Delivery of several forms of DNA, DNA-RNA hybrids, and dyes across human sclera by electrical fields

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Purpose: Iontophoresis has been used for drug delivery across the cornea for many years. We sought to test whether small charged dyes and DNA can be transferred across human sclera by an electric field.

Methods: Full-thickness human scleral fragments were embedded vertically in an agarose gel and positioned to completely span individual gel lanes. The scleral fragments were located approximately 1 cm downstream from the gel wells. DNA or dyes were loaded into the wells and electrophoresis was carried out at about 3.3 V/cm for approximately 2 h per run. Movement of DNA and dyes through the agarose and sclera was measured with either digital time-lapse photography or through DNA extraction and purification from the gel. SYBR green stain was used as a sensitive method to detect DNA.

Results: Digital time-lapse photography of agarose gel electrophoresis revealed that two dyes, xylene cyanol and bromphenol blue, passed through the sclera in the presence of an electric field. Xylene cyanol was driven through the sclera virtually unimpeded except for some spreading of the dye. Bromphenol blue was slowed markedly by the sclera, but it too eventually passed through the tissue. Small DNAs, including a single stranded 51-mer and a double hairpin 68-mer oligonucleotide, passed through the sclera as detected by SYBR green staining. Linear double stranded DNAs ranging from 50 bp to 12,000 bp passed through the sclera. The larger the DNA, the slower the rate of passage through the sclera, and the greater the band spreading. pEGFP-1 (a 3 kb plasmid) passed through the sclera but was accompanied by a great amount of band spreading. Following completion of the initial electrophoresis run, the plasmid DNA was extracted from the smeared bands in the agarose distal to the sclera and re-run on a second gel without sclera. The initially smeared plasmid bands resolved into 2 distinct bands after extraction and purification and matched well with control plasmid bands.

Conclusions: Charged molecules such as xylene cyanol, bromphenol blue, and DNAs ranging from 51 bp oligonucleotides to a 3 kb plasmid can be driven across human sclera by an electric field and directly detected. Passage of plasmids was efficient, but the plasmid bands were diffuse after transit. This technique offers promise as a noninvasive DNA delivery tool, where gene therapy can be accomplished by small RNA or DNA synthetic oligonucleotides, larger double stranded fragments, or even plasmids.

An important consideration in ocular gene therapy is the delivery of therapeutic agents to specific tissues within the eye. The sclera represents the first of several barriers to the efficient delivery of DNA to posterior segment targets. Whether the sclera is the major barrier remains to be tested. To deliver gene therapy agents to the eye, several approaches are being developed, including several forms of ocular injection, systemic injection, and surface applicants. The DNA being delivered may be "naked", conjugated with transfection agents such as liposomal agents, or packaged in hobbled viruses (for reviews, see [1-3]).

Each of these approaches has benefits and drawbacks. Surface delivery (eye drops) of therapeutic DNA is noninvasive and can be given repeatedly. It has been used to deliver virally-packaged DNA in treatment of excimer laser-induced corneal haze [4], but is unlikely to be able to deliver DNA to posterior segment targets.

Ocular injections are routinely conducted in the clinic and they allow precise delivery to a target tissue. However, in addition to the direct tissue damage caused by the needle, injections often require anesthesia (itself delivered by injection), and may result in infection or inflammatory responses. Conversely, systemic injections are non-invasive, but appear to require viral [5,6] or pegylated liposomal [7] packaging of therapeutic DNA constructs for ocular treatment.

Various forms of viral packaging may have resulted in adverse reactions in humans, including immune responses in the eye [8,9], leukemia-like symptoms [10,11], or death [12,13]. Adeno-associated virus (AAV)-mediated DNA delivery appears to have fewer side-effects [8] and has been used to rescue vision in some animal models of blindness [14-18]. However, AAV delivery may require direct ocular injection to achieve efficacy [1] and the randomness of integration of the therapeutic DNA inserts in the genome following recombinant AAV infection is of concern [19].

A completely different delivery modality is available for ocular gene therapy. This approach takes advantage of the fact that DNA is highly charged at neutral pH and can be driven through sieves such as agarose or even high concentrations of

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tightly crosslinked polyacrylamide by electric fields. A low voltage, constant-current electric field (iontophoresis) is commonly used clinically to drive charged molecules across tissue layers (notably the skin) [20] and at one time was commonly used ophthalmologically [21] and is being re-introduced into the ophthalmology clinic [22,23]. Trans-corneal and transscleral delivery of small charged compounds and DNA has been successfully tested in several animal models [24-33].

Here we report the testing of an ocular DNA delivery scheme for potential use in humans. We investigated whether single stranded DNA, a DNA-RNA hybrid molecule, a series of longer double stranded DNAs, or supercoiled plasmid DNA can be delivered across human sclera by a constant-voltage electric field (i.e., by electrophoresis).

Below are the definitions of electrophoresis, electroporation, and iontophoresis as used in this article.

Electrophoresis is defined as the movement of charged molecules under the influence of an electric field through a liquid medium in a porous support (which can take on many forms including agarose, polyacrylamide, paper, and many others). This approach is most often used in the laboratory as an analytical or preparative bench science technique rather than a clinical delivery approach.

Electroporation is a delivery technique in which a high field-strength, short-duration pulse is used to reversibly create pores in the plasma membrane of the cell. By diffusion or electroendoosmosis, molecules may cross into the cell through these transient pores. This technique is commonly used in the laboratory to introduce DNAs into eukaryotic cells in culture, bacteria, tissue explants or living animals.

Iontophoresis is the introduction of ions into tissues by an electric field. Usually the bulk of the current is carried by a charged drug itself as other ions such as those of buffers or physiological salts such as NaCl would reduce the number of drug ions delivered. This is in contrast to electrophoresis in which several buffer constituents are used to maintain a discrete band of an analyte (CF, a short DNA) migrating at a velocity different from another analyte (CF, a longer DNA).

The study design was to conduct experiments with cadaveric human sclera to assure that the same thickness and
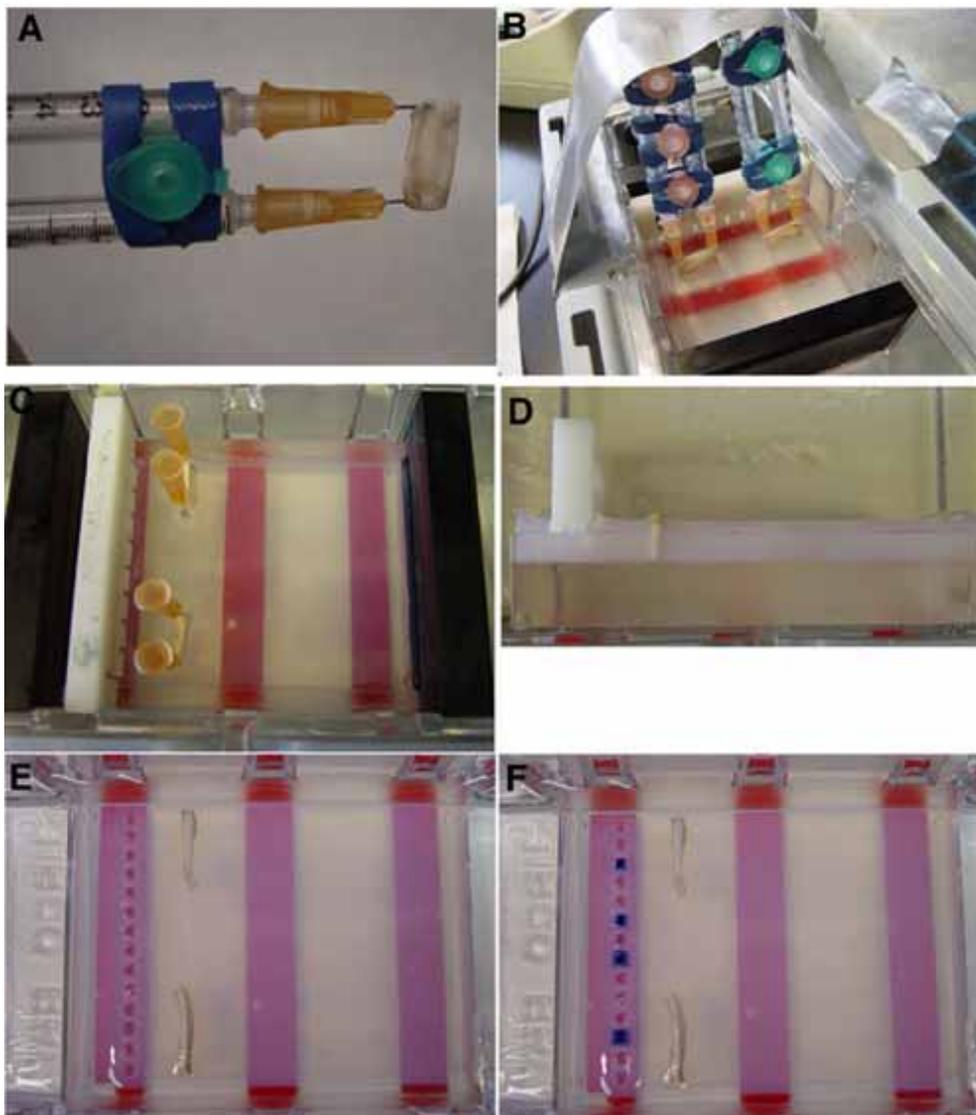

Figure 1. The experimental apparatus. The study design was to conduct experiments with cadaveric human sclera to assure that the same thickness and pore characteristics of sclera would be tested as might be required in treating a human patient. Conventional molecular biological equipment and methods for analyzing DNA were adapted to test whether DNAs of various sizes could be driven across the sclera. The arrangement and assembly of scleral fragments in an agarose gel apparatus is shown in **A** through **F**. **A**: Mounting the scleral fragments on syringe needles before embedding in agarose. **B**: Securing the mounted scleral fragments during agarose gel solidification. **C**: Removal of the syringe supports. **D**: Side view of the embedded scleral fragments. Note the plastic boosts elevating the gel comb. **E**: Top view of the embedded scleral fragments. **F**: Loaded DNA and dyes prior to electrophoresis.

pore characteristics of sclera would be tested as might be found in human patients. Conventional molecular biological equipment and methods for analyzing DNA were adapted to test whether dyes or DNAs of various sizes could be driven across human sclera.

METHODS

Cadaveric eyes from 10 donors were purchased from the Georgia Eyebank, Inc. (Atlanta, GA). Criteria for inclusion in this study included a postmortem age of 7 days or less. The average time postmortem was 4 days. Age at time of death ranged from 17 to 74 years, with a median of 54 years. Samples were excluded if there was indication of corneal or retinal disease. Eyes from individuals who had had cataract surgery were excluded.

Rectangular scleral fragments were obtained by blunt dissection with scissors, forceps, cotton swabs, and razor blade. Conjunctiva was removed from the outside of the sclera and
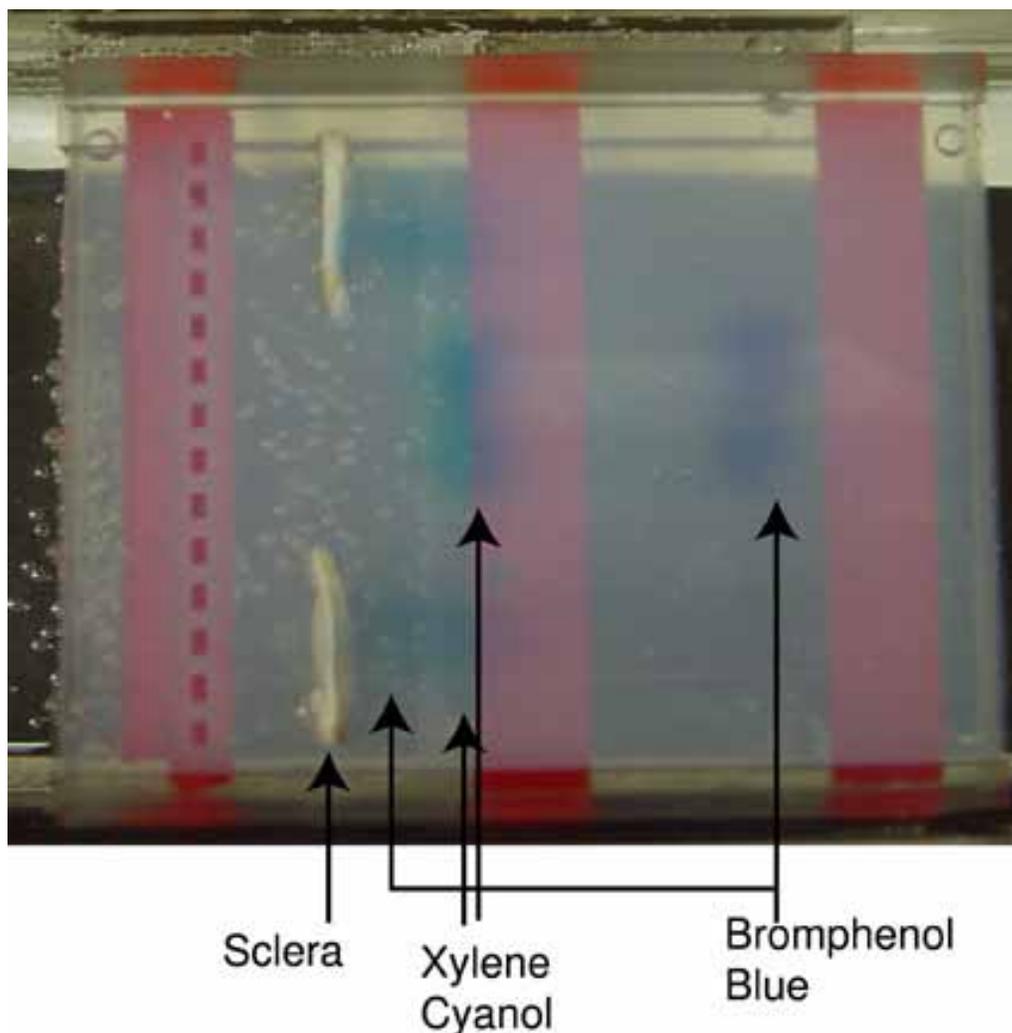

Figure 2. Electrophoresis of charged dyes through human sclera. Rectangular fragments of human cadaveric sclera were mounted vertically in a horizontal gel electrophoresis chamber. The tissue samples were oriented with the outside eye surface facing and closest to the gel wells. 2% agarose was poured into the gel chamber and allowed to solidify, which embedded the sclera and formed wells. The agarose contained 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Bromphenol blue and xylene cyanol in 1X TAE buffer and 10% glycerol were loaded into well positions 3, 6, 8, and 12. Two samples (Lanes 6 and 8 from the top down) were unimpeded by sclera, and two other wells (lanes 3 and 12) were completely occluded by human scleral fragments. The samples were subjected to an electric field of 3.3 V/cm for 114 min, and digital photographs were taken every 3 min during electrophoresis. The final picture of the series is shown. The 39 pictures of the run are shown in sequence as a movie in the online version of this article. Lanes 6 and 8 show that bromphenol blue migrated roughly twice as quickly (about 4 cm/h) as xylene cyanol (about 2 cm/h) when unimpeded by sclera. Lanes 3 and 12 show that bromphenol blue ran into the sclera first, but its passage was slowed or delayed in this encounter while xylene cyanol met the sclera second. Xylene cyanol in lanes 3 and 12 appeared to pass through the sclera relatively unimpeded except for some band spreading when compared to the movement of xylene cyanol in control lanes 6 and 8 that lacked the scleral obstacle. After the xylene cyanol passed through the sclera, bromphenol blue was observed slowly appearing in agarose on the inner side of the scleral face. The online movie illustrates that the dyes were not migrating around the sclera. Bromphenol blue was retained and slowly passed through human sclera while a different dye, xylene cyanol, simultaneously passed through the same scleral fragments with minor band spreading and nearly the same migration rate as xylene cyanol through agarose alone.

retina, retinal pigment epithelium (RPE), and choroid were removed from the inner surface. The fragments were cut from the intact globe so that one short edge of the rectangle was from the limbus and the other from the posterior pole. One of the long edges was trimmed with a razor blade to make it as flat as possible for mounting perpendicular to and in contact with the bottom agarose gel chamber surface.

Rectangular fragments of human cadaveric sclera were mounted (Figure 1A) vertically in a horizontal gel electrophoresis chamber (Figure 1B; either Model H6 or H58; GIBCO-BRL, Gaithersburg, MD) as shown in Figure 1. Care was taken to insure that the edge of the sclera was in direct contact with the bottom of the gel chamber (Figure 1C). A sample comb with 14 small teeth was positioned with a 1.5 mm space from the bottom of the gel surface to the bottom tips of the teeth (Figure 1D). The additional space was created by applying a thin piece of styrofoam to the bottom of the comb rests in order to raise the level of the teeth in the gel (Figure 1D). The scleral fragments were large enough to span multiple teeth (and subsequent gel lanes) horizontally (Figure 1E). These steps helped to assure that electrophoresed mate-

rial would not flow around or underneath the sclera, but instead would have to traverse through the sclera when subjected to an electric field. The scleral fragments were oriented with the outside eye surface facing the gel wells and the cathode. Agarose was prepared in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). As per conventional electrophoresis, the agarose was melted in a microwave oven with continued boiling for 1 min, followed by cooling to about 60 °C before pouring into the gel chamber that had the rectangular scleral fragments pre-positioned (Figure 1B). Either 1.1% or 2% (W/V) regular agarose was poured into the gel chamber and allowed to solidify (Figure 1C), which embedded the sclera and formed the wells. The thickness of the agarose gel was about 4 mm, and it was poured until just below the top edge of the scleral fragments (Figure 1D). This gel thickness assured that test materials could not traverse over the tops of the scleral fragments in the electric field. 1X TAE buffer, in addition to being used to prepare the agarose, also served as the running buffer (Figure 1F).

The dyes bromphenol blue and xylene cyanol or DNAs in loading buffer (1X TAE buffer and 10% glycerol) were pipetted into wells each in a volume ranging from 1 to 10 µl, so that the well was only partly filled. Duplicate samples were loaded into wells either unimpeded by sclera or into wells that were completely blocked downstream by human scleral fragments.
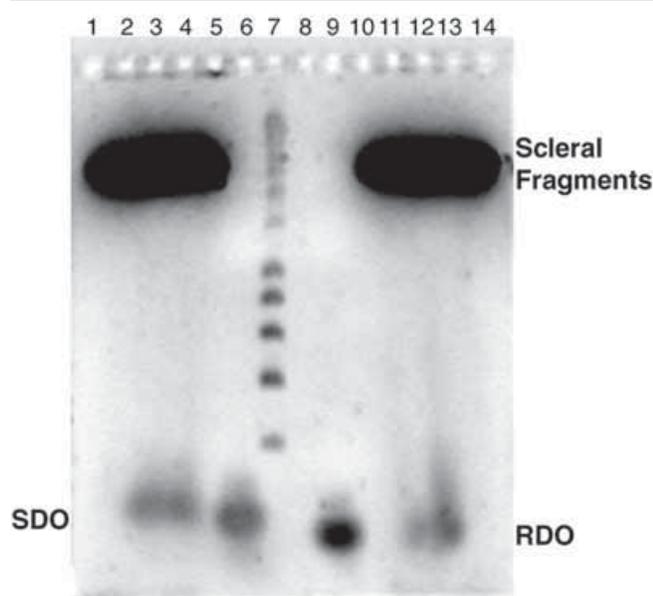

Figure 3. Electrophoresis of small oligonucleotides through scleral fragments. The same experimental apparatus and protocols were used as described in Methods and the legend for Figure 2, except small oligonucleotides were loaded into sample wells in 10% glycerol 1X TAE buffer. Two types of oligonucleotides (SDO and RDO) were loaded in the wells to test whether they could be electrophoresed through the sclera. First, a 51-base long single stranded DNA (SDO) was tested (see lanes 3 and 6). Lane 3 contained a scleral fragment and lane 6 did not. The second type of oligonucleotide was a hybrid RNA-DNA molecule (RDO) possessing a double hairpin structure synthesized as a 68-mer. RDO was loaded in lanes 9 and 12. Lane 12 contained a scleral fragment whereas lane 9 did not. The gel was run at 58 volts for 2 h and 5 min. Subsequently, the gel was soaked in SYBR Green II (which effectively stains both single stranded and double stranded DNA) for 30 min and examined by fluorescence.
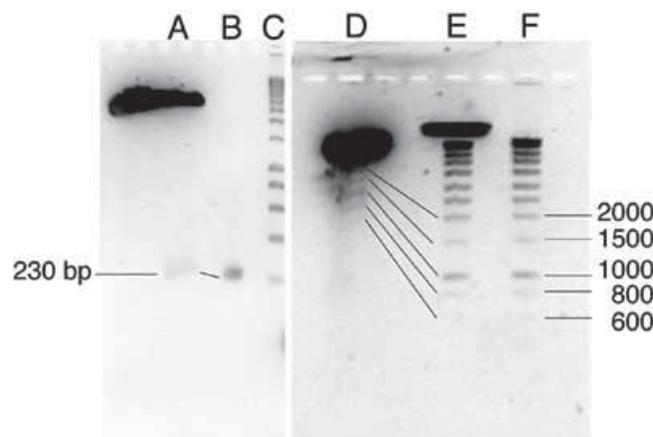

Figure 4. Trans-scleral electrophoresis of linear DNA larger than 200 bp. A 230 bp PCR product (lane A) is obstructed by sclera. Lane B is the same 230 bp DNA fragment unimpeded. Lane C is a DNA ladder ranging from 200 to 10 kb unimpeded. Lane D is a DNA ladder obstructed by sclera. Lane E is the same DNA ladder obstructed with a nitrocellulose 0.45 µm filter. Lane F is the same DNA ladder unimpeded. Lanes A, B, and C were run on one gel, and lanes D, E, and F were run on a second gel. The same conditions as in Figure 2 were employed. The data indicate that a 230-bp fragment of DNA passed through the sclera only minimally impeded (compare lanes A and B). Several fragments of a DNA ladder passed through the sclera (lane D) but had slower migration rates than the unimpeded samples. Lanes C, E, and F were loaded with the Gene Choice DNA ladder-1 (now known as HyperLadder I, Catalog number BIO-33025; Bioline USA Inc., Canton, MA).

The reservoir was filled with 1X TAE buffer to a 1-2 mm height above the surface of the gel. The gel was run as a conventional submarine gel. The voltage difference was 56-58 volts, constant voltage mode, and the current 34 or 56 mA when electrophoresis was started, depending on the particular gel box. The distance between the electrodes was about 17 cm, yielding a voltage drop of about 3.3 V/cm. The run time was usually about 2 h. Digital photographs (Sony Cybershot DSC-P9) were taken every 3 min during electrophoresis for samples containing just loading buffer. For gels in which DNA samples were loaded, the gels were subsequently soaked in SYBR Green II (Molecular Probes, Eugene, OR), which stains both single stranded and double stranded DNA, for 30 min, and examined on a fluorescent light box. Digital images of nucleic acids in gels were captured with a Gel Doc 1000 using Molecular Analyst software (Bio-Rad Inc, Hercules, CA).

To recover DNAs that had migrated through the scleral fragments, large pieces of agarose containing DNA were cut out of the gel and DNA extracted using a Quantum Prep Gel Slice Kit as per manufacturer's instructions (Bio-Rad, Inc., Hercules, CA). The agarose was dissolved in 5.7 M guanidine thiocyanate, 25 mM acetate, pH 5.2, and mixed with kit matrix. The DNA was bound by the matrix, which was collected by centrifugation, whereas the solubilized agarose remains in the supernatant. The pelleted sample was washed in isopropanol twice and resuspended in a kit wash buffer. The DNA-matrix sample was collected on a spin column and washed again. The DNA was eluted from the resin with kit elution buffer. In many cases, DNA had to be recovered from large agarose gel fragments over 3 cm along the length of a lane, having gel volumes up to about 0.5 ml.

RESULTS

Figure 2 illustrates the migration of two dyes, bromphenol blue and xylene cyanol, through scleral fragments. Unobstructed by sclera, the two dyes migrate with velocities of about 2 cm/h (xylene cyanol) and 4 cm/h (bromphenol blue) as shown in Lanes 6 and 8. For lanes impeded by scleral fragments (Lanes 3 and 12), bromphenol blue ran into the sclera first, but its passage was slowed or delayed in this encounter, and the dye transiently disappeared. Xylene cyanol reached the sclera second and passed through relatively unimpeded. It migrated at the same rate as in lanes 6 and 8, which lacked sclera, except that there was more spreading of the dye band. After the xylene cyanol passed through the sclera, bromphenol blue appeared in agarose at the inner side of the scleral
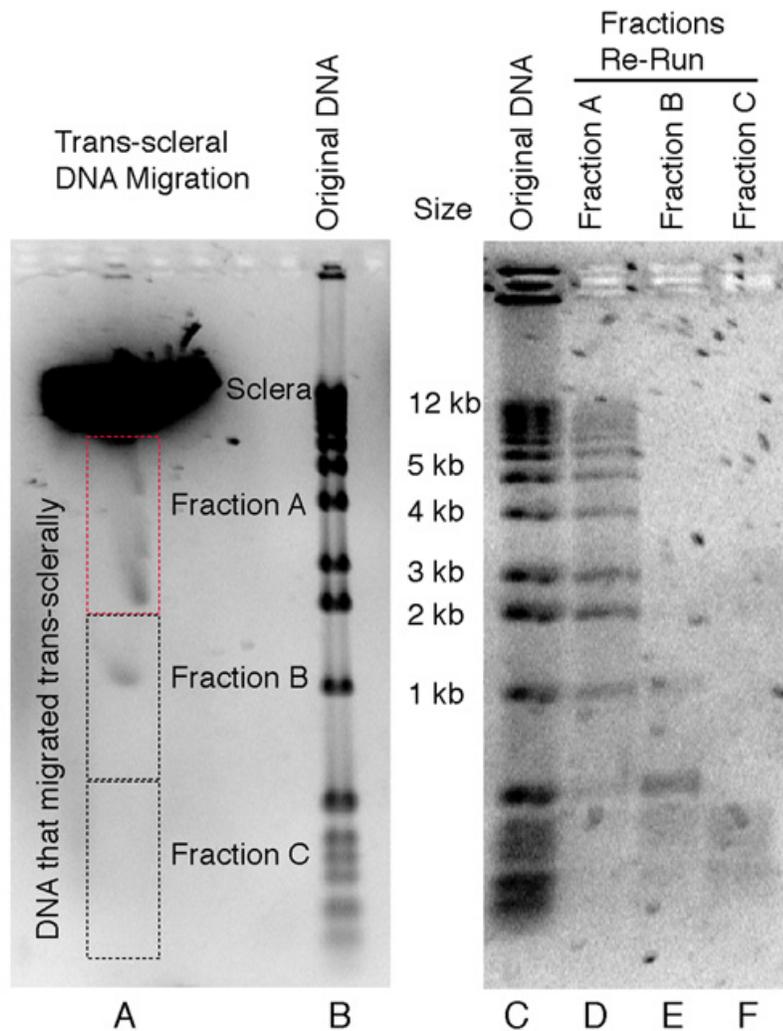

Figure 5. Electrophoresis of linear double stranded DNA fragments through the sclera. Typical results from the electrophoresis of DNA of differing sizes through human sclera. DNA (ranging from approximately 50 bp to 12 kb long) passed through the sclera in the electric field. This experiment was repeated five times; data shown are representative. Lane A shows a DNA ladder (1 kb ladder; Invitrogen Corporation, Carlsbad, CA) that passed through the sclera. Lane B shows the same DNA ladder unimpeded by sclera. The lane A material that had passed through the sclera was retrieved in three fractions labeled Fractions A, B, and C, as illustrated by the dotted lines (the Fraction A box is in red to indicate the upper boundary of the excised gel, which was about 2 mm downstream of the scleral fragment). The DNA from each agarose gel fragment was recovered and analyzed on a second gel. Wells D, E, and F were loaded with DNA extracted from Fractions A, B, and C, respectively. The original DNA ladder is shown in Lane C. There was no scleral tissue in Fraction A. Fraction A (Lane D) shows that DNA as large as 12 kbp migrated through the sclera. The mobility of the smaller double-stranded DNA fragments is largely unaffected. In larger fragments, from about 400 bp and greater, mobility is reduced in proportion to the size of the DNA.

face. The bromphenol blue band was more diffuse and was more difficult to detect by eye. Time lapse photography of agarose gel electrophoresis revealed these points more vividly (Figure 2B). Without current flow, the dyes remain stationary in the wells excepting a slow diffusion taking hours to migrate a measurable distance (about 1 mm) by eye, with no net flow (data not shown).

Figure 3 illustrates the electrophoresis of small DNA and RNA-DNA oligonucleotides through the sclera. Two types of oligonucleotides were studied. First, a 51-base single stranded DNA oligonucleotide (SDO) was tested (see Lanes 3 and 6). Lane 3 contained a scleral fragment and Lane 6 did not. The mobility of SDO was minimally altered by sclera. A hybrid 68-base RNA-DNA oligonucleotide (RDO) was loaded in Lanes 9 and 12. The latter lane contained a scleral fragment and Lane 9 was unimpeded. Again, RDO had the same mobility regardless of whether it passed through the sclera or not.

Figure 4 shows the trans-scleral electrophoresis of larger double-stranded, linear DNAs, a 230-bp PCR product (Lanes A and B), and a DNA ladder ranging from 200 to 10 kb (Lanes D, E, and F). The same conditions as in Figure 2 were employed. In comparing Lanes A and B, a 230-bp fragment of DNA passed through the sclera (Lane A) only minimally impeded compared with the unimpeded Lane B. Several fragments of a DNA ladder passed through the sclera (Lane D) but appeared to be slower in mobility compared to unimpeded DNA (Lane F). Further experiments were required to establish the largest size that passed through.

Figure 5 shows that although the passage of high molecular weight DNA could not be detected directly, following the concentration of DNA from excised agarose gel fragments from below the scleral fragment, discrete bands the same size as the original DNA were recovered. The interpretation is that the mobility of large DNA molecules passing through the sclera was not uniform, such that no discrete band could be observed. However, the agarose below the sclera contained large double stranded DNA, and this DNA could be recovered from large pieces of agarose gel using a gel extraction kit. Once purified and concentrated, each DNA had an identical mobility to the starting material, suggesting that the DNA was undamaged by its passage through the sclera. The commercial kit was able to tolerate large amounts of agarose even in extracting and recovering relatively small amounts of DNA fragments. In this experiment double stranded DNA ranging from about 50 bp to 12 kb passed through the sclera.

Figure 6 shows that electrophoresis of plasmid DNA through human sclera does not alter the DNA as determined by agarose gel migration. Plasmid DNA (pEGFP-1) was electrophoresed through agarose with or without sclera in the lane. The unimpeded plasmid showed two bands, probably due to supercoiling (Figure 6A, lane 1). Passage through the scleral slice produced smearing of the plasmid signal (Figure 6A, lane 2). Following the first electrophoresis, DNA was purified from agarose slices taken from each lane. Recovered plasmid was subjected to agarose gel electrophoresis a second time. As shown in Figure 6B, both bands from the original run without
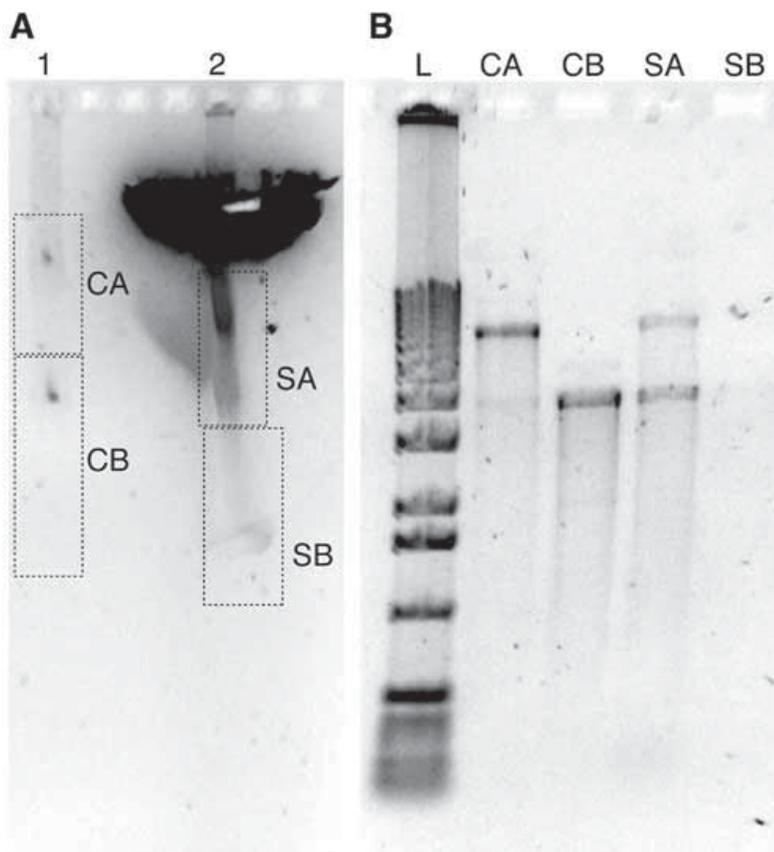

Figure 6. Electrophoresis of plasmid DNA through human sclera. **A**: Plasmid DNA (pEGFP-1) after standard electrophoresis through 1.1% agarose (lane 1) is compared to that which is impeded by a piece of human sclera (lane 2). The gel was loaded with 925 ng of pEGFP-1 in lanes 1 and 2, subjected to 56 Volts for 2.5 h in 1X TAE, and was stained with SYBR Green for 35 min. Following electrophoresis, displayed in **A**, DNA was purified from agarose slices taken from each lane (the location of each agarose slice is denoted by the boxes labeled CA, CB, SA, and SB). **B**: DNA was recovered from each gel slice from **A**, reduced in volume, loaded on a second 0.8% agarose gel, subjected to 56 V for 1.5 h, and stained with SYBR Green for 30 min. Recovered gel-purified DNA (in **B**, lane SA) reveals that the smear located below the sclera in **A** (box SA) contained DNA identical in mobility to plasmid recovered from lanes unimpeded by sclera (lanes CA and CB). Lane SB contained little DNA. These results suggest that the plasmid crossed the sclera. The lane labeled L was loaded with 1,000 ng of a 1 kb ladder (Invitrogen, Carlsbad, CA).

sclera (that is, DNA purified from lane 1) are apparent in lanes CA and CB. DNA isolated from lane 2 (which had passed through the scleral slice), visualized as two bands with identical migration to the bands in lanes CA and CB. These banding patterns suggest that both forms of the plasmid passed through the sclera unaltered in terms of this assessment.

DISCUSSION

This report establishes that charged dyes and nucleic acids pass through human sclera when driven by an electric field. Trans-scleral mobility in the electric field was monitored in a simple apparatus, a horizontal gel box with a piece of human sclera embedded vertically in an agarose gel. The voltage and current used in these experiments is probably higher than what would practically be possible in an in vivo system, but here the emphasis was to show that an electrical field could, in principle, be used to transport charged molecules through the sclera. Future experiments are planned at more physiologic conditions.

It seems remarkable that large molecules can pass through the sclera so readily, and with succeeding reports, investigators have become more ambitious. Many investigators have delivered small drugs (<1,000 daltons) through the cornea or sclera. Recently, Ambati et al. [34] delivered molecules of about 150,000 molecular weight. In this report, we delivered DNA up to about 8 million daltons.

Several approaches have been used to drive materials across the sclera. For example, Ambati et al. [35] implanted an osmotic pump to provide a long-term high concentration source of material for diffusion across the sclera. This technique though is invasive (requiring surgical implantation) and carries the potential for infection.

The sclera, though tough, thick, and rigid, has many microscopic to molecular-scale, water-filled pores [36]. The pores may allow the sclera to behave as a hydrophilic network of fibers. At neutral pH, nucleic acids are highly charged. In an electric field, they easily pass through sieves such as agarose or even high concentrations of tightly crosslinked polyacrylamide. Like agarose and polyacrylamide, the hypothesized pores in sclera appear large enough for nucleic acids to snake through under the influence of an electric field.

The electric field may help to solve one of the most difficult and important problems in gene therapy for eye diseases; the need to deliver therapeutic agents to the interior of the eye without damaging ocular tissues. Electrophoresis or iontophoresis of nucleic acids into the eye may be ideal for gene therapy because RNA and DNA are so hydrophilic and charged.

We were not able to discern any major differences among fragments of sclera from different geographic parts of the globe. Dyes and DNA passed through all the fragments, irrespective of their origins across the surface of the globe. Even so, there still may be significant differences in the "electrophoretic permeability" of the sclera from different regions as (1) the thickness of the sclera varies widely from about 0.3 mm at the equator to about 1 mm at the optic nerve and at the limbus, (2) the contents and composition of sclera may vary

regionally, and (3) our technique may not be sensitive enough to measure these potential differences with the current homemade apparatus and the given size of the scleral fragments.

We were not able to detect differences among different aged scleral preparations either postmortem or by age at death. There must be morphological and anatomical changes in the sclera that take place with death. Unfortunately, little has been published on such changes in the sclera, over time, following death. We expect autolysis of fibroblasts in the sclera postmortem, but the fibroblasts are limited in number and may not manifest much of a change in the pore structure living versus postmortem for a few days. Hence, experimental variability due to postmortem changes in the sclera is expected, but no changes in DNA mobility through the sclera over a period of a few days postmortem were found.

Both xylene cyanol and bromphenol blue passed through the sclera under the influence of an electric field. Their rates of passage were different, with xylene cyanol passing through much faster than bromphenol blue (about 30 min versus 1 h, respectively), despite the considerably faster migration rate of bromphenol blue in 2% agarose with 1X TAE buffer. That bromphenol blue was considerably slowed while xylene cyanol passed through the same sclera unimpeded suggests that both dyes actually passed through the tissue rather than around it. This differential behavior suggests that there were no large caliber "holes" in the scleral fragments (either naturally occurring or inadvertently introduced into the sclera during dissection or trimming). The sclera seems to be capable of binding large amounts of bromphenol blue. It may be that changes in the charge properties of scleral-bound versus free dye account for its apparently slower rate of transit. Xylene cyanol appeared to pass through the sclera without interacting with the scleral pore material. The only major effect on xylene cyanol transit appeared to be irregular sieving that dispersed the dye.

Small oligonucleotides electrophoresed through the sclera. Both the double hairpin RNA-DNA hybrid and the single-stranded DNA fragment passed through the scleral fragments. The mobility of the oligonucleotides passing through the sclera was slightly slower versus their respective unimpeded counterparts, and we observed extra band spreading and tailing when compared to their unimpeded counterparts. These findings suggest that DNA and RNA have approximately the same electrophoretic mobility through the scleral pores.

A series of double-stranded DNA fragments electrophoresed through the sclera. Linear double-stranded DNA passed through the sclera, though with apparently slower rates than the same DNA unimpeded. Relatively large DNA passed through the scleral fragments, suggesting that many potentially therapeutic nucleic acids, even large ones, can be electrophoretically delivered to the interior of human eyes. The relatively short time span for transit is encouraging and suggests that high doses of nucleic acids could be delivered by the electrophoretic route repeatedly. These doses and sizes of fragments suggest that gene augmentation therapy could be achieved through the sclera.

Administering an electrical current for 2 h would be very difficult in a patient setting and may cause significant toxicity [21,30]. However, the length of electrophoresis in our present experiments in the agarose gel was much longer than would be used clinically because:

(A) The DNAs had to travel about 1 cm through the agarose gel to arrive at the sclera, and then the DNA had to migrate several more centimeters beyond the choroidal side of the sclera to allow our analytical evaluations of whether and how the DNAs passed through the sclera. This time is "extra" since in a patient setting the initial distance, instead of 1 cm, would be much less, a fraction of a millimeter at most (leading to a much shorter time before the DNA would make first contact with the sclera). Similarly, the length of time after the DNA crossed the sclera would be much less in a patient, as instead of traveling several centimeters beyond the sclera in the agarose gel, the DNA would migrate only 100-200 µm into the RPE or sensory retina. Thus, in a clinical setting, the times would be much shorter (as the DNA would have to travel less than a millimeter), about 5-10 min maximum in the patient setting, (versus travelling several centimeters total in the agarose gel, which takes much longer, about 2 h).

(B) In an analogous fashion, the total current would be much reduced in the patient, because the cross-sectional area of the agarose gel is quite large in our present experimental testbed, several square cm, while treatment area of sclera in the real patient setting would be about 1-2 square cm maximum area. While the surface of the human sclera is large, about 17 square cm, we would plan the use at any one time only at most 10% of the sclera surface area available. Thus, the total current would be reduced 90% just by this proportionality.

(C) Also, the current flow is in large part a function of the "buffer strength." We used 1X TAE Buffer, a buffer used mainly in the analysis of DNA fragment mobility in standard molecular biology laboratory settings, and we used it here in a "proof of principle" context. We selected 1X TAE Buffer because it is so common in DNA electrophoresis in the laboratory, and the properties of DNA migration in this buffer and in agarose are so well understood. This buffer is unlikely to be the optimum buffer for a clinical setting because it is a relatively high ionic strength buffer, and by reducing the ionic strength and, more importantly, changing its constituents and formulation, we expect to optimize buffer performance and greatly reduce current density for a clinical setting. Such a reduction in current density will be necessary as, if it is too great, burn damage, fibrosis, and necrosis can occur [21,30]. However, buffer optimization for clinical use is a subject of another future publication.

Large amounts of supercoiled, closed circular, double-stranded DNA could be delivered across the scleral fragments via electrophoresis. Transit across the sclera was rapid, taking place in less than 1 h. No apparent damage to the plasmids occurred as the plasmids before and afterwards had identical mobilities after extraction and recovery following initial electrophoresis. The substantial extent of dispersal of the plasmids following the scleral transit was not initially anticipated,

and our preliminary interpretation was that the plasmids had not crossed the sclera at all. This interpretation was experimentally challenged by excising a larger volume of agarose from the crossed side (inner surface of the sclera). DNA was extracted from this agarose and concentrated. Any DNA in this agarose would suggest that the plasmid had crossed the scleral fragments. The amount of DNA recovered was almost as much as was applied to the well, as determined from relative fluorescent intensities of the bands. Also, the size of the DNAs (whether the transited and recovered DNA or the original starting material) matched exactly by subsequent agarose gel electrophoresis.

Our guiding hypothesis predicted that small DNAs would migrate through the sclera. The data from Figure 2 through Figure 5 indicate that this prediction is true. We were surprised that larger DNA exhibited no clear size limit beyond which DNA would no longer pass through the sclera (as shown in Figure 4 and Figure 5). These results suggest that the sclera functions as a sieve of fibers not unlike the more characterized networks of fibers in agarose or polyacrylamide. The material of the scleral sieve exhibits some selectivity in that the two dyes, bromphenol blue and xylene cyanol, exhibit different behaviors in migrating through the sclera compared to the migration rates observed in agarose or acrylamide.

The absence of a size limit in the scleral sieve may imply that fragments of DNA much larger than we tested may pass through the sclera (or even other tissues or barriers). We speculate that, much as pulsed-field or field inversion gel electrophoresis allows very large DNA fragments to transit agarose pores, DNAs thought too large for regular sieving may pass through pores in the sclera if field inversion pattern and frequency are optimized. We plan to test this hypothesis in the future. We speculate that the use of field inversion techniques may allow much larger DNA to pass through the sclera and might yield more focused DNA bands following electrophoretic transfer. Field inversion may prevent the potentially problematic dispersal of the plasmid in the agarose during passage. The use of field inversion may increase the DNA concentration in the tissue or layer of interest.

As mentioned in the Introduction, the sclera represents just one of several barriers to the therapeutic delivery of large or small nucleic acids to the retina and other ocular tissues. However, it is encouraging that the present electrophoretic approach seems to be effective for trans-scleral delivery of several forms and sizes of nucleic acids. Further work is needed to evaluate strategies to optimize crossing other barriers and transfecting the nucleic acids into the target cells.

Although many steps and barriers remain to clinical application, other investigators are testing procedures for low current iontophoresis of several classes of drugs across the conjunctiva and cornea [37-39]. We suggest that the charged nature of DNA and RNA may make virtually any size nucleic acid an excellent drug for delivery by electric fields through porous tissues.

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