Characterization of Paracellular and Aqueous Penetration Routes in Cornea, Conjunctiva, and Sclera

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**Purpose.** To characterize quantitatively the paracellular permeation routes in rabbit cornea, conjunctiva, and sclera using polyethylene glycol (PEG) oligomers.

**Methods.** Corneal, conjunctival, and scleral tissues from New Zealand white rabbits were tested individually in a modified two-chamber Ussing apparatus with the mixture of PEGs with mean molecular weights 200, 400, 600, and 1000 in glutathione bicarbonated Ringer’s solution buffer on the donor side of the chamber. The samples and standards were analyzed with high-performance liquid chromatography–thermospray mass spectrometry method. The pore sizes and the pore densities of the corneal and conjunctival epithelia were calculated using an effusion-like approach.

**Results.** The conjunctival and scleral tissues were 15 to 25 times more permeable than the cornea and the molecular size affected the conjunctival permeability less than that of the cornea. The palpebral and bulbar conjunctivas had equal permeabilities. The scleral permeability was approximately half of that in the conjunctiva and approximately 10 times more than in the cornea. The conjunctival epithelia had 2 times larger pores and 16 times higher pore density than the cornea. The total paracellular space in the conjunctiva was estimated to be 230 times greater than that in the cornea.

**Conclusions.** The conjunctival epithelium, due to its higher membrane permeability and larger absorptive and intercellular space surface areas, is the most viable route for ocular delivery of peptides and oligonucleotides. Invest Ophthalmol Vis Sci. 1997;38:627-634.

The cornea is considered to be the main route by which topically applied drugs enter the eye.1,2 Another potential route of ocular drug absorption is across the bulbar conjunctiva and sclera into the uveal tract and vitreous humor. This route has been shown to be important, for example, in the case of inulin3 (molecular weight, 5000) and α-aminoclonidine4 (molecular weight, 245.1), both of which have poor corneal permeability. Conjunctival absorption also has been suggested as a potential route to deliver bioactive peptides to the systemic circulation.

Both in the corneal and conjunctival epithelia, the intercellular space is sealed by the junctional complexes and that hinders the transport of hydrophilic compounds, like many peptides.5-8 The corneal epithelium consists of basal columnar cells, two to three layers of wings cells, and one or two layers of squamous, polygonal-shaped superficial cells. The first and second flattened apical cell layers are the rate-determining barriers in the paracellular permeation of molecules.9 For example, after intravenous administration, no horseradish peroxidase activity was seen on the apical surface of the superficial cells in corneal epithelium, but the activity was present paracellularly in the wing cell and basal cell layers.9

The conjunctival epithelium can be divided into three morphologically distinct epithelia: bulbar epithelium, which is continuous with that of the cornea; fornix epithelium; and palpebral epithelium.10 The conjunctiva contains many mucous goblet cells, and the epithelium is two- to three-cell-layers thick, with cuboidal basal cells, and it contains tight junctions only on the apical surface.11 Consequently, the epithelial layer is the rate-limiting barrier for drug penetration in the cornea and conjunctiva. Sclera is composed of collagen and mucopolysaccha-
Animals

Male and female albino New Zealand rabbits weighing between 3.0 and 4.5 kg were used. The animals were housed in standard laboratory rabbit cages and fed regular diets with no restrictions on the amount of food or water consumed. All experiments conformed to the ARVO Resolution on the Use of Animals in Research.

Materials

PEGs with mean molecular weights of 200 (Mw/Mn = 1.11), 400 (Mw/Mn = 1.07), 600 (Mw/Mn = 1.10), and 1000 (Mw/Mn = 1.05) were obtained from Chemical Pressure (Pittsburgh, PA). Polypropylene glycol 425 (Aldrich, Steinheim, Germany) was used as an internal standard. All other chemicals were of analytic grade.

Polyethylene Glycol Solutions

PEG 200 (final concentration, 2.0 mg/ml), PEG 400 (4.0 mg/ml), PEG 600 (6.0 mg/ml), and PEG 1000 (10.0 mg/ml) were weighed and dissolved in glutathione bicarbonate Ringer’s solution (GBR). On the basis of mean molecular weight, the concentrations are 0.01 M.

The osmolarity of solutions was between 300 and 309 mOsm as determined on an Osmostat osmometer (Kyoto Kaqaku, Kyoto, Japan). The pH of the solution was adjusted to 7.65 at 37°C with oxygen–carbon dioxide (95:5) bubbling.

In Vitro Permeability Experiment

The rabbits were killed by a marginal vein injection of a lethal dose of T-61-vet (Hoechst, Munich, Germany). Conjunctivas were dissected from the palpebral (lower eyelid) or bulbar side (lower cul-de-sac). The bulbar conjunctivas were dissected without Tenon’s capsule. Then, an incision was made along scleral tissue and corneas were dissected, leaving a scleral ring (4 mm laterally from the limbus). Lens and iris were removed, and the remaining sclera with episclera was detached from the orbit. The sclera samples for permeability experiments were obtained from the superior temporal quadrant. The samples were without any muscle attachments or blood vessels. Six to seven tissues were used for each permeability determination.

The tissues were positioned between two plastic rings and placed within 25 minutes from the death in the center of the modified perfusion chamber as described previously. The exposed surface areas of the conjunctiva, sclera, and cornea were 0.28, 0.28, and 1.17 cm², respectively. GBR solution (6.5 ml) was added to the receptor side. Immediately thereafter, an equal volume of PEG in GBR was added to the donor side. Constant mixing of the reservoir solution was achieved by bubbling an oxygen–carbon dioxide (95:5) mixture, which maintained the pH at 7.65. The experiments were conducted at 37°C for a period of 4 hours. Samples of 1 ml were collected from the receptor chamber at 30-minute intervals and replaced with an equal volume of blank GBR buffer.

After each permeability experiment, the cornea was removed from the mounting rings, and the remaining scleral tissue was cut away. The cornea was weighed and dried at 50°C overnight. After reweighing, the water content of the cornea was calculated. In all cases, the corneal hydration levels were in the normal range (76% to 80%).

Analytic Procedure

Pure PEG oligomers to be used as analytic standards were obtained by preparative high-performance liquid chromatography (HPLC) using Shimadzu LC-6A Liquid Chromatograph (Kyoto, Japan), equipped with a Kromasil C-8 column, 1 cm × 25 cm (Eka Nobel, Bolus, Sweden) at a flow rate of 3 ml/minute. The eluent consisted of acetonitrile/water, 15/85 for PEG 300, 20/80 for PEG 600, and 22.5/77.5 for PEG 1000 (Fluka, Buchs, Switzerland). PEG oligomers were detected at 195 nm, the fractions were collected, and the eluent was evaporated in vacuum. Each fraction of oligomer was weighed, and the molecular weights and purities (>95%) of each fraction were deter-
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were used as standards for determination of oligomer concentrations in the polydisperse PEG mixtures used in permeation studies. The HPLC system with column of 4.5 mm × 15 cm was used. The acetonitrile-water gradient was 10% to 15% in 7.5 minutes and 25% in 30 minutes. Calibration standards were made at concentrations 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml for each oligomer. Six replicate samples of the PEG solution used in the donor side of the permeation chamber were analyzed.

The samples and the quantitation standards were analyzed with the HPLC–thermospray mass spectrometry (TSP–LC–MS) method. The method has been described in detail previously.20 Model 2900-0374 solvent delivery system (Applied Biosystems), Rhodyne 7125 injector (50 μl), and PRP-1 column (150 × 4.1 mm inner diameter, 10 μm particle size; Hamilton, Reno, NV) were used. The isocratic eluent consisted of ammonium acetate (0.1 M, pH 6)-acetonitrile (79:21, vol/vol). The flow rate was 1 ml/minute.

The LC–MS system was a vacuum generator (VG) thermospray probe coupled to a VG Trio-2 quadrupole mass spectrometer (VG Masslab, Manchester, UK). The ion source was modified by changing the original blunt tip repeller electrode to a needle tip electrode located 3 mm from the ion exit aperture. The ion source was at 210°C, the vaporized temperature 175°C to 200°C, and the ion repeller potential was 300 V. Other source parameters were tuned daily with [PEG + NH₄⁺] + ions at m/z 388, 608, and 916 by injecting PEG through a loop.

The oligomers in the PEG sample mixtures were quantitated using selected ion recording. Selected ion recording was based on the ammoniated molecules (MNH₄⁺ ions): m/z 384 for internal standard, polypropylene glycol, and m/z 256, 300, 344, 388, 432, 476, 520, 564, 608, 652, 696, 740, 784, 828, 872, 916, and 960 for PEGs. The method allows direct measurement of the permeation percentage for all 17 PEG oligomers in the mixture.

The HPLC–TSP-mass spectrometer was calibrated with PEG-spectrum and reference table of PEGs. Retention times of each oligomer were controlled with standards. The internal standard (polypropylene glycol, MNH₄⁺, at m/z 384) had retention time of 8 minutes. The samples were analyzed without any extraction procedure.

The quantitation standards were prepared by diluting the donor-side PEG solution with GBR buffer. These dilutions correspond to 0.002%, 0.05%, 0.1%, 0.3%, and 1% transmembrane permeation of PEG, respectively. Four to five calibration point graphs (duplicate injections) were collected for the range corresponding to 0.01% to 0.3% permeation by plotting the ratios of analyte and internal standard peak areas versus the amounts of analytic. (r² = 0.998 to 0.999). The precision of the TSP–LC–MS system was tested using within-day and day-to-day precision. Three same samples were analyzed repeatedly on six different days. The precision of the analyzing method was best by PEG in the molecular weights ranging from 238 to 810 g/mol (RSD, 4.7% to 19.8%). The bigger the molecular weight was, the worse was the precision. The reason for weak precision (at molecular weight >810) could be the contamination of the ion source with PEG.

Estimation of the Pore Sizes and Pore Density of the Epithelia

The penetrated amounts (milligrams) of PEGs were plotted versus time (minutes). The permeability coefficient P_app (centimeters/second) was calculated as oligomer flux (Jh) divided by its initial concentration in the donor chamber (milligram/milliliter). The lag time (Tlag) of permeation is the time required to reach the steady-state concentration gradient in the membrane. This is prerequisite for steady-state flux. It was defined as the extrapolated intercept of the linear part of the permeation curve on the time axis.

Effusion approach was used to calculate the limiting pore size and pore density. It is based on the low probability of molecules to find a pore as a limiting factor and therefore, permeation is not hindered by the transport in the pore. Another criteria for effusion-like process is the small rate-limiting barrier thickness. The theory and the derivation formula are given.21

In effusion-based approach, pore size and porosity are obtained from the following relation:

\[ J_h = \frac{RT\epsilon}{12\pi\eta r_s^2N_A\lambda} \]

where Jh/C, permeability of PEGs; rs, radius of PEG oligomer molecules; ε, porosity of the membrane; λ, jump length (i.e., 3.1 Å); η, viscosity of water; R, gas law constant; T, temperature; and NA, Avogadro’s number.

This equation predicts that the measured permeability, Jh/C, inversely is proportional to the radius of the drug molecule, and from the slope of this relation, Jh/C = f(1/rs), the porosity ε can be evaluated. Furthermore, the extrapolation into the zero permeability gives information for the critical value of the molecular radius still able to permeate into the hydrophilic pore of the membrane. Number of the pores is obtained from the relation ε = Αp/Α, where Αp is the effective surface area of the hydrophilic pathways (Αp = Α/ra; m is the number of paracellular routes in the area Α, and ra is the surface area of an individual orifice).
The molecular size ($r_s$) and diffusion coefficient ($D_{oo}$) of PEGs in water are needed to calculate the pore size and pore number. The radius of PEG molecules ($r_s$) was calculated based on the radius of gyration ($r_g^2$) for a spherical solute. Diffusion coefficients ($D_{oo}$) of PEGs were based on the treatment of Chin et al. The viscosity ($\eta$) of water was used, and the jump length was determined by the solvent (water, 3.1 Å). $R$, $T$, and $N_A$ had their usual meanings.

The corneal and conjunctival clearances (microliter/minute) for PEG 238 and 942 from donor compartment were calculated using equation $CL = P \times S$, where $P$ is the corneal or conjunctival permeability (centimeter/second) and $S$ is the corneal (1.59 cm$^2$) or conjunctival (15.13 cm$^2$) surface area obtained. Consequently, the first-order rate constant for elimination from tear compartment ($K_e$ [%/minute]) was calculated from the relation $K_e = CL/V_d$, where $V_d$ is the volume of PEG distribution in tear compartment (i.e., combined volumes of eyedrop and lacrimal fluid, 50 µl). The expected noncorneal permeabilities of PEG 238 and 942 (i.e., through bulbar conjunctiva + sclera) were calculated using equation $1/P_{nc} = 1/P_{cs} + 1/P_{sc}$, where $P_{nc}$ is the permeability in the noncorneal route, $P_{cs}$ is the permeability of the bulbar conjunctiva, and $P_{sc}$ is the permeability of sclera.

The statistical significance between the groups was tested using one-way analysis of variance. Values of $P < 0.05$ were considered to represent statistically significant differences.

RESULTS

PEG oligomers permeated through the cornea, sclera, and bulbar conjunctiva at a constant rate (Fig. 1). Because of the small fraction of PEG permeation (<2%) during 4 hours, PEG concentration in the donor chamber did not change significantly during the experiment.

Cornea

Corneal permeability of PEGs decreased with increasing molecular weight (Fig. 2A). Permeability coefficient of PEG was $1.03 \times 10^{-6}$ cm/second at molecular weight 238, and almost five times smaller $0.22 \times 10^{-6}$ cm/second at the molecular weight of 942. The permeability of PEGs decreased most rapidly at molecular weights below 414. At higher molecular weights, the permeabilities decreased slower, in particular above 500. The permeated fractions (percentage) of PEG varied from 0.25% ± 0.05% (PEG 238) to 0.06% ± 0.03% (PEG 942), respectively (Fig. 2A), whereas $T_{lag}$ ranged from 47 ± 5 minutes to 96 ± 36 minutes.

Conjunctivas

In general, both palpebral and bulbar conjunctivae were approximately 15 to 25 times more permeable than was the cornea (Figs. 2A, 2B, and 2C). Similarly, permeabilities of hydrophilic β-blockers, mannitol, and fluorescein isothiocyanate–dextran (molecular weight, 4400) were shown to be higher in bulbar conjunctiva than in cornea. In our study, permeabilit-
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![Graph A](file1)

![Graph B](file2)

![Graph C](file3)

![Graph D](file4)

FIGURE 2. The permeability coefficients (cm/sec) (■) and penetrated amounts (%) of PEGs (■) in the molecular weight range of 238 to 942 g/mol. Mean ± standard error of the mean. (A) Cornea. (B) Palpebral conjunctiva. (C) Bulbar conjunctiva, and (D) sclera. The percentage values are not comparable between membranes, because corneas had different exposed area than did conjunctivas and scleras.

ies of PEGs in palpebral and bulbar conjunctiva were equal.

Although increasing molecular weight decreased the penetration of PEG in both palpebral and bulbar conjunctiva, the effect of molecular weight was minor (approximately twofold to threefold), and statistically significant differences were seen only between molecular weight extremes 238, 898, and 942 (analysis of variance, $P < 0.05$) in bulbar conjunctiva (Figs. 2B, 2C).

**Sclera**

In sclera, the permeability of PEGs between molecular weights 238 and 942 decreased nearly three times. PEG 238 had the permeability coefficient of $8.80 \times 10^{-6}$ cm/second and PEG 942 $3.08 \times 10^{-6}$ cm/second (Fig. 1D). The permeability of PEGs decreased linearly with increasing molecular weight, and the permeated amounts of PEG 238 and PEG 942 were $0.53\% \pm 0.15\%$ and $0.19\% \pm 0.08\%$, respectively.

The estimated permeability of the noncorneal route (bulbar conjunctiva + sclera) was calculated to be $6.25 \times 10^{-6}$ cm/second for PEG 238 and $2.03 \times 10^{-6}$ cm/second for PEG 942. Compared to that of the cornea, the scleral permeabilities of PEG 238 and PEG 942 were six times and nine times higher, respectively.

**Epithelial Pore Size and Pore Density**

Conjunctival epithelium has larger ($P < 0.05$) paracellular pores than does the corneal epithelium. The pore diameter in the apical rate-limiting cell layer of corneal epithelium was $2.0 \text{ nm} \pm 0.2 \text{ nm}$ ($n = 7$). In palpebral conjunctiva and bulbar conjunctiva, the pore diameters were $4.9 \text{ nm} \pm 2.5 \text{ nm}$ and $3.0 \text{ nm} \pm 1.6 \text{ nm}$, respectively. For peptides with molecular weights $<550$ (tetrapeptides and smaller), the molecular diameter is approximately 1.0 to 2.0 nm, whereas lysozyme (molecular weight, 14,000) has a diameter of 4.1 nm. Therefore, the paracellular space of the conjunctiva appears to be large enough for permeation of small peptides and oligonucleotides (molecular weight, 5000 to 10,000). In contrast, the pores in the cornea allow paracellular permeation of only small molecules (molecular weight, $<500$).
Porosities of the corneal and conjunctival rate-limiting layers were $0.13 \times 10^{-6}$ and $1.71 \times 10^{-6}$, respectively, and the number of pores in the corneal and conjunctival surfaces was $4.3 \times 10^6 / \text{cm}^2$ and $20.2 \times 10^6 / \text{cm}^2$, respectively. The intercellular spaces occupy only a fraction of $30 \times 10^{-7}$ and $2 \times 10^{-7}$ of the total surface area of the conjunctival and corneal epithelia, respectively. Consequently, in the cornea (area, 1.59 cm$^2$), the total intercellular space is 0.03 $\times 10^{-3}$ mm$^2$ and in the conjunctivae (area, 15.1 cm$^2$), it is $7 \times 10^{-3}$ mm$^2$, respectively. For comparison, it was estimated that in the highly permeable intestinal epithelium, the intercellular spaces occupy approximately 0.01% of the total surface area of the epithelium (i.e., fraction of $1000 \times 10^{-7}$). The paracellular corneal clearance values for PEG 238 and PEG 942 from tear compartment were estimated to be 0.1 µl/minute ($K_a = 0.20%$/minute) and 0.02 µl/minute ($K_e = 0.04%$/minute), respectively (assuming $V_d = 50$ µl). Conjunctival (palpebral) clearances of the PEG 238 and 942 from the lacrimal fluid were calculated to be in vivo 15.0 µl/minute ($K_a = 30%$/minute) and 6.3 µl/minute ($K_e = 12.7%$/minute), respectively, assuming 50-µl volume in tear fluid and contact with the entire membrane. Because of the great paracellular porosity and permeability, the paracellular elimination from the tear fluid through conjunctiva is expected to be much greater than the elimination through cornea.

**DISCUSSION**

The permeability result of cornea is comparable with the reported progressively decreasing urinary recovery of PEG between molecular weights 200 and 400 after dosing of PEG as a 5% (wt/vol) solution into the stomach of rat. The result, however, differs from the corneal permeation data of Liaw and Robinson, who reported essentially constant apparent permeability coefficients for PEGs 206 to 415 and substantial decrease in the permeability at higher molecular weights. The differences may be because of different experimental and analytical methods. Liaw and Robinson used approximately 10 times greater initial PEG concentrations in the donor phase, the sample bathing medium was 0.16 M sodium chloride, and the analytic method was HPLC with refractometric detection. In concentrated solutions, the PEGs are in states of association, which differ from the individual molecular species, whereas in dilute solutions, PEG chains are expanded. It is also known that the salts in the aqueous solution may affect the conformation of PEG chains.

The results of permeability studies with rabbit conjunctivae show that neutral, hydrophilic molecules with molecular weights below 1000 are able to permeate equally through bulbar and palpebral conjunctiva, and, further, the solute size has not pronounced effect on permeability. Therefore, in terms of epithelial permeability, absorption of hydrophilic compounds to the palpebral side is not favored over bulbar side. In vivo, however, there are other factors (e.g., surface area, metabolic activity, vascularization) that may affect drug absorption. It is known that more than 20% to 50% of the instilled small molecular weight drug absorbs systemically through palpebral conjunctiva. Systemic absorption of [D-Ala$^2$-metenkephalinamide (molecular weight, 647) and insulin (molecular weight, 5778) through conjunctiva was 36% and 1%, respectively. The permeability of sclera was approximately half of that in the conjunctiva and approximately order of magnitude greater than in the cornea. The sclera has been shown to be more permeable than is the cornea for inulin and hydrophilic beta-blockers. Previously, Huang et al reported that solute size has more pronounced effect on scleral permeability than on corneal permeability, so that sucrose (molecular weight, 342) permeated eightfold faster than did inulin (molecular weight, 5000) in the cornea, whereas in sclera, the difference was 16-fold. In our study, the molecular weight had a most pronounced effect on the corneal permeability, and in sclera, the permeability was less affected by the molecular weight. Our observations are in line with the data of Ahmed and Patton, which show importance of noncorneal route in the ocular absorption of hydrophilic inulin.

Intercellular space was characterized in the cornea and conjunctiva. In these membranes, the rate-limiting barrier for hydrophilic compounds is the thin, most-apical cell layer in the epithelium, and anatomically, the intercellular space is much smaller than the area of the cells. Sclera does not fulfill these criteria.

The values for the critical pore size and porosity of the ocular surface based on controlled series of permeating analogues are the first quantitative determinations. These values are in line with published permeation of molecules. Because the surface of the corneal epithelium is impermeable to horseradish peroxidase (molecular weight, 40,000) and fluorescein isothiocyanate-dextran (molecular weight, 20,000), the intercellular spaces of the apical layer of corneal epithelium should be smaller than 3 nm. Furthermore, particles of 5 nm do not penetrate to the cornea. Other previous estimates for the pore size in the corneal epithelium are the molecular diameters of glycerol (1.2 nm) and inulin (3.0 nm). No attempts to estimate the pore size or porosity of conjunctiva have been reported.

In conclusion, using effusion-like calculations and polyethylene-glycol permeation, we determined the drug absorption-limiting pore sizes and porosities of
the ocular surface tissues. Both intercellular pore size and pore density in the cornea are much smaller than in the conjunctiva. Therefore, bulbar conjunctiva and sclera may constitute a more viable route for ocular peptide and oligonucleotide delivery than would the cornea.

Key Words

intercellular space, membrane porosity, molecular weight, paracellular permeation, polyethylene glycol

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References


