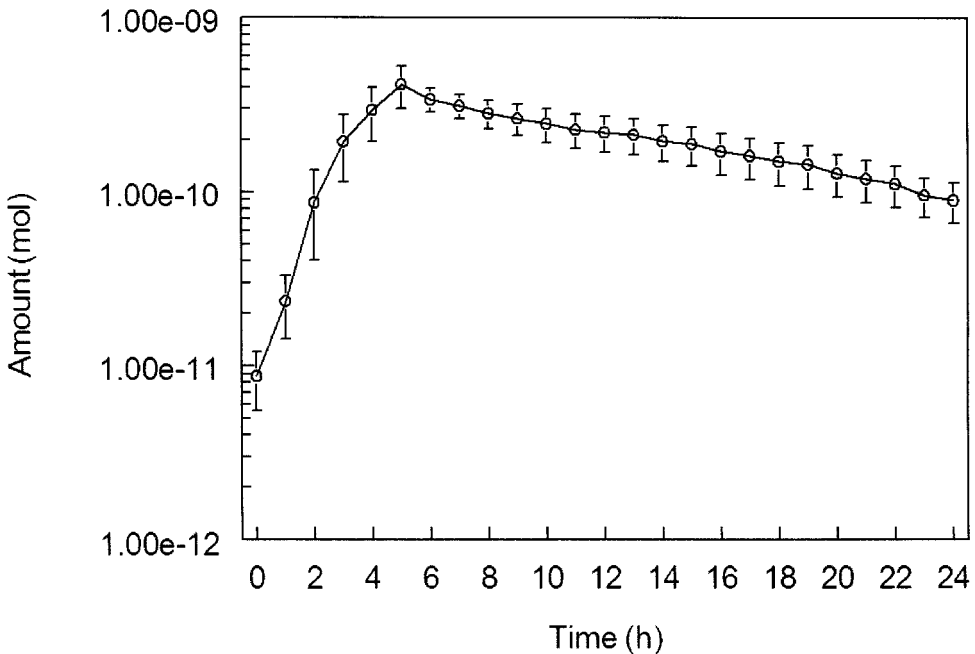


FIGURE 2. Transscleral Diffusion of a Fluorescently End-Labeled Oligonucleotide Maximum Was Reached at About 5 Hours After Initiating Diffusion. The data suggest that the DNA passively diffused a small but significant distance through a porous scleral matrix of fibers. The means of 7 independent determinations are shown. The error bars represent the standard error of the mean.



FIGURE 3. Characterization of Intrasceral Injection. Light microscopy demonstrates a pool of India ink deposited about 80% through the scleral thickness, demonstrating the depth of penetration and the location of the depot of oligonucleotide in intrasceral injection assays. The episcleral surface is to the top and the choroidal surface is to the bottom of the image. The asterisk highlights the needle tract through the sclera.

In Vitro Intrasceral Permeability

Figure 4 illustrates the diffusion of the naked, single-stranded, fluorescein-labeled oligonucleotide after intrasceral injection. The permeability constant or K_{trans} for the intrasceral diffusion of fluorescein labeled oligonucleotide was $1.32 \pm 0.42 \times 10^{-7}$ cm/second (mean \pm SEM, $N = 4$). The mean peak value of $5.36 \pm 2.73 \times 10^{-11}$ moles (mean \pm SEM, $N = 4$) was reached at 1 hour. The scleral tissue retained $27.03 \pm 7.43\%$ moles of the oligonucleotide at 24 hours. At completion, $33.26 \pm 7.02\%$ and $17.75 \pm 4.05\%$ moles were collected from the receiver and donor chambers, respectively.

DISCUSSION

In this study, we determined the transscleral and intrasceral permeability of a naked, 24-base, single-stranded, fluorescein-labeled oligonucleotide to compare *in vitro* permeability coefficients for two different delivery methods. This analysis demonstrated that diffusion of this oligonucleotide could be accomplished by both of the described methods. The ability to deliver single-stranded oligonucleotides across the sclera may be advantageous given the development of several novel therapeutic strategies that use these molecules.

Experimental strategies that use single-stranded oligonucleotides are under active investigation as potential treatments for human posterior segment diseases. Some of these strategies include antisense (19), gene therapy (16), RNAi (20), and ribozyme technologies (21). The most notable success using a single-stranded oligonucleotide for treatment of human chorioretinal disease is Formivirsen or Vitravene (Isis Pharmaceuticals, Inc., Carlsbad, CA). Formivirsen is a 21-nucleotide phosphorothioate oligonucleotide that is FDA approved for treatment of CMV retinitis in patients who fail other

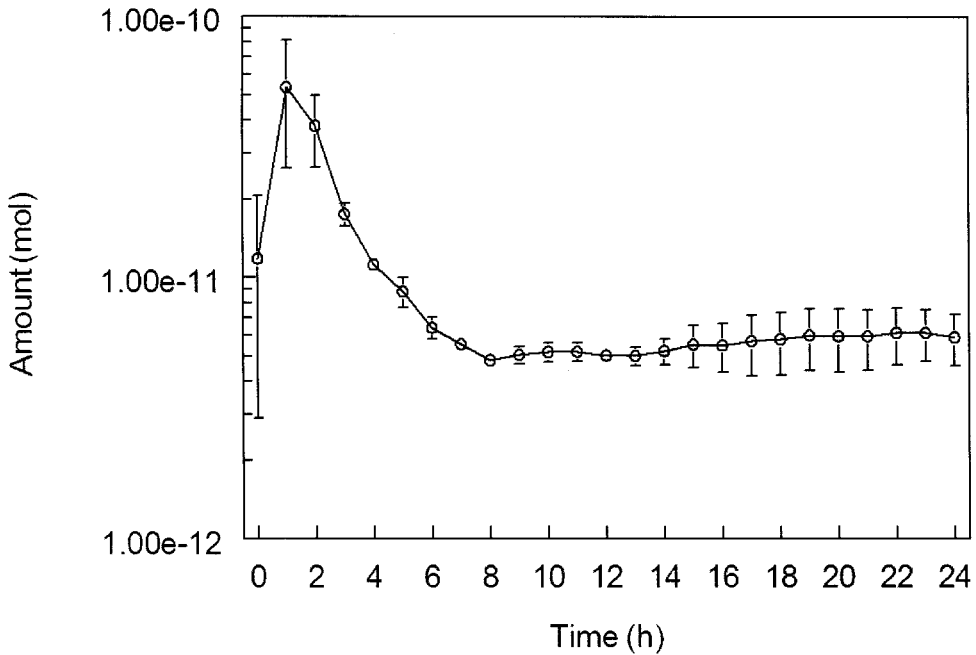


FIGURE 4. Intrascleral Diffusion of a Fluorescently End-Labeled Oligonucleotide Intrascleral Injection of Fluorescein-Labeled Oligonucleotide Allowed Diffusion Through the Remaining Sclera Toward the Choroidal Side. Similar to transscleral diffusion (Fig. 1), the oligonucleotide diffused from the intrascleral depot to the lower chamber. The 1-hour time point represents the amount of DNA that accumulated in the collection chamber during the 60 minutes following DNA intrascleral injection. This fraction contained the largest amount of DNA and suggests that the distance from the intrascleral depot to the collection point was small and that this sclera was highly porous. The log scale plot of the mean of four independent determinations is shown. The error bars represent the standard error of the mean.

anti-CMV treatments (22,23). Formivirsen demonstrates the effectiveness of oligonucleotides in treatment of retinal disease. However, clinical applications using oligonucleotides would benefit greatly by the development of less invasive delivery methods for these compounds.

Additional studies have further revealed the potential use of small, single-stranded oligonucleotides in the treatment of posterior segment diseases. Recently, promising results have been reported in a human clinical phase II trial. This study described stabilization or improvement in patients' vision 3 months after multiple intravitreal injections with the anti-vascular endothelial growth factor (VEGF) aptamer EYE001 (15). In other investigations, antisense oligonucleotides inhibit cellular and viral gene expression in the retina after intravitreal injection (24–25). Intravitreal administration of oligonucleotides targeting VEGF reduces new blood vessel growth by 25%–31% in a murine model of neovascularization (26). However, despite the effective delivery of oligonucleotides by intravitreal injection, oligonucleotides are rapidly cleared from the vitreous making repeated injections necessary to maintain effective therapy. Repeated intravitreal administration of drugs and bioactive molecules increases the risk of endophthalmitis, retinal detachment, vitreous hemorrhage, and possible retinal toxicity (27).

The initial investigation of the safety of another small, single-stranded, phosphorothioate oligonucleotide demonstrated reasonable tolerability by the choroid and retina to this type of molecule after subretinal injection (19). The only toxicity noted was described as a limited cellular infiltration (19).

Even though transscleral diffusion permits a less invasive method of oligonucleotide delivery, this method has limitations as well. Periocular injections can be too indiscriminate or transient to ad-

minister bioactive molecules to the posterior segment of the eye. Large volumes injected into the subconjunctival space may overwhelm the absorption capacity of the sclera leading to dispersion into nonocular, orbital tissues or systemic exposure and not achieve therapeutic levels in the posterior segment. Therefore, a sustained-release delivery system (i.e., fibrin sealant or biodegradable polymers) may be advantageous by allowing continuous transscleral diffusion (15).

The current studies suggest that this intrascleral delivery system can achieve diffusion of a small but significant amount of oligonucleotide across the sclera. Intrascleral injection is a new technique that has not been well studied but offers the potential to more precisely localize small volumes of an ocular drug and possibly minimize systemic side-effects. Intrascleral injection, like intravitreal injection, requires repeated administration for treatment of chronic diseases. This modality needs further investigation prior to its clinical use.

In summary, systemic therapy may be accompanied by side-effects and repeated intravitreal injections or intraocular surgery can have significant complications; therefore, less invasive methods of sustained delivery of bioactive molecules for treatment of posterior segment diseases are needed. Several therapeutic strategies using small, single-stranded oligonucleotides are actively being investigated; however, delivery of these molecules to the posterior segment using current methods carries significant risk of vision-threatening complications. We have demonstrated transscleral diffusion of small but significant amounts of naked, single-stranded oligonucleotides using two methods. Utilization of a reservoir such as fibrin sealant or biodegradable polymeric systems in conjunction with transscleral diffusion may allow sustained delivery of these molecules to the posterior segment and needs further investigation.

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