Permeation of polyethylene glycols across the tympanic membrane

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Abstract
Localized and non-invasive delivery of therapeutics across barriers in the body is challenging. Examples include the flux of drugs across the tympanic membrane (TM) for the treatment of middle ear infections, and across the round window to treat inner ear disease. With the emergence of macromolecular therapies, the question arises as to whether such delivery can be achieved with macromolecules. Here, we have used polyethylene glycols (PEGs) in solutions to investigate macromolecular permeation across the TM in the chinchilla ex vivo. As the molecular weight of PEG increased, flux across the TM decreased, with an exponential relationship between the apparent diffusion coefficient and the molecular weight of the polymers. PEG flux was further decreased if it was released from a poloxamer 407 hydrogel, and lessened with increasing hydrogel concentration. Our results provide a framework for understanding the permeation of macromolecules noninvasively across barriers.

Keywords
Polyethylene glycol; Molecular weight; Noninvasive trans-tympanic delivery; Across barriers

Introduction
Topical drug delivery, for local or systemic drug distribution, is often impeded by the stratum corneum, the outermost layer of the skin and some other tissues. The stratum corneum is impermeable to most molecules [1–2], particularly hydrophilic molecules or molecules with a molecular weight greater than 1 kDa [3]. Unlike some other biological barriers, such as the blood-brain barrier, there are no specialized systems in the stratum corneum to assist with the flux of macromolecules, such as receptor-mediated transcytosis [4]. A number of approaches have emerged to help drugs cross the stratum corneum

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These drugs have generally been small molecules; delivery of macromolecules remains challenging and could hinder the application of macromolecular therapies. Physical methods, particularly microneedles [5], and iontophoresis [6], or a combination of the two [7] have been particularly prominent in efforts to enhance transdermal delivery of macromolecules. Non-physical approaches for macromolecules have included ionic liquids [8–9], and other chemical permeation enhancers (CPEs) [10–11]. CPEs enhance the permeability of the stratum corneum by reversibly disrupting the lipid bilayers [2, 10]. CPEs have shown variable effectiveness in improving delivery of many molecules, mostly under 500 Da., for dermatological applications [12–13].

The tympanic membrane (TM), or eardrum, is a barrier that separates the external ear from the middle ear. The outer layer of the TM is a stratum corneum that is structurally similar to that in the skin, and is also impermeable to most molecules [1, 14]. There is increasing interest in drug delivery across that barrier, to treat acute and chronic diseases of the middle ear [14]. Otitis media (OM) is the most frequently diagnosed pediatric disease, with more than 12 million physician visits per year in the United States [15]. Localized delivery of active therapeutics such as antibiotics across the TM directly into the middle ear to treat OM could avoid systemic exposure to drugs, which can result in systemic side effects that can limit therapy, affect the gut microbiome, and possibly induce the development of antibiotic-resistant bacteria [16]. Compared with trans-tympanic injections [17–18], non-invasive treatment methods are very attractive [19–22]. However, the deeply recessed location of the TM, the sensitivity of the TM to touch, and the fact that many of the patients are children and therefore unlikely to remain still, make many physical means of crossing the TM unappealing. Recently, CPEs have been demonstrated effective to provide a non-invasive way to deliver small molecule antibiotics across the intact TM in a sustained ototopical drug delivery system [21–25]. Here we explore whether this CPE-based approach could also be used to enhance the delivery of macromolecules across the TM. Moreover, given the location where the CPE-polymer mixture is placed, standard means of keeping them in place (e.g., occlusive dressings) are not practical, necessitating a medium such as a thermosensitive hydrogel. The effect of that hydrogel on macromolecule flux is also studied.

Materials and methods

Materials

Sulforhodamine B sulfonyl chloride, Poloxamer 407 (Pluronic® F-127), tetrahydrofuran, triethylamine, sodium dodecyl sulfate (SDS), limonene and bupivacaine hydrochloride were purchased from Millipore Sigma (Burlington, MA), and polyethylene glycol (PEG) amines from JenKem Technology USA Inc (Plano, TX).

Preparation of sulforhodamine (SRB) labeled PEGs

PEG amine (500 mg) and triethylamine (10 equiv.) were dissolved in tetrahydrofuran (10 mL) at 0 °C, and sulforhodamine B sulfonyl chloride (1.5 equiv.) was added slowly. The reaction mixture was stirred at 0 °C for 2 h, allowed to warm to ambient temperature and stirred for 24 h. Free dye was removed by dialysis against deionized water and silica gel chromatography. Gel permeation chromatography (GPC) was performed in N,N-

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dimethylformamide with 0.04M LiBr on a Tosoh EcoSEC HLC-8320 system (Tokyo, Japan) equipped with a TSKGel GMH_H 7.8 x 300 mm column and RI/UV-vis detectors, with polystyrene standards. UV-vis absorption at 200–1100 nm was measured on an Agilent 8453 UV-vis G1103A spectrometer (Santa Clara, CA). The fluorescent emission was measured on an Agilent Cary Eclipse Fluorescence Spectrophotometer (Santa Clara, CA).

Formulations

All percentages are weight by volume percentage (w/v). Solutions of 1% PEGs without CPEs were made by simply dissolving powdered PEGs in aqueous solution, while 1% PEGs with 3CPEs were made by dissolving the powder PEGs with a stock solution of 1% SDS and 0.5% bupivacaine, followed by addition of limonene. Solutions of 10–25% P407 hydrogel formulations containing 1% PEGs and 3CPEs were made by adding powdered PEGs and P407 to a stock solution of 1% of SDS and 0.5% bupivacaine. After the polymers completely dissolved in a cold room (4 °C), limonene was added.

Study of gelation properties

Rheological properties were measured on a TA-Instrument DHR-2 (New Castle, DE) equipped with a parallel 25 mm diameter plate. The gap between the plate and the sample stage was 150 μm. In a typical temperature ramp experiment, the storage moduli (G') and loss moduli (G'') were recorded using linear oscillatory shear rheology measurements (100 rad s\(^{-1}\), 1% strain) from 5 °C to 45 °C at 1 °C min\(^{-1}\). Viscosity was measured at 37 °C as a function of shear rate between 0.1 and 1000 s\(^{-1}\).

Animal maintenance

Experiments were carried out in accordance with protocols approved by Boston Children’s Hospital Animal Care and Use Committee. Healthy adult male and female chinchillas weighing 500-to-650 g were purchased from Ryerson Chinchilla Ranch (Plymouth, OH).

Ex vivo trans-tympanic permeation experiment

The trans-tympanic permeation of SRB labeled PEGs was determined using auditory bullae harvested from healthy chinchillas, as reported [21]. Chinchillas were euthanized and decapitated, and the auditory bullae were dissected out undamaged. The bullae were placed with the external auditory canal facing up in 12-well plates containing 3 mL of PBS in each receiving chamber. Formulations (200 μL) were deposited onto the auditory bullae, placed at 37 °C, and solutions were replaced at 0.5, 1, 2, 6, 12, 24 and 48 hours. Permeation of PEGs across the TM into the receiving chambers at each time point was quantified using a BioTek Instrument Synergy Mx Microplate Reader (Winooski, VT). Standard concentrations of PEG-SRB solutions with each molecular weight were used to calculate the concentrations of the corresponding polymer across the TM to avoid deviation from possible non-labeled PEGs.

Statistics

Data are presented as means and standard deviations (n=4), calculated with Microsoft Excel. Unpaired t-tests were used to calculate the \( p \) values using Microsoft Excel.
Results and discussion

We investigated macromolecule permeation across the tympanic membrane (TM), using polyethylene glycol (PEG) as the model macromolecule. PEG was selected because different molecular weights are readily available, providing a series of macromolecules varying in polymer length (i.e., molecular weight) rather than chemical composition. PEGs and their copolymers are widely used as carriers in drug delivery systems. PEGs can also be easily labeled with fluorescent moieties for quantification. Here, the PEG molecules were labeled with sulforhodamine B (SRB). A recent work reported the possible instability of commercial PEG-amines [26]. The molecular weights and narrow distributions (Đ < 1.3) of the PEG-SRBs we synthesized were demonstrated (Figure S1 and Table S1). SRB-labeled PEGs (PEG-SRB) showed maximum light absorption at 570 nm and maximum emission at 590 nm (Fig. 1). The absorption and emission were not dependent on PEG length (Table S1).

The flux of molecules across the TM was assessed ex vivo as reported [21].

We used PEG-SRB in the ex vivo trans-tympanic permeation experiments. Chinchillas were used because their auditory system is structurally similar to that of humans [27]. Ex vivo experiments were used in this study as we have previously shown with small molecules that ex vivo flux correlated with in vivo flux across the TM [22].

Effect of CPE on trans tympanic permeation

Aqueous solutions of various molecular weights of PEG were made at 1% concentration (all concentrations are w/v), with or without a combination of chemical permeation enhancers (CPEs) termed 3CPE: 1% sodium dodecyl sulfate (SDS), 2% limonene, and 0.5% bupivacaine. This CPE combination has synergistic effects on flux across the TM [21, 23]. PEG solutions with 3CPE are referred to as PEG-3CPE.

We compared the flux of PEG across the TM from solutions with or without 3CPE. Without 3CPE, there was little PEG flux across the TM (Fig. 2a; see Fig. S3a for zoomed plot). PEG permeation increased significantly after 3CPE was introduced (Fig. 2b). Taking PEG5k as an example, permeation of PEG5k-3CPE was 40.4 ± 8.9 μg at 6h, 7.7 times higher (p < 0.05) than PEG permeation without 3CPE (5.2 ± 2.7 μg). At 48h, PEG permeation from PEG5k-3CPE was 704 ± 61 μg, which was 26.5-fold higher (p < 0.05) than that from PEG permeation without 3CPE (26.6 ± 10 μg). Similar increases of about 20-fold (p < 0.05) in PEG permeation with introduction of 3CPE were observed for PEGs with other molecular weights (Fig. 2c). When the molecular weight of PEG was higher than 20 kDa in absence of 3CPE, the flux across the TM was undetectable. These increases in PEG permeation were consistent with previous findings with the small molecule drug ciprofloxacin (~15-fold increase in flux due to 3CPE) [23]. The results suggest that the ability of 3CPE to enhance permeability extends across a broad range of molecular weights.

Effect of molecular weight on trans-tympanic permeation

In the presence of 3CPE, PEG flux decreased with increasing PEG molecular weight, at all time points. For example, increasing the molecular weight of the PEG from 1 kDa to 30 kDa (Fig. 3a; see Fig. S2 for data at 6h) decreased the polymer flux from 1246 ± 66 μg to 105 ±
We hypothesized that PEGs in formulations need two steps to cross the TM (Fig. 4): (1) diffusion of PEGs within the formulation, to the interface between the formulation and TM, and (2) diffusion of PEGs across the TM. Since molecules can move more freely in solution than in the TM, the first step should be relatively fast. Therefore, the second step would be the rate determining step. The PEG diffusion process within the TM can be described by the diffusion equation:

\[ D = \frac{k_B T}{6 \pi \eta R_h} \]  

where \( D \) is the diffusion coefficient; \( k_B \) is Boltzmann constant; \( T \) is temperature; \( \eta \) is viscosity of the medium within the membrane; and \( R_h \) is hydrodynamic radius. Because all experiments were carried out under the same condition, \( k_B \frac{T}{6 \pi \eta} \) is constant for all samples. Therefore, \( D \) is inversely proportional to \( R_h \) or

\[ D \propto \frac{1}{R_h} \]  

Flexible polymers like PEGs can be treated as random coils (spheres), whose \( R_h \) is proportional to \( R_g \) (radius of gyration) \([28]\), and the \( R_g \) of the polymers can be estimated by Eq. 3: \([29–30]\)

\[ R_g = b (N/6)^{1/2} \]  

where \( b \) is the Kuhn length of PEG; and \( N \) is the degree of polymerization. All the polymers have the same repeating unit (oxyethylene), thereby, \( b \) is constant for all groups, and \( N \) is proportional to the molecular weight (\( M \)). Therefore, the following relationship is obtained:

\[ R_h \propto R_g \propto N^{1/2} \propto M^{1/2} \]  

\[ D \propto \frac{1}{R_h} \propto M^{-1/2} \]  

or \( D = aM^{-1/2} \) (where \( a \) is a constant)

If we take the logarithm of both side of Eq. 6, then:

\[ \lg D = -1/2 \lg M + \lg a \]  

A linear relationship between \( \lg D \) and \( \lg M \) is derived, with a slope of \(-1/2\). To assess the correlation of the derivation with our experimental observations, we calculated the apparent diffusion coefficients (\( D_{exp} \)) of PEGs from the data in Fig. 2b, as described: \([22]\)
\[ D_{\text{exp}} = \frac{h^2}{(6 \times x_0)} \]  

(8)

where \( h \) is the thickness of the stratum corneum layer in chinchilla TMs, and is about 4.7 um; \( x_0 \) is the absolute value of the \( x \)-intercept determined from a linear fit of the data in Fig. 2b. As seen in Fig. 3b, \( D_{\text{exp}} \) decreased markedly with increasing molecular weight. When plotted on a logarithmic scale (Fig. 3c), using the regression equation below:

\[ \log D_{\text{exp}} = -0.51 \log M - 13.76 \]  

(9)

a linear relationship between \( \log M \) and \( \log D_{\text{exp}} \) was obtained (\( R^2 = 0.91 \)). The molecular weight of SRB was included in the calculation. The experimental value of the slope was \( -0.51 \), which was close to the derived value of \( -1/2 \) in Eq. 7. The consistency between experimental result and derivation supported our assumption that permeation of PEG solutions across the TM is mainly controlled by the diffusion. The diffusion coefficient across the TM decreased exponentially with increasing molecular weight of PEG. Exponential relationships between diffusion constant and molecular weight have been reported for polymers in solutions or melts \[31\text{-}33\].

**Effect of hydrogels on trans tympanic permeation**

Hydrogel systems can provide prolonged drug release compared to solutions \[34\]. Poloxamer 407 (P407, also known as Pluronic® F127) is a polymer with reverse thermal gelation properties that is used in drug delivery. Its solution is an injectable liquid at low temperature, which gels at around body temperature \[35\text{-}36\]. P407 has been used in several otic drug delivery systems (e.g., OTIVIDEX™) \[37\]. Our group has recently used hydrogel systems based on P407 and its derivatives as the vehicles that would hold ciprofloxacin and CPEs against the TM, to promote trans-tympanic flux of ciprofloxacin \[22, 24\text{-}25\]. Here, we studied the effect of the concentration of P407 in the hydrogel on the trans-tympanic permeation of PEG using PEG5k as the example.

The hydrogels were formulated so that 1% PEG was contained in 10% to 25% P407 in water, with 3CPE. They are referred to as PEG5k-3CPE-10% [P407], PEG5k-3CPE-15% [P407], PEG5k-3CPE-18% [P407], and PEG5k-3CPE-25% [P407]. The flux of PEGs across the TM was evaluated as above. Flux at 48 h of PEG across the TM at 37 °C was inversely related to P407 concentration: 98 µg with PEG5k-3CPE 10% [P407] and 2 µg with PEG5k-3CPE 10% [P407] (Fig. 5), both significantly lower (7 to 300-fold, \( p < 0.05 \)) than PEG5k flux from solution (no P407) over the same period (704 µg).

The dependence of PEG flux on gel concentration may be attributable to effects on mobility of the PEGs within the formulations. The impact of the gel on PEG diffusion (step 1 in Fig. 4) is seen in the following analysis. At 48h, 704 µg PEG5k out of 2000 µg crossed the TM when the PEG was in simple solution, i.e., the TM prevented 1296 µg of the total PEG from getting across the TM. In contrast, with the introduction of 25% P407, only 2 µg of PEG 5k crossed the TM (i.e., PEG flux was decreased by a further 702 µg). Thus, the gel at its highest concentration tested here contributed to 35% (702 µg / 1998 µg) of the impedance to flux across the TM.
The reduction of flux by P407 was due to gelation at body temperature, slowing PEG diffusion within the gel, so that step 1 became a substantial factor. The effect of P407 concentration on PEG release was therefore related to changes in rheological properties (Fig. S4). After immersion in a 37 °C water bath, PEG5k-3CPE-10% [P407] still flowed like a liquid, while formulations with 15% or more P407 did not. (Fig. S5). Viscosity and moduli of the gels at 37 °C increased with increasing P407 concentration, leading to decreased PEG flux at 48h (Fig. 5c). These results were consistent with previous reports that gels with higher concentration had denser polymer networks that will more effectively slower molecular diffusion in the gels [38].

Conclusion

The permeation of PEGs across the chinchilla TM was accelerated about twenty-fold by chemical permeation enhancers. This acceleration by the CPEs was seen across a range of molecular weights of PEGs from 1k Da to 30kDa in this work, as it has for small molecules such as ciprofloxacin [21–23], tetrodotoxin, and bupivacaine [25], which have different hydrophilicities. Polymer flux in the presence of CPEs decreased exponentially with increasing molecular weight. We showed that the permeation of PEG solutions across the TM was a diffusion-controlled process, where the diffusion constant was inversely related to the molecular weight. The flux also decreased when the PEG was released from P407 hydrogels, and decreased further with increasing gel concentration. The decrease in flux correlated with the increase in the storage moduli and viscosities of the gels.

This work used a series of PEGs as the model macromolecules to focus on the effect of molecular weight. Other macromolecular properties (e.g., charge, hydrophilicity) could clearly also have an effect. Nevertheless, our results provide a framework for the future design of noninvasive therapies where macromolecules have to cross barriers within the body, such as the TM, the round and oval windows (between the middle and inner ear), and others.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Sulforhodamine (SRB) labeled polyethylene glycols (PEGs). (a) General reaction scheme, (b) UV-vis absorption spectra and (c) fluorescent spectra of PEG-SRB aqueous solutions.
Fig. 2.
Effect of chemical permeation enhancers (CPEs) on \textit{ex vivo} permeation of the tympanic membrane by PEG. (a) Time course of cumulative permeation of various molecular weights of PEG (1\% in aqueous solution; 200 μL) across the TM in the absence of CPEs. See Fig. S3a for zoomed plot. (b) Time course of cumulative permeation of the same PEGs as in panel (a) across the TM, in the presence of 3CPE. (c) Increase (as a multiple) in cumulative flux at 48h due to 3CPE. All concentrations are w/v. Data are means ± SD (n = 4). 3CPE is 1\% sodium dodecyl sulfate, 2\% limonene, and 0.5\% bupivacaine.
Fig. 3.
Effect of molecular weight on ex vivo trans-tympanic permeation of PEGs in presence of 3CPE. (a) Cumulative permeation of PEGs across the TM at 48h as a function of molecular weight. * indicates $p < 0.05$. Data are means ± SD ($n = 4$). (b) Relationship between PEG molecular weight and diffusion coefficient. (c) Linear fitting of data in panel (b) in logarithmic scale.
Fig. 4.
Illustration of the diffusion process of PEG across the TM. Panel on the right is a magnified view of the outlined section in the panel on the left. Step 1 is diffusion of PEGs within the formulation, to the interface between the formulation and TM, and Step 2 is diffusion of PEGs across the TM.
Fig. 5.
Effect of the concentration of P407 hydrogels on ex vivo trans-tympanic permeation of PEGs. (a) Time course of cumulative permeation of PEG across the TM in the presence of 3CPE and various concentrations of P407 (200 μL). (b) Cumulative flux of PEG across the TM at 48h (from panel (a)) from gel and solution (non-gel) formulations. Data are means ± SD (n = 4). * indicates p < 0.05. (c) Effect of P407 concentration on decrease (as a multiple, black curve) in cumulative flux of PEG across the TM at 48h compared with PEG solution (no P407), storage moduli (G', red curve) at 37 °C, and viscosity with 200 s⁻¹ shear rate at 37 °C (blue curve). (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)