Non-invasive measurement of solute permeability in cerebral microvessels of the rat

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Introduction

The blood–brain barrier (BBB) is a dynamic barrier essential for maintaining the micro-environment of the brain. The BBB endothelial layer is different from that in the rest of body in several aspects. First, anatomically, the BBB endothelial layer is ensheathed by pericytes and astrocyte foot processes (Nicholazzo et al., 2006). Second, the BBB endothelium has much less fenestration and more intensive tight junctions, which are responsible for restricting paracellular passage of water and hydrophilic solutes from the peripheral circulation entering into the central nervous system (CNS) (Butt et al., 1990; Hawkins and Davis, 2005).

The special anatomical features of the BBB determine its protective role for the CNS. The BBB is such a complicated barrier that even after numerous attempts have been made to decode its complexity, very little has been determined conclusively (de Boer and Gaillard, 2006). One difficulty is a lack of quantitative and well-defined model system. Many in vivo methods have been developed to quantify the BBB permeability properties (summarized in Pardridge, 1998). Vital dye injection and fluorescence dye injection intravenously or through the carotid artery (Cornford et al., 1992; van Uitert et al., 1981; Zlokovic et al., 1986), quantitative autoradiography (QAR) (Nagaraja et al., 2006), peroxidase histochemistry (Kaur et al., 2006), positron emission tomography (PET) (Elsinga et al., 2004) and magnetic resonance imaging (MRI) (Wang et al., 2007) have been used to measure the brain uptake index (BUI) in animal models and in humans. Although these measurements reflect the BBB leakage, they do not represent the BBB permeability because they cannot determine the driving force for the leakage flux, nor their resolutions (hundreds µm to mm) are high enough for microvascular level imaging (0.1–1 µm). The transendothelial electrical resistance (TEER) has been measured to quantify the BBB permeability. But it only represents ion permeability.

Although it has its advantages for protecting brain from blood-born neurotoxins, however, the BBB extremely limits the therapeutic efficacy of drugs into the CNS, which greatly hinders the treatment of major brain diseases, such as Alzheimer’s disease, Parkinson’s disease and brain tumor (Pardridge, 2006). Thus, determination of the permeability of cerebral microvessels to various drugs across the BBB is important for the drug development for CNS disorders, especially at the early stage for searching new drug candidates with the ability to penetrate the BBB. In addition, the CNS disorders, such as multiple sclerosis, Alzheimer’s disease, HIV infection and brain tumors, are always associated with microvessel hyperpermeability (Pardridge, 2006), which is one of the critical steps for the abnormal transport of molecules and cells across the blood vessel wall. Therefore, understanding the mechanisms of microvessel hyperpermeability from various approaches is necessary in combating these CNS diseases.

Several in vivo rat models have been used for investigating transport across the BBB. Since it is hard to measure the BBB permeability in brain parenchyma, the microvessels in pia dura at the surface of brain are often used in in vivo BBB permeability study. The pial microvessel has been proved as a valid model for studying the BBB permeability (Allt and Lawrenson, 1997). By removing the rat skull and the dura mater and covering the exposed cerebral cortex with a glass cranial window, Gaber et al. (2004) systematically...
injected a fluorescence dye through the left femoral vein and measured the dye clearance or leakage rate out of the rat pial microvessels rather than the true permeability of the microvessels, which is defined as the solute flux over the concentration difference across the vessel wall per unit surface area of the vessel, since their method did not well determine the dye concentration difference across the vessel, i.e. the driving force, for the dye extravasation to the surrounding tissue.

Easton and Fraser (1994) proposed a method to measure the solute permeability of rat pial microvessels using the single microvessel occlusion technique. In their technique, pial microvessels were exposed after removing the skull and overlying meninges. When the solution containing a fluorescence tracer was introduced to the cerebral circulation by a bolus injection through the carotid artery, an occluded segment (100–200 μm long) of a pial post-capillary venule was formed by trapping the dye as it passed through the vessel by a glass probe to occlude the downstream of the vessel. The fluorescent intensity in this occluded segment was recorded to determine the apparent permeability of the vessel to the tracer. Using the same technique, Easton et al. (1997) found that the permeability of pial microvessels to small solute, Lucifer Yellow (MW 457 Da), was very low within 1 min after removing the meninges, and the permeability rose to 8 times in 20–60 min and to 62 times in 44–164 min following the craniotomy. The researchers suggested that these permeability increases were resulted from the BBB disruption due to the removal of the skull and the meninges.

To quantify the permeability of intact rat pial microvessels and overcome the above mentioned disadvantages, we developed in this study, a non-invasive method, without exposing the cortex, to measure the apparent permeability (P) of post-capillary venules on rat pia mater to various sized solutes. The pial microvessels were observed by a high numerical aperture objective lens through a fluorescence microscope imaging method instead of photometry method (Easton and Fraser, 1994). P was measured on individual pial venular microvessels with the perfused fluorescence tracer solution through the carotid artery by using highly sensitive quantitative fluorescence microscope imaging method.

**Materials and methods**

**General preparation**

All in vivo experiments were performed on adult female Sprague-Dawley rats (250–300 g) supplied by Hilltop Laboratory Animals (Scottsdale, PA). All procedures and the animal use have been approved by the Institutional Animal Care and Use Committee at the City College of New York. The rat (age 3–4 months) was anesthetized with pentobarbital sodium given subcutaneously. The initial dosage was 65 mg/kg, and additional 3 mg/dose was given as needed. The rat was then kept warm on a heating pad. The skull in the region of interest was exposed by shaving off the hair and cutting away the skin and connective tissue. A section of left or right frontoparietal bone, approximately ~5 mm × ~5 mm, was carefully ground with a high-speed micro-grinder (0–50,000 rpm, DLT 50KBU; Brasseler USA, GA) until a part of it (~2 mm × ~2 mm) became soft and translucent. The blood vessels could be seen under a 16× dissection microscope (Fig. 1). During the process, artificial cerebrospinal fluid (ACSF) with the room temperature was applied to the surface of the skull to remove the heat due to grinding. After grinding, the rat was laid face-up on a tray with its thinned section of the head placed in a shallow chamber formed by a glass cover slip. The thinned section was observed under the microscope and the pial microvessels were focused by carefully adjusting the focusing knob. The fluorescent solution was introduced into the cerebral circulation via the left or right carotid artery by a syringe pump at a constant rate of 3 ml/min while the images of the pial microvessels and nearby brain tissue were simultaneously collected. To repeat the measurement, the perfusate was switched to a washout solution containing no fluorescently labeled solutes to wash away the fluorescence in the microvessel.

**Microscope preparation**

A detailed description of a photometry method used to measure permeability (P) of fluorescently labeled solutes has been published elsewhere (Adamson et al., 1988; Fu et al., 1998; Huxley et al., 1987). In the current experiment, we used an imaging method instead of photometry. A Nikon TE2000-E inverted fluorescence microscope was used to observe pial microvessels. The rat was laid on a customized tray with the thinned section of the rat skull placed on a glass cover slip (view area ~20 mm diameter) which was embedded in the tray. The surface of the skull was constantly superfused with 37 °C ACSF at the rate of ~1 ml/min through a dripping tube attached to the side of the head. The vessels were observed with a 20× lens (NA=0.75, Nikon) and an illumination system (the monochromator with a xenon lamp, FSM150Xe; Bentham, U.K.), which was computer-controlled to change the excitation/emission wavelengths for various fluorescent probes. The intensity of the xenon light source was controlled by an attenuator knob on the front panel of the lamp house. The intensity of the light output can be adjusted from 0% to 100%. To reduce the tissue damage due to the exposure to fluorescence light, the light intensity was kept as low as possible. Further protection was provided by using an experimental protocol in which the time of tissue exposure to the excitation light was kept as short as possible for the permeability measurement. The excitation/emission wavelengths were set to

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**Fig. 1.** (A) The thinned frontoparietal bone on the rat skull; (B) Enlarged thinned section; (C) Exposed pial vessels by removing the thinned frontoparietal bone and the meninges (the method used in Easton et al., 1997).
475 nm/520 nm and 490 nm/520 nm for sodium fluorescein and FITC-dextrans, respectively. The images were captured using a highly sensitive 12-bit CCD camera (Sensicam QE; Cooke, MI) and then transferred to an imaging acquisition and analysis workstation. The spatial resolution of our system with 20× lens (NA=0.75) is 0.5 μm/pixel. The acquisition speed is 3 frames/s for image size 1024×1376 pixels or 8 frames/s for image size 512×672 pixels.

**Solutions and fluorescent test solute preparation**

**Mammalian Ringer solution**

Rat Ringer solution was used for all perfusates. The solution composition was (in mM) NaCl 132, KCl 4.6, MgSO4 1.2, CaCl2 2.0, NaHCO3 5.0, glucose 5.5 and HEPES 20. All these chemicals were from Sigma. The pH was buffered to 7.4–7.45 by adjusting the ratio of HEPES acid to base. In addition, both the washout solution and the fluorescent dye solution contained bovine serum albumin (BSA, A4378, Sigma) at 10 mg/ml (Fu and Shen, 2004) The solutions were made fresh on the day of use to avoid binding to the serum albumin (Adamson et al., 1994; Fu et al., 1998).

**Sodium fluorescein**

Sodium fluorescein (F6377, Sigma; mol. wt. 376, Stokes–Einstein radius ~0.45 nm) was dissolved at 0.1 mg/ml in the Ringer solution containing 10 mg/ml BSA.

**FITC-dextrans**

All FITC-dextrans, FITC-dextran-4k (FD4, Sigma; mol. wt. 4000, Stokes radius ~1.4 nm), FITC-dextran-10k (FD10 s, Sigma; mol. wt. 10,000, Stokes radius ~1.9 nm), FITC-dextran-20k (FD20 s, Sigma; mol. wt. 20,000, Stokes radius ~2.4 nm), FITC-dextran-40k (FD40 s, Sigma; mol. wt. 40,000, Stokes radius ~3.0 nm), and FITC-dextran-70k (FD70 s, Sigma; mol. wt. 70,000, Stokes radius ~3.6 nm), were used in experiments at the concentration of 1 mg/ml in Ringer solution containing 10 mg/ml BSA.

**Artificial Cerebrospinal Fluid (ACSF)**

The ACSF solution composition was (in mM) NaCl 110.5, KCl 4.7, CaCl2 2.5, KH2PO4 1.1, MgSO4·7H2O 1.25, NaHCO3 25 and HEPES 15 (Easton et al., 1997; Hu et al., 2005), and the solution was buffered to pH 7.4±0.5. All chemicals were obtained from Sigma.

**Permeability measurement**

The left or right carotid artery was cannulated with a PE50 tubing with I.D. 0.58 mm and O.D. 0.965 mm from Becton Dickinson, and the fluorescent solution was introduced into the cerebral circulation by a syringe pump at a constant rate of 3 ml/min. It took ~15 s for the dye from the cannulation site to the microvessel. Measurement of solute permeability \( P \) was made on individual post-capillary venule (20–40 μm in diameter). We followed the method described in Easton et al. (1997) for identifying post-capillary venules. When the dye was perfused into the pial microvessels, the images were recorded simultaneously. The \( P \) was determined off-line from the prerecorded images. Briefly, in recorded images, the total fluorescence intensity \( I \) in a rectangular window including the vessel lumen and surrounding tissue (Fig. 2) was measured by using the imaging analysis software (Intracellular Imaging Inc., Cincinnati, OH). The measuring window was at least 100–200 μm long and 40–100 μm wide and was set at least 100 μm from the base of the bifurcation to avoid solute contamination from the side arms. The size and placement of the measuring window were chosen to satisfy that 1) the vessel segment is straight; 2) the dye does not spread out of the window during the time for \( P \) measurement (5–30 s for the size of dyes used in our experiment); and 3) no contamination of dye from the neighboring vessels. When these conditions were satisfied, \( P \) that is defined as the solute outflow rate per unit vessel surface area divided by the concentration difference across the vessel wall, could be calculated from the relationship \( P = (1/ΔI_0) (dI/dt)_0 (r/2) \), where \( ΔI_0 \) is the step increase in fluorescence intensity in the measuring window as the test solute fills the microvessel lumen, \( (dI/dt)_0 \) is the initial rate of increase in fluorescence intensity after the solute fills the lumen and begins to accumulate in the tissue, and \( r \) is the vessel radius (Fig. 3) (Adamson et al., 1988; Fu et al., 1998; Fu and Shen, 2004; Huxley et al., 1987). We measured the diameter of the vessel and the total intensity of fluorescence in the lumen of the vessel segment from the recorded images and found no changes during the period in our experiments (up to 1 min). This indicates that there was no vasoactivity, which may induce the change in the vessel radius.

**Calibration experiments**

The primary assumption in the calculation of \( P \) with the use of fluorescent solutes is that the fluorescence intensity \( I \) is a linear function of the number of solute molecules (concentration) in the measuring field. To test the linear range of the intensity vs. the concentration, we used the same instrument settings in the *in vitro* calibration experiments as those used in *in vivo* permeability measurements. *In vitro* calibrations were performed using two chambers of different depths. One was a cell counting chamber (hemocytometer) of depth of 100 μm (Hauser Scientific, PA), and the other was the chamber constructed of glass cover slips (Adamson et

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**Fig. 2.** A pial microvessel before (A) and after (B) filled with the fluorescent solution. The fluorescence intensity in the rectangular window (in red) was measured to determine the solute permeability \( P \).
that for 0.1 mg/ml sodium fluorescence in our in vivo calibration experiments showed that the relationship between concentration and the fluorescence intensity was linear from 0.05 to 0.12 mg/ml for sodium fluorescein (Fig. 4), from 0.2 to 2 mg/ml for FITC-dextran-4k permeability, which is 0.88×10⁻⁶ cm/s. The right panel is for the permeability to FITC-dextran-70k, which is 0.12×10⁻⁶ cm/s.

al., 1994; Fu et al., 1998; Huxley et al., 1987). The latter chamber has a depth of approximately 170 µm. A large 24×50-mm cover slip formed the base of the chamber. Two small 22-mm² cover slips were laid on top of this base, ~1 cm apart, and a third small cover slip was placed on top of those to form a chamber, 170 µm deep. Solutions of fluorescence probes were applied to fill the chamber by capillarity. The chamber was cleaned before the solution of different concentration was applied. The measuring window was ~250 µm×300 µm. These in vitro calibration experiments showed that the relationship between the concentration and the fluorescence intensity was linear from 0.05 to 0.12 mg/ml for sodium fluorescein (Fig. 4), from 0.2 to 2 mg/ml for FITC-dextran with various molecular weights (not shown here) for both 100 µm and 170 µm chambers. We used 0.1 mg/ml sodium fluorescein, or 1 mg/ml FITC-dextran at 28% maximum power of illumination in our in vivo experiments.

Furthermore, we found in the in vitro photo bleaching experiment that for 0.1 mg/ml sodium fluorescein, or 1 mg/ml FITC-dextran at 28% maximum power of illumination, the intensity values fell less that 0.5% of their original values in ~1 min. One minute was typically 2 to 3 times of the exposure time required for an individual solute permeability measurement (Fig. 3). The low degree of photo bleaching was due to the reduced excitation light intensity and more sensitive camera for the image collection.

**Depth of light collection**

Since the measurements of fluorescence intensity for pial microvessels were performed on thick tissue, collection of the light from out-of-focus region would contribute to the measured value of intensity. To measure the depth of light collection of our system, we did in vitro experiments similar to Huxley et al. (1987) and Yuan et al. (1993) but using an imaging system to determine the fluorescence intensity. Four chambers with different depths, 50 µm from Intracellular Imaging Inc. (Cincinnati, OH), 100 µm (hemocytometer), 170 µm chamber formed by cover slips (described in the previous section), and 340 µm chamber with two cover slips stacked, were used to measure fluorescence intensity of sodium fluorescein solutions, which were filled in the chambers by capillarity. The experiment instrument settings were the same as in in vitro linear calibration experiments as well as in in vivo permeability measurement (20× lens, NA = 0.75, 28% maximum illumination). With the concentration-depth product kept constant, the solutions of sodium fluorescein were diluted according to the different depth of the chambers (Yuan et al., 1993). The intensity was measured for a window area ~250 µm×300 µm, and the focus was on the top surface of the solution. Results are shown in Fig. 5. Intensities for 50 µm and 100 µm deep chambers remained almost the same. However, for the chambers with depths larger than 100 µm, i.e. 170 µm and 340 µm chambers, the intensity decreased with increase of the depth of the chambers. The reason was that our experiment system can only collect the light from the solution in a smaller thickness region for the 170 µm and 340 µm chambers. Using a light collection index function defined in Yuan et al. (1993), we did the curve fitting for the measured total intensity in the chambers of different depths (Fig. 5). We found that within the depth of 95 µm (Z₀=95µm in their index function), our imaging system can collect all the light. Beyond this depth, our system can collect the light either partially or none. This in vitro experiment indicated that the depth of light collection of our system was ~100 µm (Huxley et al., 1987; Yuan et al., 1993). Within this depth of...
the light collection, the fluorescence intensity is proportional to the total number of fluorescent molecules and is independent of the chamber depth. This narrow depth of light collection in our system allowed us to minimize the influence from the light coming from other parts of the brain tissue, especially from the meninges.

Results and discussion

A summary of the apparent permeability P to various sized fluorescent tracers on individual post-capillary venules in shown in Fig. 6. Two ROIs per vessel were measured for P, and the average was the permeability of that vessel. The average of 2.5 (±0.8 SD, n=55, ranging from 1 to 4 vessels per animal) vessels were measured for each animal. Total 22 animals were used in the study. The apparent permeability of sodium fluorescein, P(FITC), was 2.71 (±0.76, SD; n=11)×10^-6 cm/s, ranging from 1.84 to 4.14×10^-6 cm/s. The apparent permeability of FITC-dextran-4k, P(extravasation) was 0.92 (±0.45, SD; n=10)×10^-6 cm/s, ranging from 0.36 to 1.79×10^-6 cm/s. The apparent permeability of FITC-dextran-10k, P(extravasation) was 0.31 (±0.13, SD; n=7)×10^-6 cm/s, ranging from 0.12 to 0.41×10^-6 cm/s. The apparent permeability of FITC-dextran-20k, P(extravasation) was 0.24 (±0.10, SD; n=6)×10^-6 cm/s, ranging from 0.11 to 0.34×10^-6 cm/s. The apparent permeability of FITC-dextran-40k, P(extravasation) was 0.19 (±0.11, SD; n=10)×10^-6 cm/s, ranging from 0.07 to 0.38×10^-6 cm/s. The apparent permeability of FITC-dextran-70k, P(extravasation) was 0.15 (±0.05, SD; n=11)×10^-6 cm/s, ranging from 0.07 to 0.27×10^-6 cm/s.

Evaluation of methods: open vs. non-open skull measurements

Table 1 compares the permeability values measured with and without the skull. For open skull measurements, the frontoparietal bone (right or left), ~5 mm×5 mm, was thinned with a micro-driller without the skull. For 5 vessels, P(open) was 2.50 (±0.65 SD)×10^-6 cm/s measured in 10 min, and 2.59 (±0.73 SD)×10^-6 cm/s in 30–60 min. No significant increase (p>0.8). For another 5 vessels, P(open) was 1.00 (±0.26 SD)×10^-6 cm/s measured in 10 min, and 1.09 (±0.33 SD)×10^-6 cm/s in 30–60 min, also no significant increase (p>0.6).

Table 1

<table>
<thead>
<tr>
<th>Solutes</th>
<th>P with bone (×10^-6 cm/s)</th>
<th>P w/o bone (×10^-6 cm/s)</th>
<th>Significance test p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>2.71 (±0.76), n=11</td>
<td>2.45 (±0.74), n=10</td>
<td>0.34</td>
</tr>
<tr>
<td>Dextran-10k</td>
<td>0.31 (±0.13), n=7</td>
<td>0.28 (±0.15), n=7</td>
<td>0.49</td>
</tr>
<tr>
<td>Dextran-20k</td>
<td>0.24 (±0.10), n=6</td>
<td>0.22 (±0.09), n=12</td>
<td>0.58</td>
</tr>
<tr>
<td>Dextran-40k</td>
<td>0.19 (±0.11), n=10</td>
<td>0.17 (±0.06), n=9</td>
<td>0.96</td>
</tr>
<tr>
<td>Dextran-70k</td>
<td>0.15 (±0.05), n=11</td>
<td>0.14 (±0.04), n=10</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Evaluation of methods: perfusion rate and RBCs

We chose the perfusion rate of 3 ml/min in injecting fluorescence solution through the carotid artery because 3 ml/min is the normal blood perfusion rate at the carotid artery for the rats we used in the experiments (García-Villalón et al., 1992). Although we completely changed the blood to the fluorescence solution from the carotid artery at this perfusion rate, there was still residue blood in the pial microvessels. The red blood cells (RBC) of the blood would induce an underestimate of the fluorescence intensity in the lumen, Δl0 in Fig. 3. We estimated that the red blood cell would account for 10% volume (see Appendix for the details) of the fluorescence solution in a pial microvessel at the perfusion rate of 3 ml/min from the carotid artery. The measured lumen fluorescence intensity would be only 90% of that of the effective solution. The effective solution means that the solute in the solution can travel across the vessel wall. The underestimated lumen intensity Δl0 would induce an 11% overestimation in the measured P. The corrected P for the influence of the red blood cells is shown in Table 2.

Evaluation of methods: free dye associated with FITC-labeled dextrans

We chose FITC as the labeling fluorophore for dextrans with different molecular weights to obtain high quantum yield (ratio of the number of fluorescence photons emitted to the number of photons absorbed) with low light excitation. However, FITC (mol wt 389.4) diffuses through capillary walls much faster than FITC-dextrans (mol wt ranging from 4000 to 70,000). A small amount of the free FITC will cause a large overestimation of the permeability to FITC-dextran molecules. We therefore measured the amount of free dye in FITC-dextran solutions. After being ultrafiltered by a clinical centrifuge
(1750 rpm, 444 g) through a centric filter (Millipore, 3000 mol. wt. cutoff) from the 1 mg/ml FITC-dextrans solutions used in our experiments, the filtrate was checked for fluorescence intensity due to free FITC ($k$). The method for measuring $k$ was the same as that described in the in vitro calibration, and the instrument settings were the same as those used for the $P$ measurements. The ratio of free dye to original solutions was less than 0.3% for all 1 mg/ml FITC-dextrans solutions. If we use measured $P$ to free dye effect (see previous section) and the weight of the average-weight dextran-20k. We used the same similar molecular weight of the average-weight dextran-40k, and the polymer of the average-weight, while the components that are overestimation in the overall permeability for the polydispersed components that are lighter than the average-weight would induce -40k and -70k (Fig. 7). Since the dextrans are polydispersed, the weight distributions of 3 largest dextrans in our study: dextrans-20k, Nano ZS (Malvern Instruments Ltd, UK) to measure the molecular conductivity $L$ of intermediate-sized and large molecules. Using the hydraulic values for the free dye are shown in Table 2.

Table 2 (Page 171)

<table>
<thead>
<tr>
<th>Solutes</th>
<th>No. of vessels</th>
<th>$P$ (measured) ($\times 10^{-6}$ cm/s)</th>
<th>$P$ (corrected for RBC) ($\times 10^{-6}$ cm/s)</th>
<th>$P$ (corrected for RBC and free dye) ($\times 10^{-6}$ cm/s)</th>
<th>$P$ (corrected for RBC, free dye and the solvent drag) ($\times 10^{-6}$ cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>11</td>
<td>2.71 ± 0.78</td>
<td>2.43 ± 0.68</td>
<td>2.43 ± 0.68</td>
<td>2.43 ± 0.68</td>
</tr>
<tr>
<td>Dextran4k</td>
<td>10</td>
<td>0.92 ± 0.46</td>
<td>0.83 ± 0.41</td>
<td>0.82 ± 0.41</td>
<td>0.82 ± 0.40</td>
</tr>
<tr>
<td>Dextran10k</td>
<td>7</td>
<td>0.31 ± 0.13</td>
<td>0.28 ± 0.12</td>
<td>0.27 ± 0.11</td>
<td>0.26 ± 0.11</td>
</tr>
<tr>
<td>Dextran20k</td>
<td>6</td>
<td>0.24 ± 0.10</td>
<td>0.22 ± 0.09</td>
<td>0.21 ± 0.09</td>
<td>0.21 ± 0.08</td>
</tr>
<tr>
<td>Dextran40k</td>
<td>10</td>
<td>0.19 ± 0.11</td>
<td>0.17 ± 0.10</td>
<td>0.16 ± 0.10</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td>Dextran70k</td>
<td>11</td>
<td>0.15 ± 0.05</td>
<td>0.14 ± 0.05</td>
<td>0.13 ± 0.04</td>
<td>0.13 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SD; RBC = red blood cells.

Effects of polydispersed dextrans

We purchased the FITC-dextrans from Sigma and used Zetasizer Nano ZS (Malvern Instruments Ltd, UK) to measure the molecular weight distributions of 3 largest dextrans in our study: dextran-20k, -40k and -70k (Fig. 7). Since the dextrans are polydispersed, the components that are lighter than the average-weight would induce an overestimation in the overall permeability for the polydispersed polymer of the average-weight, while the components that are heavier would induce an underestimation in the overall permeability. Fig. 7 shows that the lightest component of dextran-70k (11%) has similar molecular weight of the average-weight dextran-40k, and the lightest component of dextran-40k (6.1%) has similar molecular weight of the average-weight dextran-20k. We used the same formula for the free dye effect (see previous section) and the measured $P_{\text{dextran-40k}}$ (corrected for the free dye) to calculate the overestimation of $P_{\text{dextran-70k}}$ due to the size overlap. It is 3.1%. We used the measured $P_{\text{dextran-40k}}$ to calculate the overestimation of $P_{\text{dextran-40k}}$. It is only 2.1%. If we consider the underestimation of larger components for the dextran permeability, the overall overestimation will be less than these values.

Solvent drag contribution to the permeabilities for intermediate-sized and large molecules

Since solute flux can be coupled to water flow (solvent drag), the permeability coefficient $P$ measured in our experiments (apparent permeability) tends to overestimate the true diffusive permeability ($P_d$) of intermediate-sized and large molecules. Using the hydraulic conductivity $L_p$ value of the frog pial microvessel, 2.0 × 10⁻⁶ cm/s/cm H₂O, which was measured in Fraser et al. (1990), we calculated the diffusive permeability $P_d$ for FITC-dextrans (the data are summarized in Table 2) by employing the following formula in (Curry and Frokjaer-Jensen, 1984; Fu et al., 1998; Fu and Shen, 2003),

$$P = P_d \frac{P_f}{\exp(P_f) - 1} + P_d (1 - \sigma) \Delta P_{\text{eff}}$$

(1)

$$P_e = \frac{L_p (1 - \sigma) \Delta P_{\text{eff}}}{P_d}$$

(2)

$$\Delta P_{\text{eff}} = \Delta \rho \sigma \text{albumin} \Delta \sigma \text{albumin} \Delta \text{FITC-solute} - \Delta \text{FITC-solute}$$

(3)

Here $L_p$ is the hydraulic conductivity of the microvessel, $\sigma$ is the reflection coefficient of the microvessel to the solute, and $\Delta P_{\text{eff}}$ is the effective filtration pressure across the microvessel wall. $\Delta \rho$ and $\Delta \sigma$ are the hydrostatic and onotic pressure drops across the microvessel wall, respectively. Superscript FITC-solute can be either FITC-dextran-4k, FITC-dextran-10k, FITC-dextran-20k, FITC-dextran-40k, or FITC-dextran-70k.

Since no data were reported for the reflection coefficients of rat pial microvessel to FITC-dextrans, we estimated the reflection coefficients to dextrans based on those found in rat mesenteric microvessels to albumin (mot. wt. 67,000) and $\alpha$-lactalbumin (mot. wt. 14,176). $\sigma_{\text{albumin}}$ and $\sigma_{\alpha$-lactalbumin} of the rat mesenteric microvessel are 0.94 (Kendall and Michel, 1995) and 0.4 (Michel and Curry, 1999), respectively. Therefore, $\sigma_{\text{dextran-70k}}$ was assumed as 0.6, 0.5, 0.3, and 0.2, respectively. In the calculation, $\Delta P$ in the rat pial microvessel was ~10 cm H₂O (Mayhan and Heistad, 1986). $\Delta \rho_{\text{albumin}}$ was 3.6 cm H₂O for 10 mg/ml BSA. Using these parameters, the diffusive permeability of dextran-4k $P_{\text{dextran-4k}}$ was calculated as 0.82 × 10⁻⁶ cm²/s, which was 99% of its corrected apparent permeability (corrected for the RBC and the free dye); the diffusive permeability of dextran-10k, $P_{\text{dextran-10k}}$, was 0.26 × 10⁻⁶ cm²/s, which was 97% of its corrected apparent permeability; the diffusive permeability of dextran-20k, $P_{\text{dextran-20k}}$, was 0.13 ± 0.04 cm²/s, which was 95% of its corrected apparent permeability; and the diffusive permeability of dextran-40k, $P_{\text{dextran-40k}}$, was 0.13 ± 0.04 cm²/s, which was 93% of its corrected apparent permeability.
permeability of dextran-20k, \( P_{dextran-20k} \), was calculated as \( 0.21 \times 10^{-6} \text{ cm/s} \), which was 97\% of its corrected apparent permeability; and the diffusive permeability of dextran-40k, \( P_{dextran-40k} \), was calculated as \( 0.16 \times 10^{-6} \text{ cm/s} \), which was 97\% of its corrected apparent permeability. If the reflection coefficient of the pial microvessel is estimated as 0.99 instead of 0.94, the difference in corrected \( P_{dextran-70k} \) is 0.47\%.

Comparison of \( P \) of the pial microvessel and \( P \) of the mesenteric microvessel

In Fig. 8, we plot the diffusive permeability of the rat pial and mesenteric microvessels to various sized solutes. The measured permeability data for rat mesenteric microvessels are from Fu and Shen (2004) for a small molecule, sodium fluorescein, an intermediate-sized molecule, \( \alpha \)-lactalbumin and a large molecule, albumin. Fig. 8 shows that the solute permeability of rat pial microvessels is about an order of magnitude lower than that of rat mesenteric microvessels, from 1/11 for a small solute, sodium fluorescein, to 1/6 for a large solute, albumin or dextran 70k. We also plotted the permeability data for a small molecule, Lucifer Yellow (mol. wt. 457), measured by Easton and Fraser (1994) and Easton et al. (1997) using an open-skull method. We can see from Fig. 8, there is a good

![Fig. 8. Comparison of solute permeability \( P \) for rat pial microvessels ( ■ Current study; ▲ Easton et al., 1997) with that for rat mesenteric microvessels ( ●) (Fu and Shen, 2004). Values are means±SD.](image)

Fig. 9. A compartment model for estimating RBC concentration in pial microvessels during perfusion. (A) model schematic showing the perfusion to the half brain through the carotid artery: (B) concentration of RBC in microvessels as a function of perfusion time under two extreme conditions: 1) the perfusate is well mixed with the blood in the half brain and 2) the perfusate is not mixed with the blood.
agreement between Easton and Fraser's data and our results from a non-invasive measurement.

In summary, we developed a non-invasive method for measuring solute permeability in rat pial microvessels. This non-invasive method overcomes the disadvantage of previous in vivo methods due to exposing the brain tissue to the atmosphere, which induces the increase in microvessel permeability in longer time experiments. We will use this method in the future to quantify the cerebral microvessel permeability changes due to varied physiological and pathological conditions and to develop better strategies for drug delivery across the blood–brain barrier.

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Appendix

The compartment model developed in the appendix is used to estimate the RBC volume concentration in the pial microvessel during the dye perfusion through the carotid artery. Fig. 9A shows the schematic for this compartment model. The blood volume in half brain is denoted as \( V \), the RBC concentration is \( C \). The initial concentration of RBC is \( C_0 = 40\% \) (normal RBC volume concentration in the blood). The perfusion rate at the carotid artery is \( Q \).

(1) Well-mixing case: When the perfusate is well mixed with the blood in the brain, the RBC concentration at the brain circulation exit is the same as that in the circulation (in microvessels), which is \( C \). Conservation of RBC gives:

\[
\frac{dC}{dt} = -\frac{Q}{V} C
\]

(\text{A1})

With the initial condition, \( C(t=0) = C_0 = 40\% \), the solution for the RBC concentration in the microvessel,

\[
C = C_0 \exp \left( -\frac{Q}{V} t \right)
\]

(2) No-mixing case: When the perfusate is not mixed at all with the blood in the brain, the RBC concentration at the brain circulation exit is \( C_0 \). The \( C \) in the right hand side of Eq. (A1) should become \( C_0 \) and the solution for this no-mixing case is,

\[
C = \begin{cases} 
C_0 \left( 1 - \frac{Q}{V} t \right) & t \leq \frac{V}{Q} \\
C_0 & t > \frac{V}{Q}
\end{cases}
\]

Fig. 9B shows the RBC concentration as a function of time for these two cases when the perfusion rate \( Q = 3 \text{ ml/min} \) in our experiment and the blood volume in the half rat brain \( V = 0.5 \text{ ml} \) (Sandor et al., 1986).

We can see that in a perfusion time of \( \approx 15 \text{ s} \), the RBC concentration will be \( \approx 9\% \) if the perfusate is well mixed with the blood while it will be \( 0\% \) if there is no mixing. Therefore, in the real case the RBC concentration in the microvessel will be some value between \( 0\% \) and \( 9\% \). Taking into consideration of variations in the perfusion time and the blood volume in the brain, \( 10\% \) for the RBC concentration will be a reasonable upper estimate in our experiment.

References


