Clearance kinetics and clearance routes of molecules from the suprachoroidal space after microneedle injection

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Supplementary Information

Materials & Methods

Fluorescent labeling of monoclonal antibody

Bevacizumab (Avastin, Genentech, South San Francisco, CA) was fluorescently labeled with fluorescein isothiocyanate (FITC) using previously described methods. Briefly, 40 mg FITC was added to a 1.0 mL sodium bicarbonate (pH = 9) solution, and 3 mL of bevacizumab solution (25 mg/mL) was added to the FITC solution and stirred in the dark for 2 h at room temperature. The solution was dialyzed against phosphate buffered saline (PBS) using dialysis tubing with a cutoff of 30 kDa (Spectra/Cor, Spectrum Laboratories, Rancho Dominguez, TX). The PBS was changed daily for 5 days to remove unreacted FITC.

Determination of SCS collapse rate by ultrasonography

High-frequency ultrasound B-scan (U/S; UBM Plus, Accutome, Malvern, PA) was used to determine the rate of SCS collapse after a microneedle injection of 50 µL Hank’s Balanced Salt Solution (HBSS; Mediatech, Manassas, VA) into the SCS. Three sagittal views were acquired: (a) supranasal, over the injection site; (b) superior, 45° lateral to the injection site; and (c) nasal, 45° nasal to the injection site. Image acquisition occurred every minute for 10 min, and then every 2 min for 1 h.

Off-line post processing was performed on the U/S views to determine the SCS thickness. For each U/S image, a line segment 5 mm posterior to the scleral spur and perpendicular to the sclera was found. The line started at the outer surface of the sclera and ended at the inner surface of the retina, which were readily identifiable on the U/S images. The sclera and chorioretina were included in the measurement to ensure the line was perpendicular. SCS thickness was determined as the length of the line segment minus the thickness of the sclera and chorioretina. The characteristic time of SCS collapse was determined by modeling SCS thickness versus time as an exponential decay.

Determination of SCS clearance kinetics by fundus imaging

To study the effect of molecular radius on clearance from the SCS, a 50 µL microneedle injection of the following formulations were tested: [i] 0.025% (w/v) fluorescein sodium in HBSS; [ii] 0.5% (w/v) 70 kDa FITC–dextran in HBSS; [iii] 0.5% (w/v) 500 kDa FITC–dextran in HBSS; [iv] 0.5% (w/v) 2 MDa FITC–dextran in HBSS; [v] 1.5% (w/v) FITC–bevacizumab in HBSS; and [vi] 1% (w/v) 20 nm green-fluorescent particles (Excitation: 505 nm, Emission: 515 nm; FluoSpheres, Life Technologies, Carlsbad, CA) in HBSS. Prior to injection, FITC was tagged to bevacizumab using methods described above.

The clearance rate of injected fluorescent material from the SCS was estimated by taking fluorescence fundus images over time. Topical eye drops of tropicamide and phenylephrine (Akorn, Lake Forest, IL) were administered prior to each imaging session to dilate the eye. A RetCam II (Clarity Medical Systems,
Pleasanton, CA) with the 130° lens attachment and the built-in fluorescein angiography module was used to acquire the images. Multiple images were taken with the blue light output from the RetCam II set at 0.0009, 1.6, or 2.4 W/m². In an attempt to capture the entire interior surface of the ocular globe, nine images were captured: central, supranasal, superior, supratemporal, temporal, infratemporal, inferior, infranasal, and nasal. This allowed imaging into the far periphery. Imaging was done 3 min after injection, at 1 h, every 3 h for 12 h, and every other day post-injection for up to 28 days.

The relative concentration of the injected fluorescent molecules was estimated for each eye at each time point by comparing the average fluorescence intensity with aliquots of known concentrations imaged using the same lighting conditions for calibration. The characteristic times of SCS clearance rate and total clearance time were determined by modeling these processes as exponential decays in fluorescein concentration in the SCS over time.

**Intraocular pressure measurements**

A custom-designed pressure measurement system was used to measure pressure in the vitreous humor (VH) and in the SCS after either an IVT or SCS injection. The animal was terminally anesthetized with a subcutaneous injection of a ketamine and xylazine cocktail. A 33 gauge, 0.5 inch hypodermic needle was penetrated through the pars plana into the VH, and a 33 gauge microneedle was penetrated through the sclera 3 mm posterior to the limbus to access the SCS. Both needles were connected by polyethylene tubing (I.D. 1.14 mm, Becton Dickinson and Company, Sparks, MD) to a T-valve (i.e., to switch between the SCS and VH) that was in fluid communication with a second T-valve. The second T-valve allowed switching between either a 1 mL syringe (to inject HBSS into either the SCS or the VH) or a pressure transducer (Honeywell 142PC01, Morris Plains, NJ). The pressure transducer was calibrated against a static water column and zeroed to the height of the eye, and the height difference of the needle openings was also found (<10 mm height difference). A custom LabVIEW (National Instruments, Austin, TX) script was used to record the pressure trace.

A 50 µL SCS or IVT injection (N=4 per injection site) was made either in the SCS or the VH, respectively. The pressure in the SCS and VH was measured by switching the T-valve between the two sites every few minutes. The pressure was monitored until the pressures had reached their original baseline values from before the injection (i.e., ~15 mmHg). After the measurements, the animal was euthanized with a lethal dose of pentobarbital injected intravenously. A second set of SCS and IVT injections was made in the animal postmortem. In the postmortem measurements, pressure was only measured in the tissue space (i.e., SCS or VH) where the injection was made.

The characteristic times of elevated pressure in the SCS and VH were determined by modeling these processes as exponential decays in pressure over time.

**Collection of fluorescein by different clearance routes**

For this terminal experiment, the rabbit (N=4 eyes per group) was anesthetized with a subcutaneous injection of ketamine and xylazine before microneedle injection; additional injections were given every 30 min to maintain anesthesia. A subcutaneous injection of 60 mL saline was also given on the rump to counteract fluid loss. The amount of fluorescein exiting the eye by four routes was determined by collecting samples over time from the [i] sclera anterior to the equator; [ii] anterior sclera with injection site plugged; [iii] sclera posterior to the equator; and [iv] posterior sclera with vortex vein transected.
Prior to the microneedle injection, the conjunctiva in the supranasal quadrant was carefully dissected off the sclera. A 50 µL microneedle injection was performed supranasally 4 mm nasal to the superior rectus muscle and 3 mm posterior to the limbus. In the [i] anterior sclera and [iii] posterior sclera conditions, samples were collected every minute for 10 min, and every 2 min for 1 h by swabbing the space with a 1 cm x 1 cm paper tissue (Kimwipe, Kimberley-Clark, Irving, TX) for the entirety of time between each time point (i.e., one or two minutes). Care was taken to swab only anterior or posterior to the equator, depending on the condition. The tissue was then placed in 1 mL of HBSS until analysis.

To determine the amount of fluorescein leaving [ii] the anterior sclera with injection site plugged, a similar experiment was performed. Immediately post-injection, the site of injection was plugged by carefully applying cyanoacrylate glue (Loctite 4013, Düsseldorf, Germany) with a 30 gauge needle to the microneedle hub to seal the gap between the microneedle and surrounding scleral tissue. After the microneedle injection occurred, the microneedle was left in the eye. Other methods were the same as above.

For the eyes that had a vortex vein cut [iv], the superior vortex vein was transected prior to injection. Heparin (5 mL of 10,000 IU/mL; Hospira, Lake Forest, IL) was given intravenously prior to the start of the experiment to prevent coagulation. The vortex vein was found under the nasal edge of the superior rectus muscle. The rectus muscle was lifted off the ocular surface to expose the vortex vein. The vortex vein was confirmed by verifying its path, i.e., originating from within the sclera and traveling posteriorly along the ocular surface towards the optic nerve. A transfer pipette was used to collect the blood exiting the vortex vein every 1 min post-injection for 10 min, and every 2 min for 1 h. The volume of collected blood collected was recorded and HBSS was added to reach a final volume of 2 mL per sample for analysis.

Immediately after the last time point, all animals were euthanized with an injection of pentobarbital through the marginal ear vein. The eyes were enucleated to measure the amount of fluorescein remaining in the vitreous humor and within the tissue (including the SCS). An incision in the cornea was made so the aqueous humor, vitreous humor, and lens could be removed from the ocular globe and collected. The remaining ocular tissues (with undisturbed SCS) were collected. The Kimwipe paper tissue and ocular tissue samples were placed in HBSS at 4 °C for 2 days to allow the fluorescein to diffuse out and equilibrate with the HBSS. It is possible that fluorescein bound to the Kimwipe tissue paper but it should have done so equally for all conditions tested. The amount of fluorescein in all samples was measured using a multiplate reader (Synergy H4, BioTek, Winooski, VT) with parameters set to excitation = 494 nm and emission = 521 nm.

Further analysis was performed using Matlab (MathWorks, Natick, MA) and Prism (Graphpad, La Jolla, CA) software. The characteristic times of clearance rate via each route were determined by modeling these processes as exponential decays in fluorescein concentration over time.

**Modeling clearance from the SCS**

We developed a two-dimensional model (2D) of small-molecule transport after microneedle injection into the SCS to corroborate our experimental results. A Cartesian coordinate system was used such that the x-direction corresponded to the radial direction in a spherical coordinate system centered in the eye and the y-direction corresponded to the circumferential direction. This effectively ignored the curvature of the eye, an acceptable approximation over small distances as used in the model.
The convection-diffusion equation (Eq. 1) describes the unsteady transport of a species, and includes effects of diffusion (first term), convective flow (second term), and reaction or clearance (third term). The equation is as follows:

\[
\frac{\partial c}{\partial t} = \nabla \cdot (\nabla Dc) - \nabla \cdot (v_f c) - Bc
\]

Eq. 1

where \( c \) is the concentration of the species of interest, \( t \) is the time, \( D \) is the diffusion coefficient of the species through tissue, \( v_f \) is local fluid velocity, and \( B \) is a reaction or clearance coefficient.

Due to the complex ocular anatomy and different tissues, an analytic solution of the above equation was not sought. Instead, this model utilized a modified random walk algorithm to study the position and movement of molecules by displacing the molecule at each time point in 2D based on the characteristic diffusional length a molecule would move in that time period, as well as additional rules as defined below. We defined the regions in the eye model to be the choroid, SCS, and sclera, which were set as 0.1, 0.05, and 0.3 mm thick, respectively (Figure 6A(i)).

Rules governing molecule movement over each time step (\( \Delta t \)) of the random walk algorithm were as follows. The diffusivity within the choroid and sclera (both of which are limited largely by the proteoglycan extracellular matrix) was set to 5x10^{-11} m^2/s (diffusion coefficient of fluorescein in sclera), corresponding to a characteristic diffusional length of 0.1 mm in 1 min. The diffusivity in the SCS was 5x10^{-10} m^2/s (diffusion coefficient of fluorescein in water) corresponding to a characteristic diffusional length of 0.34 mm in 1 min.

Furthermore, we defined additional behaviors for regions in the model corresponding to ocular anatomy and physiology, in particular (region i) the choroid, (region ii) the sclera, and (region iii) a drainage pathway through the sclera analogous to the injection site (Figure 6A(ii)). The choroid (region i) was subdivided into an inner and outer region, corresponding to the choriocapillaris and Haller’s/Sattler’s layers, respectively. The choriocapillaris (as opposed to Haller’s and Sattler’s layers) possessed fenestrae capable of clearing molecules, which we modeled with a clearance rate \( B \), whereas we assumed that no clearance occurred in Haller’s and Sattler’s layers.

The sclera (region ii) was able to bind fluorescein (and prevent diffusion or convection) with second-order kinetics, as described by the following equation:

\[
C_b = \frac{B_{max} C}{K_D + C}
\]

Eq. 2

where \( C_b \) is the concentration of bound fluorescein (which had zero displacement during that time point), \( C \) is the concentration of free fluorescein (which had nonzero displacement) during that time point, \( B_{max} \) is the apparent maximum binding capacity of the sclera (experimentally determined to be 1440 \( \mu \)M by Lin et al.) and \( K_D \) is the apparent equilibrium dissociation constant (experimentally determined to be 110 \( \mu \)M by Lin et al.). Since the scleral binding of molecules was hypothesized to be mediated by collagen, no binding to the choroid was included in the model.

In addition, the sclera had convective flow directed outwards due to the pressure drop of the IOP across the sclera. We found the convective velocity using Darcy’s Law:

\[
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\[ v_f = -\frac{K_h}{\mu} \nabla P = -\frac{K_h}{\mu} \frac{dP}{dx} \]  
Eq. 3

where \( K_h \) is the hydraulic conductivity of the flow pathway medium (10\(^{-18}\) m\(^2\) for rabbit sclera\(^{11-13}\)), \( \mu \) is the viscosity of the fluid (7x10\(^{-4}\) Pa s for water at 37°C\(^{14}\)), \( P \) is the pressure and \( x \) is position in the radial direction. We only considered pressure changes in the radial direction and not in the circumferential direction. Because there was no (or very little\(^{15}\)) pressure differential between the vitreous humor and the SCS, there should be little pressure-driven flow into the choroid. The significant pressure drop was therefore between the SCS and the extraocular environment across the sclera. We could assume the pressure drops linearly across the sclera, i.e., across a representative distance of 300 μm (Ref \(^4\)). We used the experimental data (Figure 4) to describe the pressure drop over time as an exponential decay with a maximum of 100 mmHg and a time constant of ~5 min.

There was also convective flow via the drainage pathway (region iii), which had a y-dimension equivalent to the outer diameter of a microneedle (i.e., 0.25 mm), and had a convective flow two orders of magnitude greater than that in the rest of sclera.

Discussion

Sensitivity analysis of model predictions

To provide greater insight into the transport phenomena, we performed sensitivity analysis on the model to determine the relative influence of different parameters to the overall clearance from the eye (Figure S1). In general, the model predicted that small changes (within 1 order of magnitude) in parameter values did not significantly change the model results. The rank order of parameter sensitivity was, from most sensitive to least: diffusivity of the molecule in the SCS and in the tissue (Figure S1A), scleral binding capacity (Figure S1B) and equilibrium dissociation constant (Figure S1C), vascular clearance rate (Figure S1D), scleral hydraulic permeability (Figure S1E), size of leakage sites (Figure S1F), \( \tau_{\text{pressure}} \) (the rate at which elevated IOP dissipated, Figure S1G), and baseline physiological IOP (Figure S1H). Since the model suggested that the rate limiting step in SCS clearance was the trans-scleral transport, it stood to reason that the parameters that modified the trans-scleral transport rate affected the clearance time constant and total clearance time the most. Nevertheless, as evidenced by the graphs in Figure S1, the clearance time constant was a linear combination of the trans-scleral and vascular clearance time constants.

Diffusivity (D) had the greatest effect on clearance times (Figure S1A); for example, reducing D (the diffusivities of fluorescein) by one order of magnitude to one-tenth the diffusivity of fluorescein (0.1xD) resulted in a doubling of the clearance time constant. Intuitively, changes in diffusivity will have a significant effect on clearance time since the characteristic time for a molecule to move across the sclera (300 μm in the radial dimension) increases with the diffusivity. As seen in Figure S1A and in our experimental results (Figure 3D), the model correctly predicted nonlinearity in the clearance time at 0.01xD. Experimental data suggested that the nonlinearity occurs at >500 kDa, which should correspond to a diffusion coefficient of ~0.01xD based on the Stokes-Einstein equation.
Figure S1 – Sensitivity analysis of parameters used in theoretical model of molecule transport from the SCS. Parameters were varied at least one order of magnitude from the value used in the model (value used in base condition highlighted in gray). The clearance time constant (black square) and time to clear 99% (open squares) of molecules are plotted against each variable. In addition, the time constants for intravascular clearance ($\tau_{\text{blood}}$, triangle) and trans-scleral transport ($\tau_{\text{sclera}}$, open circle) were also found. Note that the y-axes of the first three graphs (A–C) are on a different scale than the others (D–H).

Scleral binding of molecules (as modulated by $B_{\text{max}}$ and $K_D$; Figure S1B and C, respectively) also had a major effect on clearance times. Intuitively, increasing scleral binding effectively behaved like a decreased diffusivity by drastically slowing molecule transport. However, scleral binding affected molecule transport on short time scales (before saturating the sclera) whereas diffusivity equally...
affected transport at all times. Unlike the results seen with changing D, changes in $B_{\text{max}}$ and $K_D$ had a biphasic effect whereby small changes resulted in almost no change in clearance time but a large change caused a disproportionate increase in clearance time. This could be due to changes in the binding saturation point of the sclera.

The choroidal clearance rate had a moderate effect on characteristic clearance time constant and a minor effect on total clearance time (Figure S1D). At high clearance rates by choroidal vasculature, the choroidal perfusion contributed at most 50% of total clearance of molecules from the eye. We reasoned that this 50% made sense since molecules leaving the SCS have an approximately 50% chance to enter the choroid (as opposed to the sclera). With clearance rates set to low or nonexistent (time constants excluded in Figure S1D), most or all molecules left via trans-scleral pathways, which set an upper limit on the clearance time (i.e., it took ~25 h for all molecules to leave the eye trans-sclerally).

The scleral hydraulic permeability (Figure S1D) and size of the leakage pathways (Figure S1E), had a moderate effect on clearance time. These parameters were indirectly related to trans-scleral transport. Parameters that increased the convective velocity ($T_{\text{pressure}}$ and physiological baseline IOP) had a modest effect on clearance time. This suggested that convective flow through the sclera was not a major contributor to clearance.

Of the parameters tested, diffusivity and size of the leakage site could be changed by optimizing formulation or microneedle injection technique, respectively and thereby modulate clearance rate. Diffusivity was the most sensitive variable and changes by an order of magnitude could change the clearance time constant from 5 min to 18 h. The Stoke-Einstein equation indicated that diffusivity could be modulated by changes in formulation viscosity and the molecular radius (which is in turn a function of molecular weight). In addition, using a smaller-bore microneedle did have a moderate effect on the clearance times. On the other hand, changes in physiological parameters (e.g., physiological IOP, scleral hydraulic conductivity, and clearance rate) did not drastically change the behavior of the model, and clearance changed by less than 2-fold with up to 10-fold changes in the parameter value. This suggests that the model may be applicable to other species, like humans.

**Limitations of the model**

A limitation of the model was that it only investigated 2D transport using Cartesian coordinates, although actual clearance processes occurred in three dimensions in spherical coordinates. This limited the ability of the model to predict distribution. To simplify the model, variation in SCS area and thickness were not accounted for, as these additional considerations would complicate the model with dynamic boundary conditions. Many other simplifying assumptions were also employed, such as no convective flow simulating the process of injection, no aqueous turnover via uveoscleral outflow\(^{16}\), and no influences of biomechanical tissue properties. Despite these limitations, model predictions were in general agreement with experimental data.
References