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Influence of molecular shape, conformability, net surface charge, and tissue interaction on transscleral macromolecular diffusion

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ABSTRACT

Purpose: To study the influence of molecular shape, conformability, net surface charge and tissue interaction on transscleral diffusion.

Methods: Unfixed, porcine sclera was clamped in an Ussing chamber. Fluorophore labelled, neutral, albumin, dextran, or ficoll were placed in one hemi-chamber and the rate of transscleral diffusion was measured over 24 hours using a spectrophotometer. Experiments were repeated using dextrans and ficoll with positive, or negative, net surface charges. Fluorescence recovery after photobleaching

(FRAP) was undertaken to compare transscleral diffusion with diffusion through a solution. All molecules were 70 kDa.

Results: Using FRAP, mean $\pm$ SD diffusion coefficient (D) was highest for albumin, followed by ficoll, then dextran ($p = 0.0005$). Positive dextrans diffused fastest, followed by negative, then neutral dextrans ($p = 0.0005$). Neutral ficoll diffused the fastest, followed by positive then negative ficoll ($p = 0.0008$). For the neutral molecules, transscleral D was highest for albumin, followed by dextran, then ficoll ($p < 0.0001$). D was highest for negative ficoll, followed by neutral, then positive ficoll ($p < 0.0001$). By contrast, D was highest for positive dextran, followed by neutral, then negative dextran ($p = 0.0021$).

Conclusions: Diffusion in free solution does not predict transscleral diffusion and the molecular-tissue interaction is important. Molecular size, shape, and charge may all markedly influence transscleral diffusion, as may conformability to a lesser degree, but all need to be considered when selecting or designing drugs for transscleral delivery.

Drugs directed against vascular endothelial growth factor (VEGF) have proven efficacy in some of the most common retinal diseases. The ANCHOR and MARINA studies demonstrated efficacy in wet age-related macular degeneration (AMD),^{1,2} and studies such as CRUISE, BRAVO^{3,4} and several from the DRCnet Group have shown favourable results in retinal vein occlusion and diabetic macular oedema. Whilst the data in these studies have led to improved clinical care, the need for regular intravitreal injections represents a burdensome regimen of treatment. A less invasive mode of delivery would have potential advantages in terms of reduced cost, discomfort, and complication rate.

Systemic administration is impeded by the blood-aqueous and blood–retinal-barriers. High drug concentrations may be required to overcome these barriers to diffusion, with the attendant risk of systemic side effects.⁵

A topical route would have obvious advantages. Delivery to the retina is however difficult for several reasons, such as lacrimation, low corneal permeability to large molecules, counter directional intraocular convection, and importantly the long diffusional distance.⁵³ Blood flow in the conjunctiva, episclera and choroid can also reduce drug concentration.^{33,50}

Notwithstanding these potential difficulties, transscleral delivery has a number of potential advantages.^{6,7,8,9} The sclera has a large and accessible surface area, and a high degree of hydration, making it conducive to water-soluble substances. It is also hypocellular and thus the proteolytic enzymes are less likely to degrade drugs.

The scleral matrix consists of collagen and elastin fibres and the arrangement of these fibres is thought to influence drug diffusion. The posterior scleral fibres are less tightly packed than the anterior sclera. The scleral fibres tend to have a lateral orientation, and this may explain why diffusion is slower in the transverse direction³. The proteoglycans in the scleral matrix are negatively charged, and this may explain why negatively charged solutes may diffuse faster across the scleral matrix than positively charged solutes, which may be bound to the negatively charged matrix.^{4,5} Several authors have shown that the sclera is more permeable to hydrophilic molecules than lipophilic ones.⁴⁰⁻⁴²

Inomata studied the transscleral diffusion of radio- or dye-labelled albumin, and a 40 kDa red dextran following injection into the suprachoroidal space of rabbits. Both substances were detected in the extra-ocular tissues. Subsequent experiments demonstrated that thorotrast

particles of 10 nm diameter were able to cross the sclera in the cynomolgus monkey, suggesting that the sclera contains relatively large pores.⁴³

These experiments suggest that it would be possible to deliver relatively large drugs across the sclera and *in vivo* experiments subsequently have provided proof in principal. Ambati *et al* used an osmotic pump to deliver high molecular weight (MWT), fluorescently labelled IgG (150 kDa) across rabbit sclera.⁸ The pump was secured against bare sclera, and resulted in relevant concentrations in the choroid and retina for up to four weeks, with minimal systemic absorption. Further experimental studies have shown transscleral delivery of various macromolecules including PLGA microspheres containing an anti-VEGF RNA aptamer (EYE001),¹⁰ 70-kDa dextrans,¹¹ and FITC-albumin.¹² Industry backed clinical trials of topical anti-VEGF drugs are now underway (clinicaltrials.gov identifier: NCT01362348).

There are several drug-related factors that may influence diffusion across biological tissue, including MWT, shape, net surface charge, conformability, and water solubility, amongst others. The effect of MWT has been relatively well studied in the sclera, but there are fewer studies investigating surface charge, and to date no studies specifically examining the influence of molecular shape and conformability.

The polysaccharides ficoll and dextran have been extensively used in renal research as molecular probes to demonstrate permselectivity across a glomerular membrane. They are used largely due to their inert nature. When comparing dextrans and ficoll to other molecules of an equivalent *in vitro* Stokes-Einstein (SE) radius both molecules are hyperpermeable, and it has been proposed that this is due their relative conformable nature.²²

Lavrenko *et al* studied the mass of ficoll, its viscosity, and their effect on the diffusion coefficient in the kidney.²³ Bohrer *et al* used light scattering techniques and suggested that ficoll was shaped more like a sphere than a rod.²⁴ Ohlson *et al* and Oliver *et al* reached the same conclusion.^{25,26}

Venturoli *et al.* reviewed the literature and concluded that ficoll also exhibits "nonideal" properties due to an open, deformable structure and thus that it deviates from an ideally hard sphere in nature.² More recently, Fissel *et al* used multi-angled light scattering (MALS) to study the shape and conformability of ficoll. He proposed that ficoll may behave more like a random coil than a sphere, or it might be a mixture of both. He also considered that ficoll may

have many intermediate shapes, or that it may have the ability to change its shape under pressure.²⁷

Dextrans are thought to be linear, flexible molecules, with a loose, open structure, and a high degree of deformability, which renders them more permeable than a more rigid globular protein such as albumin, and indeed even more permeable than ficoll, at least with respect to the glomerulus.²²

These studies indicate that, in combination, dextran, ficoll and albumin serve as useful molecules with which to study the effect of shape and conformability on diffusion across tissue.

This study explores the transscleral diffusion of dextrans, ficoll, and albumin of the same MWT, and compares the results to diffusion in a free solution using fluorescence techniques. In particular, this study investigates factors other than MWT that might influence diffusion, namely net surface charge, molecular shape, and the degree of molecular conformability. By comparing transscleral diffusion to that in free solution it is possible to determine the intrinsic diffusional characteristics of each molecule, and how this might change when interacting with scleral tissue. Identifying factors that influence transscleral diffusion will assist in the design and selection of drugs that can cross the sclera to treat posterior segment disease.

METHODS

All reagents were labelled with fluorescein isothiocyanate (FITC). Neutral bovine serum albumin (Sigma-Aldrich, Poole, UK), neutral dextran and neutral ficoll were used to assess the effects of shape on diffusion. In a separate study, molecules of differing net surface charge were prepared, to determine the effect of charge on diffusion. Two molecules were selected, in case the effect of charge differed across molecules, and the results in one might not therefore be generalizable to others. Neutral, cationic and anionic FITC-dextrans and FITC-ficoll were designed and supplied for this study by TDB Consultancy, Sweden. The anionic reagents were carboxymethylated (CM) and the cationic agents were prepared using a diethylaminoethyl (DEAE) compound. The substitution levels were 5% for CM groups and 3-4% for DEAE groups. The same reagents were used for both transscleral diffusion, and

diffusion in free solution. All experiments were conducted at 20°C. Ficoll, dextran, and albumin all had a MWT of 70 kDa.

FREE DIFFUSION IN SOLUTION

Fluorescence recovery after photobleaching (FRAP) was used to determine the diffusion of molecules in free solution, to determine the intrinsic diffusional characteristics of each molecule. Test molecules were prepared in PBS at concentration of 1mM. Each 1mM concentration of test reagent was combined with 4 different viscosities of glycerol (Sigma-Aldrich) to create a variable viscosity gradient for the experiments.

A mean recovery time and diffusion coefficient was calculated for each viscosity. This data was plotted as a scatter chart of inverse viscosity ($\text{Pa}^{-1} \cdot \text{s}^{-1}$) against diffusion coefficient ($\text{cm}^2 \cdot \text{s}^{-1}$) and from this the diffusion coefficient in water ($1.003 \times 10^{-3} \text{ Pa} \cdot \text{s}$ at 20°C) was extrapolated.

The FITC-labelled molecules were inserted into 300 μL wells (GE Healthcare, Amersham, UK) and FRAP was undertaken using three different bleach areas.

For a circular bleach area the fluorescence recovery time, τ_d , can be calculated by a monoexponential fit of the recovery curve. The relationship between τ_d and the translational diffusion coefficient, D , is given in equation 1:

$$1. D = \gamma w^2 / 4\tau_d^{30}$$

Where γ is a constant and w the bleach radius.

All experiments were undertaken using an inverted confocal laser scanning microscope (Leica TCS SP2 Wetzlar, Germany). In all individual FRAP experiments the sample was measured using a low intensity ($<1\text{mW}$) continuous Ar-ion laser at 488nm through a 63x water immersion objective (Leica) with numerical aperture of 1.2. Ten pre-bleach scans were measured at low intensity, followed by 1 bleach scan in a region of interest (ROI) using the same laser at high power ($\sim 4\text{mW}$) and then 100 post-bleach scans of the sample.^{31,52} This was repeated six times for each sample viscosity. A bleach radius of 7.5, 10 and 12.5 μm was initially selected for the experiments but raw images were used to calculate the radius as explained later. The intensity in the ROI was monitored as a function of time during the scan

sequence and the resulting recovery curve was fitted to a simple single-exponential model from which the translational diffusion coefficient was determined using equation 2:

$$2. \quad I(t) = A(I - e^{-kt}) + C^{30}$$

Where I is the fluorescence intensity, A is the final plateau intensity after recovery minus the initial intensity after bleaching, that is $F_{\infty} - F_0$, C is F_0 and k is a time constant ($\tau_{1/2} = \ln 2/k$)³⁰ (Figure 1). A high specification software package (Origin 8, OriginLab, Northampton, USA) was used to facilitate calculations.

The line scan speed for each experiment was 800 Hz and images were recorded with 512 x 512 pixels such that the acquisition for each frame, including the bleach frame, was ~0.82 s.

The ratio of bleach time to diffusion time was less than 1:15 for all experiments.³² A subtraction of background intensity was incorporated to correct for any external fluorescence intensity loss during the experiments. Experiments were repeated six times for each bleach area, with three bleach radii per viscosity and four different viscosities per molecule.

As shown in the literature, underestimated values for D are often obtained and can vary depending on bleaching spot sizes,^{34,35} thought to be due to long scanning times of the confocal laser scanning microscope when viewing fast diffusing molecules³⁶; this was minimised by using the raw bleach images to obtain the bleach radius. Images extracted from the Leica software were analysed with a line profile using Image J (Image J 1.45s, Wayne Rasband, National Institute of Health, USA) across the bleach area in 10 different orientations going through the centre. The 10 diameters were then averaged and divided by 2 to produce a radius used for further calculations.

Our data for albumin were used to validate the methodology as the diffusion coefficient and radius of albumin are well documented in literature. Table 1 demonstrates the results for albumin diffusion coefficients calculated for each bleach radius at each viscosity. The plot for this is demonstrated in Figure 2. The gradients for these three different bleach radii were substituted into equation 3 below:

$$3. \quad D = KT / 6\mu\pi R$$

Where D is the diffusion coefficient, K is the Boltzmann constant, T the temperature in Kelvin, μ the viscosity and R is the radius of the molecule.

Experiments were conducted at 20 °C (293.15 K). The diffusion coefficient (and permeability coefficient) was adjusted to 37 °C using Einstein's relationship (equation 4), to allow comparison with others' data, if required:

$$4. D_{37} = D_{20} (T_{37} / T_{20}) (\mu_{20} / \mu_{37})$$

Where,

D_{20} and D_{37} = diffusion coefficient at 20 °C and 37 °C ($\text{cm}^2.\text{s}^{-1}$).

T_{20} and T_{37} = absolute temperature in Kelvin at 20 °C and 37 °C.

μ_{20} and μ_{37} = viscosity of water at 20 °C ($1.003 \times 10^{-3} \text{ Pa.s}$ at 20 °C) and 37 °C ($0.692 \times 10^{-3} \text{ Pa.s}$).

Statistical tests

Mean values of each of the three comparison groups were tested using nonparametric ANOVA (Kruskal-Wallis), using SPSS (Version 20, IBM, Portsmouth, UK). Comparisons across two groups were undertaken using Mann-Whitney. A p value less than 0.05 was considered significant.

TRANSSCLERAL DIFFUSION

Species selection and tissue preparation

Porcine eyes were selected as the most appropriate animal model. Compared to primate, murine, feline, and bovine sclera, porcine sclera has greater homology to human sclera. Studies have shown its similarity in terms of both its anatomic thickness, and ultrastructure.⁷ In particular, porcine sclera has a similar collagen organisation. Since the interfibrillary spaces may define pore size this fact may be important when studying transscleral

permeability. Studies in retina also suggest the pig may be an appropriate model for studies of human ocular diffusion.¹⁴

The extra-ocular muscles were dissected free and the anterior segment was removed by cutting circumferentially behind the limbus. The globe was then cut into two halves and the vitreous, episclera, and choroid were carefully dissected away using fine forceps and spring scissors. The underlying RPE was carefully removed and the inner surface of the sclera was rubbed gently with a cotton tipped swab. The equatorial sclera was then dissected into multiple, full-thickness, 8 by 9 mm specimens, avoiding any scleral emissaria. The scleral thickness was measured for each specimen using a digital Vernier calliper (PR5638, Toolspot, Tiverton, UK). In accordance with other researchers,^{7,14} specimens were frozen at -80°C and then defrosted when required.

Diffusion testing apparatus

Sclera was clamped in an Ussing chamber.¹³ The circular interchamber aperture measured 6 mm in diameter. The two hemi-chambers were locked together using locating screws that were tightened using the minimum force necessary to prevent leak, and yet preserve the integrity of the tissue used. Previous studies have demonstrated the integrity of tissue clamped in this Ussing chamber.^{13,15,16,17}

A small glass encased magnetic stirrer was added to each hemi-chamber. Previous studies have demonstrated that stirring does not increase the scleral permeability of high MWT compounds.^{18,19} The top of each hemi-chamber was sealed by insulation tape to avoid evaporative loss, but the tape was pierced by a needle to prevent a pressure gradient from building across the chambers if the fluid levels differed in any way. The samples were protected from light for the duration of the experiments, which were conducted at room temperature (20°C).

The hemi-chamber facing the orbital surface of the sclera was filled with 1 ml of phosphate-buffered saline (PBS; sodium chloride 137 mM, potassium chloride 2.7 mM, phosphate buffer 10 mM, pH 7.4 at 25°C; Sigma-Aldrich), with streptomycin 100 mg.L⁻¹ (Sigma-

Aldrich). The other hemi-chamber was filled with 1 ml of PBS and the FITC labelled test molecule at 0.1 mM.

Measurement of diffusion

Diffusion was tested over 48 hours. The concentration in the recipient chamber was determined using a nanodrop spectrophotometer (Nanodrop 2000, ThermoScientific, Wilmington, DE), measuring absorption at 490 nm. The scleral sample was removed and examined to ensure it remained intact at the end of the experiment. The concentration of each sample was calculated from the linear portion of predetermined standard curves, using linear regression analysis.

Calculation of diffusion coefficient and permeability coefficient

The calculation of the diffusion coefficient (D)($\text{cm}^2.\text{s}^{-1}$) is based on Fick's first law (equation 5):

$$5. \quad D = \frac{R.L}{A.C}$$

Where,

R = diffusion flux (nanomoles. s^{-1})

L = scleral thickness (cm)

A = surface area of interchamber aperture (cm^2)

C = concentration gradient (as the concentration in the receiver chamber was typically about 1% of that in the donor chamber, the concentration in the donor chamber was taken as the concentration gradient, in nanomoles/ cm^3)

The relationship between diffusion coefficient (D) and permeability coefficient (P) is given by equation 6:

$$6. \quad D = \frac{P.L}{K}$$

Where,

K = partition coefficient (0.62 at ambient and body temperature)²⁰

L = Scleral thickness (cm).

Statistical tests

Mean values of each of the three comparison groups were tested using nonparametric ANOVA (Kruskal-Wallis test with Dunn's multiple comparison test), using InStat (GraphPad, La Jolla, CA). Comparisons across two groups was undertaken using Mann-Whitney. A p value less than 0.05 was considered significant.

DYNAMIC LIGHT SCATTERING AND LASER DOPPLER VELOCIMETRY

To establish the net surface charge of molecules, samples were analysed using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) that uses laser Doppler velocimetry (LDV) to measure the zeta potential of particles in a solution. The system passes a 633nm laser through the electrically charged sample to measure the velocity of particles in an applied electrical field of known value. Samples were diluted in PBS to a final concentration of 5mg/1mL, to avoid multiple scattering, and placed in a disposable zeta cell for measurement. A 4-mW He-Ne 633 nm laser was shone onto the sample and the intensity of light scattered at an angle of 173° was detected by an avalanche photodiode.

RESULTS

FREE DIFFUSION IN SOLUTION

The radius of albumin at the three different bleach radii were calculated to be 3.42, 3.31 and 3.13 nm, giving a mean value of 3.29 ± 0.15 nm. At the viscosity of water, at 20 °C, the diffusion coefficients calculated from the three line equations were 6.27, 6.48 and 6.85×10^{-7} cm².s⁻¹, with a mean value of $6.53 \pm 0.025 \times 10^{-7}$ cm²s⁻¹. These values are in agreement to a published radius of 3.0, 3.5 and 3.59nm,^{37,38,51} and a diffusion coefficient³⁹ in water at 25°C of

$6.40 \pm 0.40 \times 10^{-7} \text{ cm}^2.\text{s}^{-1}$ ($5.59 \pm 0.35 \times 10^{-7} \text{ cm}^2.\text{s}^{-1}$ at 20 °C based on Einstein's relationship). The data and plot are shown in Table 1 and Figure 2 respectively.

Tables 2 to 4 summarise the diffusion coefficients of all molecules in PBS, at 20°C and 37 °C. Figure 3 shows combined results in PBS at 20 °C.

Of the neutral molecules, the diffusion coefficient was highest for albumin, followed by ficoll and then dextran ($p = 0.0005$) (Figure 3). Albumin diffusion was significantly faster than dextran ($p = 0.002$) but only marginally faster than ficoll. The difference between dextran and ficoll also reached significance ($p = 0.002$).

Alterations in the surface charge of dextrans significantly altered the diffusion coefficient ($P = 0.0005$), but the magnitude of difference was small (Figure 3). Positive dextrans diffused the fastest, followed by negative then neutral dextrans. Individual comparisons across all three groups were significant (all $p < 0.05$).

By contrast, alteration in the surface charge of ficolls produced a large magnitude of difference ($P = 0.0008$), and the pattern of effect was exactly opposite, in that negative molecules diffused faster than positive molecules (Figure 3). Individual comparisons across all three groups were significant (all $p < 0.05$).

TRANSCLERAL DIFFUSION

Diffusion Coefficients

Figure 3 shows the diffusion coefficients in sclera, alongside those in free solution.

Of the neutral molecules, the diffusion coefficient was highest for albumin, followed by dextran and then ficoll ($p < 0.0001$). Albumin diffusion was significantly faster than both ficoll and dextran ($p < 0.001$ for both), but the difference between dextran and ficoll did not reach significance.

Positive dextrans had a higher diffusion coefficient than neutral dextrans, which were higher than negative dextrans ($p = 0.0021$). There was a significant difference comparing positive

and negative dextrans ($p < 0.01$), but the difference did not reach significance when comparing neutral dextrans with either positive dextrans or negative dextrans.

Negative ficoll had a higher diffusion coefficient than neutral ficoll ($p < 0.01$), and positive ficoll ($p < 0.001$). Neutral ficoll had a faster diffusion than positive ficoll ($p < 0.01$).

Permeability Coefficients

Figure 4 shows the permeability coefficients in sclera. The results showed a similar pattern to the transscleral diffusion coefficients.

The mean permeability coefficient of neutral molecules was highest for albumin, followed by dextran and then ficoll ($p < 0.0001$). The difference between albumin and dextran, and between albumin and ficoll were significant ($p < 0.001$ for both), but the difference between dextran and ficoll did not reach significance.

Whereas negative ficoll showed faster diffusion across sclera than positive ficoll, the results were reversed in the dextran studies. Positive dextrans had the highest mean permeability coefficient, followed by neutral dextrans and then negative dextrans ($p = 0.0043$). Negative dextrans had significantly slower diffusion than both neutral dextrans ($p = 0.0036$) and positive dextrans ($p = 0.0055$). The difference between neutral and positive dextrans did not reach significance.

The permeability coefficient of neutral ficoll was compared to that of negative ficoll and positive ficoll. Negative ficoll had the highest permeability coefficient, followed by neutral ficoll and then positive ficoll ($p < 0.0001$). The difference was significant across all intergroup comparisons ($p = 0.0017$ to $p < 0.0001$).

DYNAMIC LIGHT SCATTERING AND LASER DOPPLER VELOCIMETRY

Results are summarised in Table 5. As shown in this table, both the molecules designed to be neutral possessed a negative charge, but this was not as negative as the molecules designed to be negatively charged. This observation concurs with other researchers.²² It is known that FITC holds an approximately -1mV charge,⁴⁵ hence FITC-labelled molecules are often

slightly more anionic than unlabelled molecules, with the final charge depending on the ratio of labelling.

DISCUSSION

This study was designed to investigate the effect of net surface charge, shape, conformability and tissue interaction on the diffusion of macromolecules across the sclera. Albumin, ficoll and dextrans were selected as test agents not because they have therapeutic potential, but because their molecular configuration may offer insight into the factors that influence transscleral diffusion. In all cases the MWT of each molecule was 70 kDa.

Diffusion in free solution

Initial FRAP experiments were undertaken to determine the diffusion in free solution, independent of any tissue interaction. These studies were designed to show the intrinsic diffusional characteristics of each molecule. Neutral albumin and ficoll had similar rates of diffusion, and both were faster than dextran. These diffusion coefficients predict a molecular radius of 3.29 nm, 3.39 nm and 5.35 nm for albumin, ficoll and dextran respectively. Reported values in the literature are 3.0 to 3.5 nm,^{37,38,51} 1 to 7 nm,⁸ and 6.4 nm,⁴⁸ respectively. The similarity between albumin and ficoll suggests that, in solution, ficoll exists as a sphere.

When dextrans were manufactured with a positive or negative charge, there was relatively little change in their diffusion coefficient, whereas either an anionic or cationic charge slowed the diffusion of ficoll to an appreciable degree. This suggests that a net surface charge may alter the size of ficoll, most likely changing it from a compact sphere to perhaps a branching dendrimer or random coil.²⁷ This is consistent with reports showing the ficoll becomes more open when a negative charge is added.²⁸ This is most likely due to the opposing negative electrostatic charges repelling each other, forcing the structure to open. Given that neutral dextrans exist in a relatively open structure, they may have been less likely to change their configuration in response to an altered surface charge.

Effect of molecular size and shape on scleral permeability

Several authors have shown that scleral permeability declines with an increasing MWT,^{6,7,8,21} but others have postulated that molecular radius is a better predictor of permeability.⁸ It has been reported that the globular protein albumin, with a MWT of 69 kDa and radius of 3.5 nm, has higher scleral permeability than a linear dextran of 40 kDa and 4.5 nm radius.⁵ Ambati *et al* also showed that rabbit sclera is more permeable to bovine serum albumin and IgG than to dextrans of comparable MWT.⁸

These results are consistent with our findings. Our data suggest that the maximum molecular radius is a better predictor of scleral permeability than MWT. Hence small globular proteins such as albumin diffuse across the sclera more effectively than branching or linear molecules with significant asymmetry in their shape.

To our knowledge, this is the first time ficoll has been used to study ocular diffusion. Ficoll has however been used by several authors to investigate glomerular permselectivity. Ventruli and Rippe reviewed the diffusion of dextrans, albumin and ficoll across the glomerular membrane of the kidney.²² Unlike the results of the present study, they noted that ficoll and dextran diffused quickly across the glomerulus. They speculated that this was due to the linear shape of dextrans, with high molecular-size-asymmetry, and the deformability of both dextrans and ficoll.

It is interesting to speculate as to why the sclera behaved differently to the glomerulus, in that dextrans and ficoll both diffused across the sclera more slowly than albumin. The glomerular capillary wall is characterised by a network of small (3.7-5.5 nm) and large (10 nm) pores, with a thickness of 200-400 nm.²² By contrast, the sclera is composed of numerous layers of meshwork fibres and a mean thickness of 0.53 mm.⁴⁶ Although some studies have shown that molecules as large as 10 nm can traverse the sclera,⁴³ others suggest a pores size of 5 nm.⁴⁷ Therefore, although the pore sizes may be comparable to the glomerulus, the pathlength for diffusion is much higher in sclera. It is possible that this entraps linear or branching molecules such as dextran and ficoll. Another possibility is that the large hydrostatic pressure gradient that exists across the glomerulus, to enable filtration, also exerts a force that compresses both dextran and ficoll, exploiting their conformable nature and enhancing clearance. Such a gradient did not exist in the present experimental set up, and would not exist in the context of transscleral drug delivery.

Net surface charge

To determine if net surface charge alters diffusion across the sclera, molecules of negative, neutral and positive net surface charge were manufactured. To ensure that findings were not peculiar to a given molecule, both dextran and ficoll were tested.

It is generally held that negatively charged molecules diffuse faster across biological barriers than positive or neutral molecules. This stems largely from the renal literature, and in particular, studies using ficoll. Asgeirsson *et al* demonstrated an increased permeability of the glomerular capillary wall to negatively charged carboxymethylated ficoll, relative to neutral ficoll.²⁸ This occurs because the negatively charged ficoll becomes larger,²⁸ and whilst the increased molecular radius might be anticipated to slow diffusion across tissue, the molecule instead becomes less dense and consequently more flexible (compressible), making it hyperpermeable across the glomerular barrier. By contrast, dextran did not show this marked increase in hyperpermeability when carboxymethylated. This suggests that charge has an indirect effect on permeability by altering the structure of the molecule and its conformability, rather than a direct effect as was previously assumed.

Scleral tissue is known to have a negative charge.⁴⁷ In general, this would be expected to slow the diffusion of positively charged molecules, which may be retained in the scleral substrate. Unexpectedly, this was not the case with cationic dextrans in the present study. The diffusion coefficient of negatively charged ficoll was very high and it is interesting to speculate whether or not this was simply due to the charge itself, of whether the combination of a negatively charged molecule and negatively charged environment somehow resulted in ficoll assuming its more spherical shape.

Conclusion

The present study investigated the effect of molecular shape, charge, and conformability. Of the three variables studied, it seems that shape is the most critical, followed by charge, and then conformability. Diffusion in free solution does not reliably predict how molecules will diffuse across the sclera, indicating that the molecule's interaction with sclera tissue is important. An understanding of the variables that influence scleral permeability may guide the selection or design of drugs for transscleral drug delivery.

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FIGURE LEGENDS

Figure 1. The top schematic showing a typical FRAP curve. Region *a* is the initial fluorescence intensity of unbleached solution in the region of interest. Region *b* occurs after photobleaching, which reduces the measured fluorescence. As unbleached molecules diffuse into the region of interest the fluorescence increases(*c*), which continues to show the recovery and once *d* is reached there is a stabilisation in the recovery and the curve flattens. The increase in fluorescence after photobleaching reflects the speed with which molecules diffuse into the bleached area, and can be used to determine the diffusion coefficient. The insert shows a representative example, using neutral albumin.

Figure 2. The graph plots the diffusion coefficient of albumin for each bleach area and over a range of different viscosities. The gradient of the lines of best fit, forced through the y axis at 0, was used to calculate the diffusion coefficient of the 70kDa albumin molecule. The mean predicted molecular radius of $3.29 \pm 0.15\text{nm}$ fits with the reported size of albumin, suggesting that the methodology was appropriate for this, and by extension, other molecules.

Figure 3. Graph shows the mean diffusion coefficient of all molecules in solution, calculated at the viscosity of water, and measured at 20°C. The darker gray columns show the results in sclera, also at 20°C. Statistical comparisons are provided in the text.

Figure 4. Graph shows the permeability coefficients of all molecules, in sclera, at 20°C. Statistical comparisons are detailed in the text.

TABLES

Table 1. Albumin diffusion coefficient in relation to viscosity and bleach radius.

Inverse viscosity ($\text{pa}^{-1}.\text{s}^{-1}$)	20.03			14.01			10.82			8.77		
Radius (μm)	10.47	12.05	14.59	10.47	12.05	14.59	10.47	12.05	14.59	10.47	12.05	14.59
Diffusion coefficient $\pm$ SD $\times 10^{-8} \text{ cm}^2\text{s}^{-1}$	1.42 $\pm$ 0.01	1.30 $\pm$ 0.08	1.25 $\pm$ 0.08	0.91 $\pm$ 0.04	0.92 $\pm$ 0.05	0.87 $\pm$ 0.05	0.74 $\pm$ 0.01	0.70 $\pm$ 0.05	0.69 $\pm$ 0.05	0.58 $\pm$ 0.02	0.56 $\pm$ 0.02	0.57 $\pm$ 0.02

Table 2. Diffusion coefficients of neutral molecules in solution and sclera.

Neutral Molecules	Diffusion in Solution		Transscleral Diffusion			
	Diffusion coefficient $\pm$ SD, 20°C ($\times 10^{-7} \text{ cm}^2\text{s}^{-1}$)	Diffusion coefficient $\pm$ SD, 37°C ($\times 10^{-7} \text{ cm}^2\text{s}^{-1}$)	Diffusion coefficient $\pm$ SD, 20°C ($\times 10^{-7} \text{ cm}^2\text{s}^{-1}$)	Diffusion coefficient $\pm$ SD, 37°C ($\times 10^{-7} \text{ cm}^2\text{s}^{-1}$)	Permeability coefficient $\pm$ SD, 20°C ($\times 10^{-6} \text{ cm}^2\text{s}^{-1}$)	Permeability coefficient $\pm$ SD, 20°C ($\times 10^{-6} \text{ cm}^2\text{s}^{-1}$)
Albumin	6.53 ± 0.025	10.01 ± 0.038	3.88 ± 2.62	6.08 ± 4.11	2.82 ± 1.30	4.15 ± 1.91
Dextran	4.01 ± 0.065	6.15 ± 0.10	1.63 ± 1.18	2.44 ± 1.77	1.24 ± 0.64	1.86 ± 0.96
Ficoll	6.33 ± 0.082	9.71 ± 0.13	1.32 ± 0.49	1.92 ± 0.71	0.88 ± 0.35	1.37 ± 0.54

Table 3. Diffusion coefficients of neutral, positive and negatively charged dextran.

Dextran Molecules	Diffusion in Solution		Transscleral Diffusion			
	Diffusion coefficient $\pm$ SD, 20°C (x10 ⁻⁷ cm ² s ⁻¹)	Diffusion coefficient $\pm$ SD, 37°C (x10 ⁻⁷ cm ² s ⁻¹)	Diffusion coefficient $\pm$ SD, 20°C (x10 ⁻⁷ cm ² s ⁻¹)	Diffusion coefficient $\pm$ SD, 37°C (x10 ⁻⁷ cm ² s ⁻¹)	Permeability coefficient $\pm$ SD, 20°C (x10 ⁻⁶ cm ² s ⁻¹)	Permeability coefficient $\pm$ SD, 20°C (x10 ⁻⁶ cm ² s ⁻¹)
Neutral	4.01 $\pm$ 0.065	6.15 $\pm$ 0.10	1.63 $\pm$ 1.18	2.44 $\pm$ 1.77	1.24 $\pm$ 0.64	1.86 $\pm$ 0.96
Positive	4.74 $\pm$ 1.71	7.27 $\pm$ 2.62	3.44 $\pm$ 1.76	3.44 $\pm$ 1.76	1.59 $\pm$ 0.84	2.44 $\pm$ 1.29
Negative	4.13 $\pm$ 1.07	6.33 $\pm$ 1.64	1.20 $\pm$ 0.50	1.20 $\pm$ 0.50	0.53 $\pm$ 0.15	0.79 $\pm$ 0.22

Table 4. Diffusion coefficients of neutral, positive and negatively charged ficoll.

Ficoll Molecules	Diffusion in Solution		Transscleral Diffusion			
	Diffusion coefficient $\pm$ SD, 20°C (x10 ⁻⁷ cm ² s ⁻¹)	Diffusion coefficient $\pm$ SD, 37°C (x10 ⁻⁷ cm ² s ⁻¹)	Diffusion coefficient $\pm$ SD, 20°C (x10 ⁻⁷ cm ² s ⁻¹)	Diffusion coefficient $\pm$ SD, 37°C (x10 ⁻⁷ cm ² s ⁻¹)	Permeability coefficient $\pm$ SD, 20°C (x10 ⁻⁶ cm ² s ⁻¹)	Permeability coefficient $\pm$ SD, 20°C (x10 ⁻⁶ cm ² s ⁻¹)
Neutral	6.33 $\pm$ 0.082	9.71 $\pm$ 0.13	1.32 $\pm$ 0.49	1.92 $\pm$ 0.71	0.88 $\pm$ 0.35	1.37 $\pm$ 0.54
Positive	6.32 $\pm$ 2.3	9.69 $\pm$ 3.53	0.49 $\pm$ 0.18	0.73 $\pm$ 0.27	0.43 $\pm$ 0.19	0.62 $\pm$ 0.27
Negative	5.97 $\pm$ 0.57	9.15 $\pm$ 0.87	6.12 $\pm$ 2.16	9.29 $\pm$ 3.28	4.38 $\pm$ 1.58	6.81 $\pm$ 2.46

Table 5. Zeta potentials for ficoll and dextran in the three electronic states

Sample	Zeta potential (mV)
Neutral ficoll	-4.79 ± 2.05
Positive ficoll	7.31 ± 0.639
Negative ficoll	-11.2 ± 1.28
Neutral dextran	-2.68 ± 0.561
Positive dextran	8.86 ± 0.393
Negative dextran	-11.6 ± 0.404

FIGURES

Figure 1. Schematic of fluorescence recovery after photobleaching (FRAP) curve.

Figure 2. Diffusion coefficients of albumin versus viscosity

Figure 3. Diffusion coefficients in solution and sclera.

Figure 4 Permeability coefficients in sclera.