Permeation of Proteins, Oligonucleotide and Dextrans Across Ocular Tissues: Experimental Studies and a Literature Update

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ABSTRACT: Proteins and oligonucleotides represent powerful tools for the treatment of several ocular diseases, affecting both anterior and posterior eye segments. Despite the potential of these compounds, their administration remains a challenge. The last years have seen a growing interest for the noninvasive administration of macromolecular drugs, but still there is only little information of their permeability across the different ocular barriers. The aim of this work was to evaluate the permeation of macromolecules of different size, shape and charge across porcine ocular tissues such as the isolated sclera, the choroid Bruch's membrane and the cornea, both intact and de-epitelialized. Permeants used were two proteins (albumin and cytochrome C), an oligonucleotide, two dextrans (4 and 40 kDa) and a monoclonal antibody (bevacizumab). Obtained data and its comparison with the literature highlight the difficulties in predicting the behavior of macromolecules based on their physicochemical properties, because the interplay between the charge, molecular radius and conformation prevent their analysis separately. However, the data can be of great help for a rough evaluation of the feasibility of a noninvasive administration and for building computational models to improve understanding of the interplay among static, dynamic and metabolic barriers in the delivery of macromolecules to the eye. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2190–2202, 2015

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INTRODUCTION

Macromolecular drugs, such as proteins and oligonucleotides, represent nowadays important therapeutic tools in the treatment of ocular diseases. For instance, bevacizumab (149 kDa), ranibizumab (48 kDa), pegaptanib (50 kDa) and aflibercept (115 kDa) are currently in use (approved or off-label) for the treatment of neovascularisation in both anterior and posterior segment eye diseases; CNTF (ciliary neurotrophic factor, 23 kDa) is in clinical trial for the treatment of retinitis pigmentosa and macular telangiectasia (Phase II).[1] Several other proteins are under investigation,[2,3] as well as oligonucleotides and other nucleic acids, as the inhibition of gene expression also represents a valuable therapeutic option for the treatment of diverse ocular pathologies for both the anterior[4] and posterior segment.[5] It is possible to expect that the understanding of the molecular basis of the pathologies will further increase the number of possible drug candidates in next years.

Drug administration to the eye is a difficult task because of the peculiar structure of this organ and the presence of static and dynamic barriers protecting the internal tissues. Presently, drug bioavailability to the anterior chamber after topical application is very limited (lower than 5%) because of the short residence time of the formulation on the ocular surface and the very low permeability of the cornea. Cornea consists of an external epithelium, a collagenous layer (stroma), and an internal endothelium (Fig. 1). The posterior segment of the eye is even more difficult to target: topical application is not efficient and systemic administration is hindered by the presence of the blood–retinal barrier. Bioavailability problems related to the posterior segment make the intravitreal injection the present option for drug administration. However, many studies are ongoing to evaluate the trans-scleral route as a possible noninvasive alternative to target the posterior segment. In this case, to reach the retina, drugs must diffuse across the sclera, choroid, Bruch's membrane and retinal pigmented epithelium (RPE) (Fig. 2).

Many papers on permeability of ocular barriers toward small drugs have been published, in order to understand how physicochemical characteristics such as the molecular weight (MW), lipophilicity and ionization can impact on the flux.[6] Permeability data are of utmost importance as it provides the basis for the development of *in silico* models, which are crucial for predicting the rate of drug delivery to the eye and ocular bioavailability.[7–10] One of the problems in the building and validation of *in silico* models for macromolecules is the lack of an extensive collection of their permeability data. Such a collection should be based on data obtained from several laboratories and for macromolecules with a wide range of physicochemical properties.

The aim of this work was to evaluate the permeation of macromolecules of different size, conformation and charge

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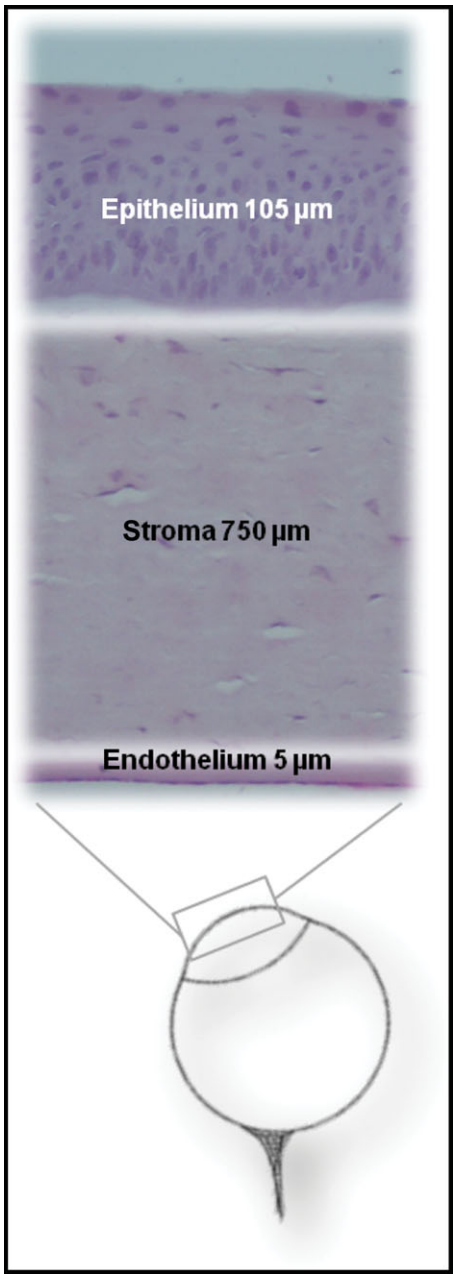

Figure 1. Schematic representation of the barriers involved in the permeation across the cornea. The outermost layer is the epithelium, followed by a connective tissue (stroma) and endothelium. The image is not to scale. The thickness reported is relative to porcine tissues.

across porcine ocular tissues, in order to collect data of the molecular characteristics impacting their permeation. In particular, the isolated sclera, the bilayer choroid Bruch's membrane, and the cornea will be studied as barriers. As some corneal pathologies compromise the integrity of the corneal epithelium, permeation across de-epithelialized cornea will also be performed. Permeants used will be two model proteins (albumin, 66 kDa and cytochrome C, 12.4 kDa), a model oligonucleotide (7.9 kDa), two dextrans (4 and 40 kDa) and bevacizumab (149 kDa), a monoclonal antibody with the therapeutic rationale. The accumulation of bevacizumab and oligonucleotide in the cornea and sclera after short application times (30 min) will also be investigated using fluorescence microscopy. All collected data will be then compared with the literature and referred to *in vivo* and *in vitro* permeation experiments through human and animal ocular barriers. The availability of this permeability data can be of great help for the development of accurate pharmacokinetic models, an important prescreening tool to estimate the likelihood of obtaining therapeutic concentrations at the target site.[11]

MATERIALS AND METHODS

Materials

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma–Aldrich (St. Louis, Missouri), as well as bovine serum albumin (BSA), fluorescein isothiocyanate conjugate (FITC-BSA, MW 66 kDa), fluorescently labeled dextrans (FD-4, MW 4 kDa; FD-40, MW 39 kDa), cytochrome C (CYTC, MW 12.4 kDa) and melanin from *Sepia officinalis*. A 24-mer single-stranded DNA (OLIGO, 5′-dAdCdC dTdGdG dGdAdC dAdTdC dGdTdT dCdCdA dTdTdC dAdTdA-3′, MW 7287.8 Da) was synthesized and labeled at the 5′ end with fluorescein at the Centre for Drug Research, University of Helsinki (Helsinki, Finland). Avastin® (Roche Pharma, Basel, Switzerland) was used as a source of bevacizumab (BEVA, MW 149 kDa). Composition: 25 mg/mL bevacizumab; 60 mg/mL α,α-trehalose dihydrate; 0.4 mg/mL polysorbate 20; 5.8 mg/mL sodium phosphate monobasic monohydrate; 1.2 mg/mL sodium phosphate dibasic anhydrous (pH 6.2).[12] Bevacizumab FITC conjugation occurs through the free amino groups of bevacizumab, forming a stable thiourea bond.[13] The derivatization procedure was explained in detail in a previous paper.[14] Briefly, 250 µL of a FITC solution (1 mg/mL) in 0.1 M carbonate/bicarbonate buffer (pH 9) were added to 1 mL of bevacizumab (5 mg/mL) in 0.1 M carbonate/bicarbonate buffer. The mixture was incubated at 20 ± 2°C for 2 h protected from light. Then, the labeled bevacizumab was separated from the free FITC on a Sephadex G-25M column (Sigma–Aldrich). The concentration of bevacizumab [$\text{Beva}_{(\text{mg/mL})}$] after conjugation was determined by measuring the absorbance at 280 nm (A_{280}), assuming that the extinction coefficient ($E_{280}^{0.1\%} = 1.4$) was not altered by conjugation[15] and taking into account the correction factor because of the absorbance of FITC at 280 nm ($0.35 \times A_{495}$)[15,16]:

$$\text{Beva}_{(\text{mg/mL})} = \frac{A_{280} - (0.35 \times A_{495})}{E_{280}^{0.1\%}} \quad (1)$$

Buffer solutions were HEPES-buffered saline (5.96 g/L HEPES, 9.0 g/L NaCl pH 7.4 with NaOH 5 N) and phosphate-buffered saline (PBS; 0.19 g/L KH_2PO_4, 5.98 g/L $Na_2HPO_4 \cdot 12H_2O$, 8.8 g/L NaCl pH 7.4 with H_3PO_4). All the other chemicals used were of analytical grade.

Tissue Preparation

Fresh porcine eyes were isolated from Landrace and Large White (age 10–11 months, weight 145–190 kg, both female and male animals) and were supplied from a local slaughterhouse (Annoni S.p.A., Parma, I). The eyes were kept in PBS at ±4°C until the dissection carried out within 2 h from the enucleation. In the first step, muscular and connective tissues around the eye-bulb were completely removed. The isolation of the cornea