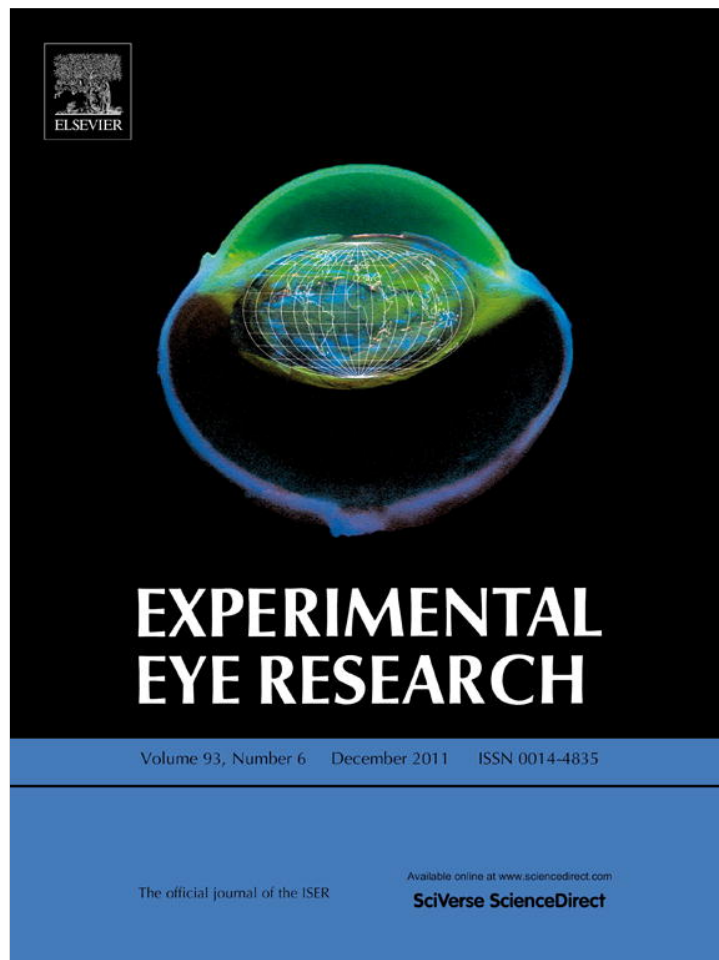


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Passage of low-density lipoproteins through Bruch's membrane and choroid[☆]

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ABSTRACT

Plasma lipoproteins are thought to transport cholesterol, vitamins and carotenoids to the retinal pigment epithelium (RPE) for ultimate use by the photoreceptors. However, to reach the RPE, these lipoprotein particles must cross Bruch's membrane. We examined the reflection coefficient of Bruch's membrane (BrM) to low-density lipoprotein (LDL). Bruch's membrane and choroid were removed from 47 bovine eyes. Specimens were placed in a Ussing chamber and perfused with phosphate-buffered saline (PBS) with (31 specimens) or without (16 specimens) fluorescent low-density lipoproteins (DiI-LDL). The hydraulic conductivity of the tissue was determined for both calf and cow eyes. In the perfusions with DiI-LDL, the fluorescence intensity emitted by DiI-LDL in the efflux was measured and the reflection coefficient of BrM/choroid preparations to DiI-LDL determined. Leakage tests were done to confirm tissue integrity. Several specimens were examined using scanning electron microscopy (SEM) to examine tissue integrity before and after perfusion. Leak testing confirmed that BrM was intact both before and after perfusion. The average hydraulic conductivity of BrM/choroid perfusion of calf eyes with PBS alone was $1.42 \pm 0.55 \times 10^{-9}$ m/s/Pa (mean \pm SD, $n = 11$). The average hydraulic conductivity of the cow eyes was $4.94 \pm 1.48 \times 10^{-10}$ m/s/Pa ($n = 5$), nearly a 3-fold decrease with age. While the flow rate remained constant during the PBS perfusions, it decreased as a function of time during perfusion with DiI-LDLs. Our major finding was of fluorescence in the effluent collected in all perfusions with DiI-LDLs, demonstrating passage of LDL through the tissue. The average reflection coefficient of calf BrM/choroid preparations to DiI-LDL was 0.58 ± 0.25 ($n = 23$); a similar distribution of reflection coefficients was seen in tissue from cow eyes (0.51 ± 0.33 , $n = 8$). Our data suggested that the DiI-LDL was modestly hindered and/or captured by the tissue. This might explain the progressive decrease of hydraulic conductivity with continued perfusion of DiI-LDL.

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1. Introduction

Lipoprotein particles have been implicated in delivery of carotenoids, vitamin E and cholesterol to the retinal pigment epithelium (RPE) for use by the photoreceptors (Curcio et al., in press; Tserentsoodol et al., 2006). These particles must pass through Bruch's membrane (BrM) in their transit from the choriocapillaris to the RPE. Local production of lipoprotein particles by the RPE has also been described, and these particles must presumably pass back through BrM to be returned to the circulation (Wang et al., 2008; Curcio et al., 2009, 2010).

Furthermore, significant lipid accumulation has been described to occur in BrM with aging (Curcio et al., 2001; Holz et al., 1994; Huang et al., 2008, 2007; Johnson et al., 2007; Ruberti et al., 2003). However, the transport characteristics of BrM to lipoproteins have not been previously examined.

Previous studies have proposed a molecular weight (MW) exclusion limit of BrM to macromolecule transport of 66–200 kD (Hussain et al., 1999; Moore and Clover, 2001), although more recent work suggests no such exclusion limit (Hussain et al., 2010). This exclusion limit is similar to the size of high density lipoproteins (MW: 180–360 kD; size: 4–10 nm) (Rifai and Warnick, 2006) but much smaller than LDL (MW: 2400–3900 kD; size: 19–23 nm) (Fisher et al., 1975; Rifai and Warnick, 2006) or the lipoprotein particles (size: 40–100 nm) (Curcio et al., 2009, 2010; Huang et al., 2008) that are seen to accumulate with age in BrM. As such, questions arise as to how lipoproteins enter BrM and whether they can pass through this tissue.

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The reflection coefficient (σ) is a parameter that indicates the extent to which a macromolecule is rejected by a membrane or tissue. The magnitude of the reflection coefficient varies from zero (no rejection) to one (complete rejection) (Kim et al., 1991; Silberberg, 1980; Taylor and Granger, 1984). The goal of this study is to determine the reflection coefficient of a tissue preparation of BrM and choroid to lipoproteins.

Low-density lipoprotein (LDL) is a spherical particle with its major protein apoB, wrapped around and embedded within the lipid (Rifai and Warnick, 2006). We chose to use LDL in this study as its size is greater than the proposed molecular weight exclusion limit of BrM and its lipid composition is very similar to that of the lipoproteins that accumulate with age in Bruch's membrane (Curcio et al., 2009, 2010; Wang et al., 2008).

LDL is available as a fluorescent preparation (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate labeled low-density lipoprotein, DiI-LDL) that has been widely used in studies on LDL-receptor activity (Gross and Webb, 1986; Stephan and Yurachek, 1993), LDL uptake (Brannian et al., 1991; Jaakkola et al., 1988; Traill et al., 1987; Traore et al., 2005), and LDL aggregation (Llorente-Cortes et al., 1998). In the current study, the fluorescence emission of DiI-LDLs was used to facilitate LDL quantification. We perfused DiI-LDL through a Ussing chamber (Clarke, 2009; Mardones et al., 2004; Moore et al., 1995; Starita et al., 1996) containing the tissue preparation and collected the downstream effluent such that we could evaluate the reflection coefficient of this tissue to LDL.

2. Materials and methods

Our experimental procedure involved perfusing DiI-LDL through bovine BrM/choroid preparations placed in a Ussing chamber. Bovine, rather than human tissues were chosen because bovine eyes are readily available, and the structure of the bovine BrM is very similar to that of the human (Nakaizumi, 1964). Both consist of five layers: the basal lamina of the RPE, the inner collagenous layer, the elastic layer, the outer collagenous layer, and the basal lamina of the choriocapillaris. However, the bovine BrM is much thinner (less than 1 μm) than is the human BrM (3 μm in young eyes).

By collecting the DiI-LDL effluent that had passed through the BrM/choroid preparation and comparing its concentration to that of the DiI-LDL suspension upstream of the tissue, we could determine the reflection coefficient of the tissue to the DiI-LDL and thus assess the extent to which this macromolecule could pass through Bruch's membrane. Although both diffusion and convection might affect transport through the BrM/choroid region (Hughes et al., 1998), the flow used to transport the DiI-LDL through the tissue in the current study was not meant to model the physiological transport (Kimura et al., 1996; Tsuboi, 1987). We chose to use a small flow to transport the DiI-LDL across the tissue preparation rather than rely on diffusion due to the very small diffusion coefficient of LDL (Fisher et al., 1971) and the unstirred layer effect. We estimated that diffusion of LDL through the Ussing chamber used in our experiments could take as long as a week to obtain a measurable concentration of LDLs in the downstream efflux without the small flow (Huang, 2007).

Studies were also done to examine the hydraulic conductivity of the BrM/choroid preparations, both in calf and cow eye samples. The latter were examined because preliminary studies indicated a higher hydraulic conductivity for calf eyes than were found in a previous study of bovine eyes (Hillenkamp et al., 2004b), and we wondered whether this might be due, in part, to an aging effect such as is found in human eyes (Moore et al., 1995; Starita et al., 1996).

2.1. Bovine eye tissues and tissue processing

Bovine eyes of young (calf) animals (18–26 weeks, from Chiappetti Lamb and Veal, Chicago IL) and older (cow) animals (22–24 months, from Aurora Packing Co., Aurora IL) were obtained within 8 h post-mortem. After removal of the anterior segment and vitreous, the posterior segment was immersed in Dulbecco's phosphate-buffered saline (PBS; Mediatech Inc., Herndon VA), and then the retina was carefully removed. A 2 \times 2 cm non-tapetal region, including RPE, BrM, choroid, and sclera, adjacent to the optic disc, was cut from the remaining tissue. The non-tapetal region was immersed in PBS, and the RPE cells were gently brushed away with a camelhair brush (SPI Supplies, West Chester PA). The tissue preparation containing BrM and the choroid was then carefully separated from the sclera using fine forceps, washed in PBS and then used as described in Sections 2.3 and 2.4.

2.2. Preparation of the fluorescent low-density lipoprotein solution

PBS with protease inhibitors (pH 7.4, EDTA, 10 mM; benzamidine hydrochloride, 10 mM; N-ethylmaleimide, 10 mM; and phenylmethanesulfonyl fluoride, 1 mM) was pre-filtered through a 0.2 μm filter (Pall Life Science, East Hills NY). DiI-LDL (Invitrogen, Carlsbad CA) was added to the PBS to a concentration of 0.05 mg/ml. The solution was centrifuged at 11,000 rpm, 4 $^{\circ}\text{C}$ for 20 min (Type 70.1 Ti rotor and L7-55 Ultracentrifuge, Beckman, Fullerton CA) to remove any possible large LDL aggregates. A set of standard DiI-LDL solutions (0.05, 0.01, 0.005, 0.001, and 0.0005 mg/ml) were prepared in order to evaluate DiI-LDL concentration of solution samples collected after each perfusion experiment.

2.3. Measurement of the hydraulic conductivity of Bruch's membrane and choroid

A total of 16 bovine eyes, 11 from young (calf) and 5 from older (cow) animals, were used to evaluate the hydraulic conductivity of bovine BrM/choroid preparations. The tissue was placed in a Ussing chamber (U-9500, Warner Instruments, Hamden CT) to which we made custom modifications (Fig. 1). The tissue was placed with BrM facing upstream. An o-ring was pushed against the tissue approximately 3.1 mm peripheral to the flow pathway in order to prevent leaking, similar to the sealing method used by other investigators studying the transport characteristics of this system (Hussain et al., 2002; Moore and Clover, 2001; Moore et al., 1995). A spacer of 250 μm thickness (slightly thinner than the tissue) was placed in-between the two compartments of the Ussing chamber to limit tissue compression at its edges. The region of the tissue through which flow passed was not compressed. The Ussing chamber was attached to a perfusion system including a computer controlled syringe pump (Harvard Apparatus, Holliston MA) and a pressure transducer (Part #142PC05D, Honeywell, Golden Valley MN) (McCarty and Johnson, 2007).

The upstream chamber, tubing and syringe were filled with PBS containing protease inhibitors as described above. The downstream chambers and tubing were filled with the same PBS solution. To measure the hydraulic conductivity (L_p) of bovine BrM/choroid preparation, the system was perfused at a pressure drop (ΔP) of 10 mmHg while the flow rate was monitored and recorded. The system was perfused for 16–21 h. In the period that ΔP remained at a relatively constant 10 mmHg, L_p was determined based on ΔP and average flow rate (Q) through the tissue (cross-sectional area $A = 0.11 \text{ cm}^2$) facing the flow (McCarty et al., 2008):

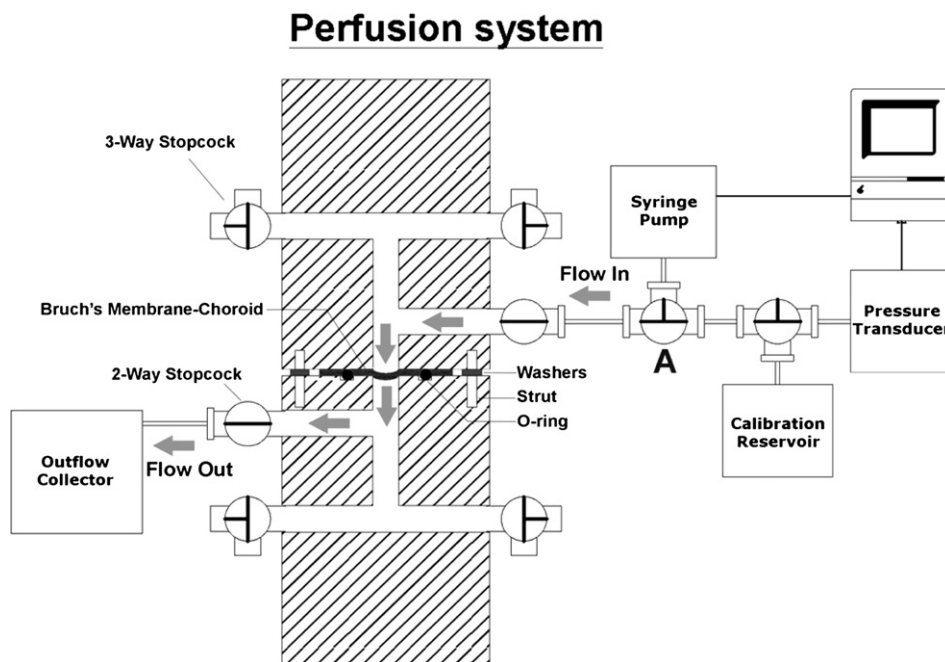


Fig. 1. The perfusion system used in the current study.

$$L_p = \frac{Q}{\Delta PA} \quad (1)$$

2.4. Perfusion of DiI-LDL through Bruch's membrane and choroid

A total of 23 calf eyes and 8 cow eyes were used to evaluate the transport of DiI-LDL through BrM/choroid preparations. For 15 of the 23 calf eyes and all of the cow eyes, the sample tissue was placed with BrM facing the upstream. Tissues from 8 calf eyes were placed with choroid facing the upstream. DiI-LDL solution was used to fill the upstream chamber, tubing, and the syringe. The downstream chambers and tubing were filled with PBS that contained the protease inhibitors. The DiI-LDL solution was then perfused through the tissue at a 10 mmHg pressure drop for 17–24 h and collected downstream as described below.

We also examined the possibility that the fluorescence detected in the collected downstream solution might have been emitted by dissociated DiI fluorophores from DiI-LDLs. To test for this possibility, the BrM/choroid preparation was replaced with a membrane filter (Diaflo XM50, Amicon Inc, Beverly, MA) with a molecular weight cutoff of 50 kD, much smaller than LDL but much larger than the molecular weight of DiI (C₅₉H₉₇ClN₂O₄, MW: 934 Da). The DiI-LDL solution was perfused at a constant flow rate of 1.5 μl/min (comparable to the flow rate used in BrM/choroid preparation perfusions) for 20 h. The perfusion condition of this control experiment was selected to ensure that both the solution volume perfused through the filter and the perfusion time scale were similar to other perfusion experiments.

2.5. Measurement of fluorescence intensity emitted by DiI-LDL

At the conclusion of the perfusion, upstream and downstream fluids were collected separately. All collected DiI-LDL solutions and also the PBS solution used to dilute DiI-LDL were examined in a PC1 spectrofluorometer (ISS, Champaign IL) excited by a 520-nm light source. The intensities of the 570-nm fluorescence emitted by each DiI-LDL solution was determined by subtracting the 570-nm

emission of PBS from the emission of DiI-LDL solutions. The fluorescence of collected upstream and downstream DiI-LDL solutions was compared to the standard DiI-LDL solutions in order to determine the DiI-LDL concentration in the upstream (C₁) and downstream (C₂) fluid samples.

2.6. Determining the reflection coefficient of Bruch's membrane and choroid

The reflection coefficient (σ) of the BrM/choroid preparation to LDL perfusion was determined using the Kedem–Katchalsky equation (Curry, 1984; Taylor and Granger, 1984):

$$J_s = Q(1 - \sigma)C_1 + PA\Delta C \quad (2)$$

where J_s is the flux of DiI-LDL across BrM and choroid, $\Delta C = C_1 - C_2$ is the change in concentration of the DiI-LDL across the tissue, and P is the diffusional permeability of the membrane to the macromolecule. The two terms on the right hand side of equation (2) respectively represent the contribution of particle transport from convection and diffusion.

The Peclet number characterizes the relative contribution of convection to diffusion in a transport process:

$$Pe = \frac{vL}{D} \quad (3)$$

where v is the velocity of the macromolecules as they are transported through the fibril network in Bruch's membrane, L is the length scale over which this transport occurs and D is the diffusion coefficient of the macromolecule in the medium. In the absence of a restricting medium through which the macromolecule is being transported, v would be the velocity of the saline (Q/A), and D would be the diffusion of the macromolecule in saline (D_0). D is related to the diffusional permeability of the medium as $P = \phi D/L$ where ϕ is the partition coefficient of the medium (Curry, 1984).

The medium slows the velocity v from Q/A and reduces D from D_0 . Studies on the transport of compact particles through solutions of chain-polymers have shown that the reduction of both sedimentation rate and diffusion rate of compact particles through such

networks are similar (Laurent et al., 1963; Ogston et al., 1973), suggesting that the Peclet number for transport of macromolecules through such media can be estimated as:

$$Pe = \frac{(Q/A)L}{D_0} \quad (4)$$

D_0 of LDL has been reported as 2×10^{-11} m²/s (Fisher et al., 1971) Q/A can be determined using equation (1), the pressure drop across the tissue (10 mmHg) and the value of L_p measured in this study. Using the lowest measured L_p of BrM/choroid preparations in the current study (0.12×10^{-9} m/s/Pa), we determined that the lowest value of the velocity v in this study was 0.17 μ m/s.

Several length scales could be used to characterize the Peclet number (Huang, 2007). While transport across Bruch's membrane itself is dominated by diffusion due to its small length scale (approximately 1 μ m), the LDL needs also cross the length of the BrM/choroid preparation (0.25 mm), and be transported far enough away from the preparation and into the downstream tubing (roughly 1 mm) so that it can be collected. It is the longest length scale that is relevant for characterizing the process of collecting DiI-LDL that has passed through this system ($L = 1.25$ mm).

Using these values, we found that the Pe would always be much greater than one with a ΔP of 10 mmHg. This indicated that convection was dominant in transporting DiI-LDL across the BrM/choroid preparations in the current study. Therefore, equation (2) was simplified as:

$$J_s = Q(1 - \sigma)C_1 \quad (5)$$

J_s (the average flux of DiI-LDL passing through the tissue per unit time) was determined based on the downstream DiI-LDL concentration (C_2), the collected downstream fluid volume (V), and the perfusion time (T):

$$J_s = \frac{C_2V}{T} \quad (6)$$

We measured the PBS volume in the downstream chamber and tubing ($V_2 = 1.43$ ml) and combined this with the cumulative fluid volume that passed through the tissue (Q^*T) to determine the total collected downstream fluid volume (V) at the end of perfusion:

$$V = QT + V_2 \quad (7)$$

As such, the reflection coefficient could be determined using the following equation:

$$\sigma = 1 - \frac{C_2V}{C_1QT} = 1 - \frac{C_2}{C_1} - \frac{C_2V_2}{C_1QT} \quad (8)$$

2.7. Hydraulic conductivity of Bruch's membrane and choroid with LDL perfusion

In each of the experiments described in Section 2.4, we also evaluated the change in the hydraulic conductivity of the bovine BrM/choroid tissue due to the DiI-LDL perfusion. Once ΔP stabilized at around 10 mmHg, the initial L_p value (L_{pi}) was determined based on the flow rate and the pressure at that time point using equation (1). The final L_p value (L_{pf}) was determined at the end of the perfusion also using equation (1).

2.8. Tissue integrity of RPE basal lamina

Since the basal lamina of RPE was the outermost layer of the bovine tissue preparations used in this study, we examined its

integrity in order to ensure that the tissue was not damaged by the process of RPE removal or by the process of placing the sample in the perfusion chamber and then perfusing it. Five samples were examined using scanning electron microscopy (SEM) after removal of the RPE with a camelhair brush, and five other samples were examined following the completion of a buffer perfusion study of the tissue.

After removal of the RPE, small blocks of the non-tapetal tissue, containing BrM, choroid and sclera were fixed for at least 1 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 10 mg/ml calcium chloride with a final pH of 7.4. After fixation, the sclera was removed, and the remaining BrM/choroid thin sheets were used for further processing. The BrM/choroid samples processed for SEM after perfusion were removed from the chamber and fixed together with the o-ring to prevent folding or tearing of the tissue. Small sheets containing the perfused area were isolated from the fixed tissue. Following fixation, all samples were dehydrated in a series of ascending concentrations of ethanol in water and then critical point dried (Polaron Critical Point Dryer, Queensland, Australia). The dried samples were sputter coated (Denton Desk III TSC Sputter Coater, Moorestown NJ) with gold/palladium and then viewed on a Hitachi S3400N-II (Hitachi High Technologies America Inc, Pleasanton CA) SEM.

2.9. Leak testing

Two additional procedures were used to test for leaks in the system following some of the perfusion experiments. In the first, we generally followed the method of Hillenkamp (Hillenkamp et al., 2004b). For those tissues perfused with DiI-LDL, after completion of a perfusion experiment, the downstream chamber fluid was exchanged with 0.83-mM dextran (150 kDa) in PBS containing protease inhibitors. Since the transport of this molecule across intact tissue would be hindered due to its size, its presence in the downstream chamber would create an osmotic pressure gradient that would drive fluid from the upstream into the downstream chamber (Hillenkamp et al., 2004b). Two fluid reservoirs, connected to either the upstream or downstream chamber, were set at the same height at the start of the leak test. The test was carried out overnight, and the difference in the fluid levels of the reservoirs was recorded the next day. The test was considered successful if the fluid level in the downstream reservoir had risen above that of the upstream reservoir, thus demonstrating osmotically-generated flow. Evaporation effects were minimized by placing wet cotton balls at the reservoir outlets. Five of the eleven perfusions of calf eyes with PBS alone and four of the eight perfusions of cow eyes with PBS alone were leak tested. For those tissues perfused with DiI-LDL, 4 out of the total of 23 were leak tested.

In five cases, a second leak test was performed that allowed us to visualize the flow using red microspheres to mark the flow pathway and demonstrate whether or not these particles entered into BrM (which they should not, unless there is a leak). After completion of the osmotic leak test, the downstream chamber was rinsed with 50 ml PBS containing protease inhibitors to remove any remaining dextran. The upstream chamber fluid was then exchanged with a 0.05% red latex microsphere (size: 200 nm) solution in PBS containing protease inhibitors. The system was then set to perfuse at a constant flow rate of 0.5 μ l/min for at least 6 h. Since the microspheres are larger than any openings that should be in an intact Bruch's membrane, they were expected to accumulate on the upstream tissue surface, with the tissue itself and the downstream fluid remaining clear of these particles. After completion of the test, the downstream fluid was inspected for changes in color that would indicate a break in the tissue. The upstream surface of the tissue was inspected for any presence of red coloring outside the perfusion area that would indicate a leak at

the chamber/tissue interface. The test was considered successful if the downstream fluid remained clear and the red microspheres accumulated only within the area used for perfusion on the upstream tissue surface.

2.10. Statistical analysis

Linear regression was used to investigate correlations between variables and to determine correlation coefficients (r) and significance of such correlations (p). All data sets were tested using the Lilliefors test and found to exhibit a normal distribution. Therefore, statistical comparisons between groups were performed by using a two-tailed Student's t -test. We considered results as significant for $p < 0.05$.

3. Results

3.1. Tissue integrity and leak testing

We followed the tissue preparation procedure of other studies (Moore et al., 1995) and used a camelhair brush to remove the RPE. To ensure this procedure and the subsequent mounting and perfusion of BrM in the Ussing chamber did not damage Bruch's membrane, particularly the basal lamina of the RPE, and thus alter its transport characteristics, we examined the surface of a BrM/choroid preparation both before and after perfusion. All samples showed good preservation of BrM, with no signs of breaks that would reveal the fibrils of the collagenous layer underlying BrM. Fig. 2A shows a SEM micrograph of bovine tissue after removal of the RPE (before perfusion) demonstrating a tight microfibrillar network characteristic of the basal lamina of the RPE (Huang et al., 2007). Also seen is a small region where the RPE was not removed; it was very rare to see remaining RPE, but we included this micrograph to better appreciate the intact surface of Bruch's membrane. We did not observe any region of the tissues showing the large fibrils that are seen in the collagenous or elastic layer of BrM (Huang et al., 2007).

Fig. 2B shows a typical example of an SEM image of the surface of bovine BrM after perfusion with no signs of damage caused by either mounting into the perfusion chamber or by the perfusion. The micrograph of the surface is very similar to that seen by other investigators (Moore et al., 1995), and showed no signs of fibrils, even at much higher magnifications.

Leak tests confirmed the histological findings that the tissue remained intact following perfusion of the tissue. Addition of 150-kDa dextran to the downstream fluid in all cases so tested created an osmotic pressure that resulted in fluid motion through BrM. Addition of 200-nm latex microspheres to the upstream fluid resulted in accumulation of the particles only on the upstream tissue surface over the perfusion area and no traces of the red particles were found downstream of that area or outside of the perfusion zone for all samples tested. The successful completion of these tests confirms that there were no breaks in the tissue before or after completion of the perfusion experiments. This confirmed the conclusion of other studies that the RPE can be removed from these preparations and these tissue can be mounted in a Ussing chamber and perfused without damaging Bruch's membrane (Ahir et al., 2002; Hillenkamp et al., 2004a, 2004b; Hussain et al., 2002; Kumar et al., 2010; Moore and Clover, 2001; Moore et al., 1995; Starita et al., 1996, 1997; Ugarte et al., 2006).

3.2. Hydraulic conductivity

Typical results of perfusions through BrM/choroid preparations are shown in Fig. 3. Fig. 3A shows the flow rate across this

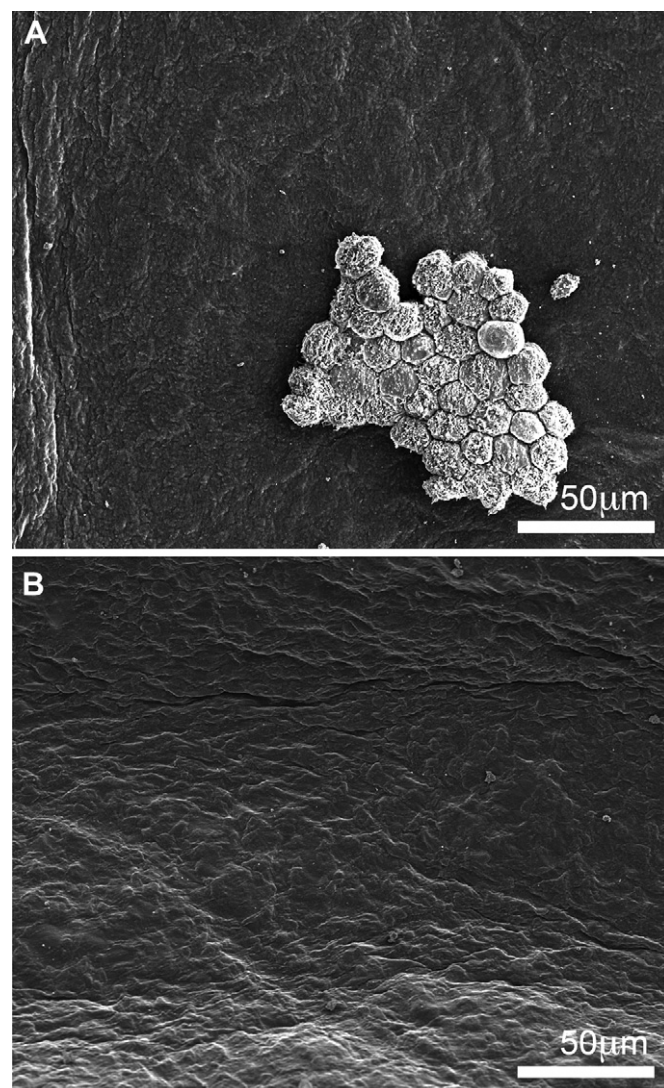


Fig. 2. (A) SEM image of calf BrM before perfusion; (B) SEM of calf BrM after perfusion.

preparation when perfused with PBS at a pressure drop averaging 10 mmHg. After a brief transient period, the flow rate across the tissue stabilizes and remains relatively constant to the end of the perfusion (the small cyclical oscillations in pressure are due to variations in the machining of the lead screw that drives the flow). The stability of the hydraulic conductivity during the buffer perfusions indicated that the use of protease inhibitors successfully prevented tissue autolysis during this period.

After determining the average flow rate and pressure drop during this stable period, we used equation (1) to find the hydraulic conductivity of the bovine BrM/choroid preparations from calf eyes to be $1.42 \pm 0.55 \times 10^{-9}$ m/s/Pa (mean \pm SD, $n = 11$). The hydraulic conductivity of BrM/choroid preparation from cow eyes was nearly 3-fold lower ($4.94 \pm 1.48 \times 10^{-10}$ m/s/Pa, $n = 5$). This value was statistically different from the calf eye results ($p = 0.003$).

Fig. 3B shows typical results for flow rate and pressure drop as functions of time during the constant pressure perfusion of DiI-LDL in PBS through the calf BrM/choroid preparations. Unlike the PBS perfusions, the flow rate did not stabilize but gradually decreased during the perfusion in all cases. Table 1 lists the initial and final values of hydraulic conductivity (L_{pi} and L_{pf} , respectively) of each calf and cow BrM/choroid preparation used in DiI-LDL perfusions. We found that the hydraulic conductivity of the calf BrM/choroid

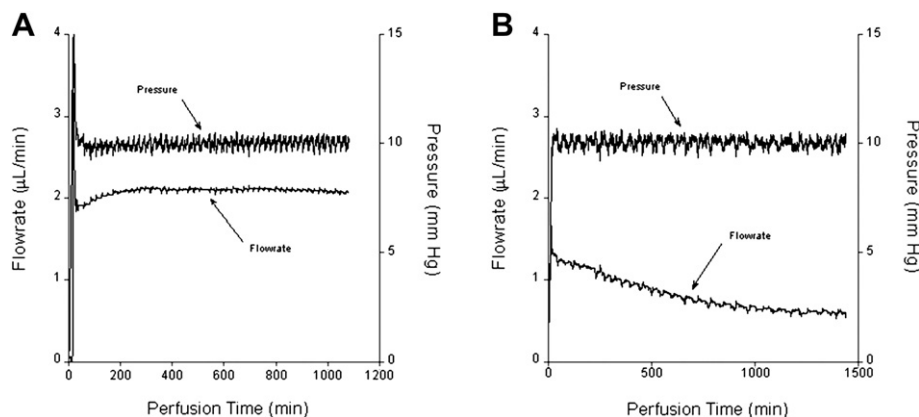


Fig. 3. The flow rate and the pressure drop as a function of perfusion time in (A) PBS perfusion of calf tissue and (B) DiI-LDL perfusion of calf tissue.

preparation (with BrM facing upstream) decreases by $1.56 \pm 0.97\%$ per hour (mean \pm SD, $n = 15$) during DiI-LDL perfusion, equivalent to an approximately 31% total decrease in L_p for an average 20-h perfusion period in the current study. The hydraulic conductivity of the cow BrM/choroid tissues decreased by $1.08 \pm 1.06\%$ per hour (mean \pm SD, $n = 8$), or approximately a 22% total decrease for a 20-h perfusion period. The decreases in L_p for calf and cow samples were not statistically different from one another ($p = 0.29$).

We also found that, as in our hydraulic conductivity measurements with buffer alone, the hydraulic conductivity of the calf eyes perfused with DiI-LDL was much higher than in the cow eyes. The average initial hydraulic conductivity (L_{pi}) of the calf eyes from the DiI-LDL perfusions (with BrM facing upstream) was $1.60 \pm 0.75 \times 10^{-9}$ m/s/Pa (mean \pm SD, $n = 15$), while in the cow tissue, this value was $4.95 \pm 2.80 \times 10^{-10}$ m/s/Pa (mean \pm SD, $n = 8$), more than 3-fold lower. The difference between these two groups was highly significant ($p = 0.00006$).

3.3. Reflection coefficient

At the end of these experiments, we collected the downstream fluid and compared the DiI-LDL concentration in it to the solution upstream of the tissue. The DiI-LDL concentration was assessed by the peak intensity at 570-nm of typical fluorescence spectrum (540–610 nm) emitted by the lipoproteins. When the tissue was replaced with a 50-kD membrane filter, the collected sample did not emit significant fluorescence, indicating that the detected fluorescence collected in the tissue perfusion experiments was not from dissociated DiI fluorophores.

The reflection coefficients of calf BrM/choroid preparations to DiI-LDL transport, determined by using equation (8), are listed in Table 1. On average, the reflection coefficient (σ) of calf BrM/choroid preparations (with BrM facing upstream) to this DiI-LDL perfusion was 0.66 ± 0.25 (mean \pm SD, $n = 15$), and in cow eyes this value was found to be 0.51 ± 0.33 (mean \pm SD, $n = 8$). There is no significant difference between these two data sets ($p = 0.22$). The average value of σ of the calf preparations to DiI-LDL perfusion with choroid facing upstream was 0.43 ± 0.20 (mean \pm SD, $n = 8$), a little lower than facing the other direction ($p = 0.036$). The average reflection coefficient for all calf BrM/choroid tissues perfused with DiI-LDL was 0.58 ± 0.25 (mean \pm SD, $n = 23$).

A linear relationship was seen between the reflection coefficient and the initial hydraulic conductivity (L_{pi}) of the calf BrM/choroid preparation to the perfusion of DiI-LDLs, with BrM facing upstream (Fig. 4A, $p = 0.002$, $r = 0.73$, $n = 15$). The higher the σ value, the lower the initial tissue hydraulic conductivity. However, this

relationship was not seen in the DiI-LDL perfusions of calf tissues with the choroid facing upstream (Fig. 4B, $p = 0.50$) or in cow tissues ($p = 0.53$).

4. Discussion

In this study, we found that the reflection coefficient of BrM and choroid to DiI-LDL was approximately 0.58 and 0.51 in calf and cow eyes, respectively. For comparison, the reflection coefficient of arterial endothelium to LDL is 0.998 and of arterial intima to LDL is 0.827 (Yang and Vafai, 2006). Our results showed that while the DiI-LDL (MW: 2400–3900 kD) did not pass freely through the BrM/choroid preparation, it could nonetheless pass and was only

Table 1
Reflection coefficients (σ) and the initial and final hydraulic conductivities (L_{pi} and L_{pf}) of BrM/choroid to DiI-LDL perfusion.

Tissue ID#	σ	L_{pi} (10^{-9} m/s/Pa)	L_{pf} (10^{-9} m/s/Pa)
<i>BrM facing upstream (calf)</i>			
120407	0.86	1.29	1.06
120607	0.62	2.81	1.91
121107	0.50	1.63	0.94
010308	0.78	1.40	0.90
010808	0.88	1.38	0.62
011008	0.11	2.99	1.43
011508	0.87	1.61	1.32
011708	0.42	2.74	1.21
012208	0.72	1.33	1.02
012408	0.28	1.88	1.19
050808	0.69	1.67	1.03
060210	0.78	0.78	0.72
061710	0.50	1.16	0.95
062210	0.90	0.77	0.73
071310	0.96	0.51	0.45
<i>Choroid facing upstream (calf)</i>			
040808	0.50	1.14	0.78
041008	0.34	0.68	0.58
042108	0.62	0.81	0.50
051208	0.37	2.85	1.86
0908110	0.46	0.31	0.22
0908111	0.01	1.05	0.68
0927110	0.55	0.52	0.38
0927111	0.59	0.31	0.28
<i>BrM facing upstream (cow)</i>			
0408110	0.61	0.27	0.20
0408111	0.77	0.26	0.12
0414110	0.21	0.20	0.19
0414111	0.87	0.42	0.41
0825110	0.88	0.52	0.50
0825111	0.32	0.62	0.61
0906110	0.38	1.06	0.61
0906111	0.00	0.61	0.45

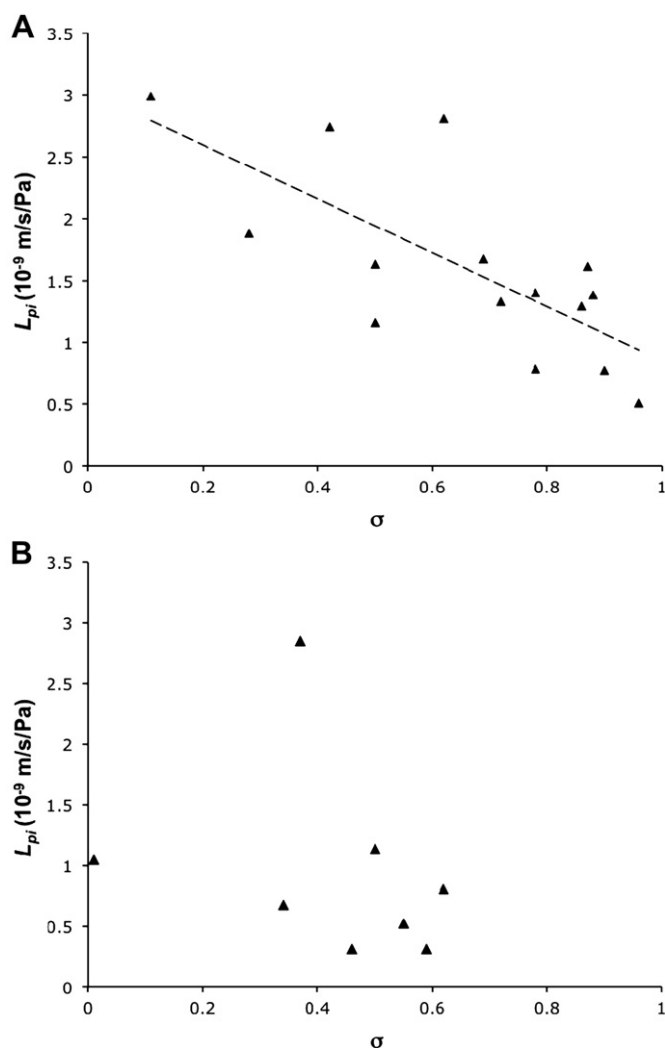


Fig. 4. The initial tissue hydraulic conductivity (L_{pi}) of the calf BrM preparations as a function of the DiI-LDL reflection coefficient (σ). (A) BrM facing upstream. (B) Choroid facing upstream. The dashed line in 5A is a linear regression to the data with $p < 0.02$ and a correlation coefficient of 0.73.

modestly inhibited in its transport through this tissue. This further indicated that the molecular exclusion limit of this membrane was much greater than the value of 66–200 kD suggested in earlier studies (Hussain et al., 1999; Moore and Clover, 2001), although a recent study indicates that, if such a limit exists, it is greater than 500 kD (Hussain et al., 2010). It has been reported that RPE cells were capable of internalizing plasma LDL from the choroid (Gordiyenko et al., 2004; Tserentsoodol et al., 2006). As such, it is perhaps not so surprising that DiI-LDL can be transported across Bruch's membrane.

We measured an average hydraulic conductivity of the BrM/choroid preparations from calf eyes (22–26 weeks) to PBS of 1.42×10^{-9} m/s/Pa, close to the hydraulic conductivity found in young human Bruch's membrane/choroid (Moore et al., 1995; Starita et al., 1996). The hydraulic conductivity of cow eye tissues (22–24 months) was significantly lower, 4.9×10^{-10} m/s/Pa. It should be noted that the cow eyes used in our experiments were adult eyes (2 years old), but still quite young considering the lifespan of these animals (20–25 years). Therefore, the overall decrease in hydraulic conductivity over the entire bovine lifespan is likely to be significantly larger than that found in these experiments. This is perhaps a reflection of a similar process seen in

human eyes in which a 100-fold decrease of the hydraulic conductivity of macular BrM/choroid preparation occurs over a lifetime (Moore et al., 1995; Starita et al., 1996). Hillenkamp et al. (2004b) reported the hydraulic conductivity of bovine BrM/choroid preparations from 18- to 24-month-old animals to be 3.45×10^{-11} m/s/Pa. This value was much lower than our results and may reflect differences in the aging process in different breeds.

The age-related decrease of hydraulic conductivity is caused by a very significant accumulation of lipids and other debris, and an increase in the thickness of Bruch's membrane (Ethier et al., 2004; Marshall et al., 1998). Given the very low concentration of DiI-LDL in the perfusion fluid used in this study (0.05 mg/ml), there was a surprisingly significant decrease of hydraulic conductivity (20–30%) with continued perfusion of DiI-LDL, both in calf tissue and in cow. This is consistent with our previous finding that trapping LDL in an extracellular matrix causes a surprisingly large decrease in its hydraulic conductivity (McCarty et al., 2008).

It is worthy of note that while the cow tissue had a much lower hydraulic conductivity than did the calf, the reflection coefficient of both of these tissues to DiI-LDL was similar. This suggests that the location of particle hindrance (likely the small openings of the basal lamina of the RPE) was distinct from the principal site of flow resistance in the tissue, which has been shown to reside in the inner collagenous layer (ICL) of Bruch's membrane (Starita et al., 1997). This parallels findings by Huang et al. (2007) in human eyes of minimal age-related changes of the RPE basal lamina as compared to those seen in the ICL (Huang et al., 2007).

We examined whether there was a relationship between the hydraulic conductivity of the BrM preparations and their reflection coefficients as one might anticipate that a tissue with a lower hydraulic conductivity might also pose a higher restriction for the transport of large solutes like LDLs. Indeed, such relationship was observed in the current study such that samples having low initial values of hydraulic conductivity also had high reflection coefficients.

In young human eyes, the anterior part of BrM, including the basal lamina of RPE, the inner collagenous layer, and the elastic layer, are composed of a high density of fibril components (Huang et al., 2007). As such, these layers may represent the major site of resistance to lipid transport through this tissue. The DiI-LDLs passing through the BrM/choroid preparations in the current study are likely to have been hindered in their transport through this region. This hindrance is likely also responsible for the observed decrease in the flow rate that progressively occurred during perfusion of the DiI-LDL solutions.

Such hindrance might be responsible for the lipoprotein accumulation in Bruch's membrane observed with age in human eyes (Curcio et al., 2009, 2010; Huang et al., 2008, 2007). In our previous reports, we found the accumulation of lipoprotein and other macromolecules starts to fill the interfibril spacing in the center part of Bruch's membrane in eyes of middle age (Huang et al., 2007). With an increase of age, the region filled by lipoproteins and other macromolecules gradually extends into the anterior part of the tissue, eventually leading to the formation of a "lipid wall" between the inner collagenous layer and the basal lamina of RPE (Curcio et al., 2009, 2010; Huang et al., 2008, 2007; Ruberti et al., 2003). While such a sequence of events is consistent with the hypothesis that the lipoproteins are secreted from RPE cells, the choroid is also a possible source of the lipoproteins that accumulate with age in Bruch's membrane (Curcio et al., 2001; Haimovici et al., 2001; Holz et al., 1994; Li et al., 2005; Mullins et al., 2000; Sivaprasad et al., 2005; Wang et al., 2008).

A potential criticism of this study is that non-physiologic convection was used to minimize the time scale of the experiment. It is important to note that transport across Bruch's

membrane itself was still dominated by diffusion as the Peclet number for transport across that tissue was always well less than one in these experiments. Furthermore, previous studies have shown that both diffusive transport of macromolecules and sedimentation of these macromolecules (analogous to convection) are similarly hindered by the presence of extracellular matrix molecules (Laurent et al., 1963; Ogston et al., 1973). No difference in reflection coefficient to either convection- or diffusion-dominant solute transport was found for transport through nanofiltration membranes (Lee et al., 2002).

The *in vitro* model used in this study might be an ideal platform to investigate the source from and mechanisms by which lipids accumulate with age in BrM. As shown in this study, LDL can be transported into the tissue with either BrM or choroid facing upstream. By using electron microscopy techniques such as freeze-etch (Huang et al., 2008, 2007; Ruberti et al., 2003), the accumulation of hindered lipoproteins within the tissue preparations can be characterized. Comparison of the lipid deposition pattern in such experiments with that seen physiologically might give insights into the mechanism by which lipid accumulate with age in this tissue.

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