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Supervisor: Assistant Prof. Ivan Pepić, PhD

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BIOFARMACEUTSKA ISPITIVANJA OFTALMIČKIH EKSCIPIJENSA NA *IN VITRO* I *EX VIVO* MODELIMA ROŽNICE

DOKTORSKI RAD

Mentor: doc. dr. sc. Ivan Pepić

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SUMMARY

Bioavailability of topical ophthalmic products is, apart from drug properties, substantially determined by the type and concentration of excipients. In addition to the use in conventional ophthalmic formulations, excipients are increasingly utilized in the development of innovative nanosystem-based ophthalmic formulations, in which excipients are supramolecularly organized into nano-sized structures, which can specifically interact with the anterior eye segment barriers and enhance the drug eye-related bioavailability. Evaluation of the drug transcorneal permeability is a critical step in predicting eye-related bioavailability of topical ophthalmic drugs. In this thesis, the effect of unimers and supramolecular aggregates of the ophthalmic excipients on corneal barrier properties and ophthalmic drug permeability was evaluated, using *in vitro* (HCE-T cell-based) and/or *ex vivo* (excised porcine cornea) corneal models. Histological and functional characterization of the *in vitro* corneal model revealed variability of the HCE-T barrier phenotype, which highlights the important aspects to be considered in the further model development. HCE-T cell-based model of a specific histological structure, but showing improved barrier tightness and a very strong correlation of ophthalmic compounds permeability with excised porcine cornea, was shown to be suitable for the ophthalmic drug permeability evaluation considering the influence of ophthalmic excipients. Biopharmaceutical evaluation of melatonin-loaded nanosystems using HCE-T cell-based models revealed significant influence of chitosan on nanosystems biopharmaceutical properties. In lecithin/chitosan nanoparticles the presence of chitosan ensured mucoadhesive properties and prolonged melatonin release, controlling melatonin *in vitro* transcorneal permeation, which may provide enhanced melatonin eye-related bioavailability and therapeutic effect. The *in vitro*/*ex vivo* evaluation of the effect of nonionic surface active ophthalmic excipients on the ophthalmic drug permeability revealed their concentration-dependent permeability-decreasing effect, which was potentially related to the association of ophthalmic drugs with self-aggregates of surface active ophthalmic excipients. The results of this thesis provide valuable insight into the effect of ophthalmic excipients on ophthalmic drug transcorneal permeability, which may contribute to more directed and economical development of both conventional and innovative topical ophthalmic products.

KEYWORDS: ophthalmic drugs, ophthalmic excipients, surfactants, nanosystems, drug permeability, eye-related bioavailability, *in vitro*/*ex vivo* corneal models, HCE-T.

SAŽETAK

Uvod: Razvoj topikalnih oftalmičkih pripravaka veliki je izazov farmaceutske industrije zbog različitih anatomskih i fizioloških barijera prednjeg segmenta oka koje značajno ograničavaju apsorpciju i bioraspoloživost topikalno primijenjenog oftalmičkog lijeka. Zbog izravne primjene topikalnog oftalmičkog lijeka na površinu rožnice preko koje se odvija permeacija lijeka do ciljnog mjesta djelovanja, sastav oftalmičke formulacije, odnosno vrsta i udio oftalmičkih ekscipijensa, značajno utječe na permeabilnost i bioraspoloživost topikalno primijenjenog oftalmičkog lijeka. Osim u konvencionalnim oftalmičkim oblicima, ekscipijensi sve više primjenu pronalaze u razvoju inovativnih oftalmičkih oblika temeljenih na terapijskim nanosustavima za dostavu lijeka. Naime, uslijed interakcija u određenim uvjetima ekscipijensi tvore supramolekulske strukture nanodimenzija, za koje je pokazano da mogu povećati bioraspoloživost lijeka prevladavanjem barijera prednjeg segmenta oka. Detaljno poznavanje utjecaja oftalmičkih ekscipijensa na permeabilnost oftalmičkih lijekova kroz barijeru rožnice može stoga značajno pridonijeti usmjerenosti i ekonomičnosti razvoja konvencionalnih i inovativnih oftalmičkih pripravaka. Cilj ovog doktorskog rada je korištenjem *in vitro* staničnog HCE-T modela epitela humane rožnice i *ex vivo* tkivnog modela rožnice svinje evaluirati biofarmaceutske značajke unimera i supramolekulskih agregata oftalmičkih ekscipijensa s obzirom na njihov potencijal promjene bioraspoloživosti topikalnih oftalmičkih lijekova, temeljem utjecaja na barijerna svojstva *in vitro* i *ex vivo* modela rožnice i permeabilnost oftalmičkih lijekova.

Metode: *In vitro* HCE-T model epitela humane rožnice uzgajan je prema standardiziranom protokolu korištenjem komercijalno dostupnih Transwell® ploča. Za potrebe *ex vivo* ispitivanja permeabilnosti korištene su svježe izolirane rožnice svinje uz upotrebu vertikalnih difuzijskih ćelija. Histomorfološka analiza *in vitro* HCE-T modela epitela humane rožnice provedena je svjetlosnom i fluorescencijskom mikroskopijom prethodno fiksiranog i obojenog modela. Karakterizacija barijernih svojstava *in vitro* i *ex vivo* modela rožnice provedena je mjerenjima transepitelnog električnog otpora (TEER) te ispitivanjima permeabilnosti oftalmičkih supstancija (timolol maleat, diklofenak natrij, kloramfenikol, deksametazon, fluorescein natrij) različitih fizičko-kemijskih svojstava. Fizičko-kemijska karakterizacija priređenih nanosustava provedena je dinamičkim raspršenjem svjetlosti te laserskom Doppler anemometrijom, dok je za ispitivanje oslobađanja melatonina iz nanočestica primijenjena metoda dijalize. Biokompatibilnost nanočestica ispitana je korištenjem MTT testa vijabilnosti stanica na monosloju HCE-T stanica. Mukoadhezivnost

nanočestica određena je ispitivanjem adhezije nanočestica na monosloju HCE-T stanica. Agregacijska svojstva oftalmičkih površinski aktivnih ekscipijensa određena su dinamičkim raspršenjem svjetlosti. Za kvantitativno određivanje oftalmičkih supstancija korištena je spektrofluorimetrija pomoću čitača mikrotitarskih ploča, kromatografija visoke djelotvornosti (HPLC) te kromatografija ultra-visoke djelotvornosti (UPLC).

Rezultati: *In vitro* HCE-T model humanog epitela rožnice uzgajan je prema standardiziranom protokolu uz upotrebu dvije vrste polikarbonatne membrane različite veličine pora. Histomorfološkim ispitivanjima utvrđeno je postojanje dva različita fenotipa *in vitro* HCE-T modela humanog epitela rožnice, ovisno o veličini pora membrane. Model I (veličina pora 0,4 μm) građen je od lipofilnog višeslojnog epitela HCE-T stanica na apikalnoj strani membrane. Model II (veličina pora 3,0 μm) građen je od lipofilnog monosloja HCE-T stanica u apikalnom dijelu te dodatnog lipofilnog monosloja migriranih HCE-T stanica u bazolateralnom dijelu membrane. Slaba barijerna svojstva te značajno viša permeabilnost oftalmičkih supstancija u usporedbi s *ex vivo* modelom rožnice svinje ukazuju na relativnu neprikladnost Modela I u ispitivanju permeabilnosti i predviđanju bioraspoloživosti topikalnih oftalmičkih lijekova. Model II karakterizira značajno čvršća epitelna barijera te vrlo dobra korelacija permeabilnosti oftalmičkih supstancija s *ex vivo* modelom rožnice, što upućuje na prikladnost Modela II za ispitivanje permeabilnosti i predviđanje bioraspoloživosti topikalnih oftalmičkih lijekova. Utvrđena međuovisnost temperature, TEER vrijednosti modela i permeabilnosti paracelularnog i transcelularnog obilježivača ukazuju na prikladnost Modela II za evaluaciju interakcije oftalmičkih ekscipijensa s epitelnom barijerom rožnice i posljedičnog utjecaja na permeabilnost oftalmičkih lijekova kroz barijeru rožnice. Rezultati ispitivanja ukazuju, međutim, na određenu varijabilnost *in vitro* HCE-T modela humanog epitela rožnice te potrebu za identifikacijom i standardizacijom čimbenika uzgoja u smislu utjecaja na barijerna svojstva modela i permeabilnost oftalmičkih supstancija.

Model II korišten je u biofarmaceutskoj karakterizaciji dviju vrsta nanosustava za topikalnu oftalmičku primjenu melatonina, u okviru koje je evaluiran utjecaj pojedinih ekscipijensa na biofarmaceutska svojstva nanosustava. Ispitane su polimerne lecitinsko-kitozanske nanočestice (MLC) te Pluronic® F127/kitozanske micele (MFC) s uklopljenim melatoninom. Polimerne nanočestice bile su karakterizirane većom veličinom čestica (241,8 ± 0,8 nm), višim zeta potencijalom (22,7 ± 0,7 mV) te sporijim oslobađanjem melatonina ($t_{50\%}$ = 1,75 h) u odnosu na micele (20,7 ± 0,3 nm, 4,3 ± 0,9 mV, $t_{50\%}$ = 0,75 h). MTT ispitivanje vijabilnosti HCE-T stanica ukazuje na biokompatibilnost obje vrste nanosustava s epitelnim stanicama

rožnice te sigurnost njihove topikalne oftalmičke primjene. Ispitivanjima adhezivnosti nanosustava na monosloju HCE-T stanica utvrđena su značajna mukoadhezivna svojstva polimernih nanočestica, koja su vrlo vjerojatno osigurana površinskim pozitivnim nabojem nanočestica posredovanim prisutnošću kitozana. Uklapanje melatonina u nanosustave rezultiralo je smanjenjem permeabilnosti melatonina preko HCE-T epitelne barijere rožnice u odnosu na kontrolnu otopinu melatonina, pri čemu je smanjenje permeabilnosti bilo izraženije za polimerne nanočestice (za oko 60%) nego za micele (za oko 30%). Rezultati ispitivanja permeabilnosti ukazuju na značajan utjecaj kitozana na permeabilnost melatonina. Uklapanjem kitozana u Pluronic® F127 micele umanjen je utjecaj Pluronic® F127 na povećanje permeabilnosti melatonina, dok je prisutnost kitozana u polimernim nanočesticama značajno smanjila brzinu oslobađanja melatonina, za koju je pokazano da kontrolira brzinu permeacije melatonina preko HCE-T epitelne barijere rožnice. Rezultati provedenih fizičko-kemijskih i biofarmaceutskih ispitivanja ukazuju na značajan utjecaj kitozana na biofarmaceutska svojstva lecitinsko-kitozanskih nanočestica, koje temeljem mehanizama mukoadhezije te kontroliranog oslobađanja i permeacije melatonina mogu potencijalno pospješiti bioraspoloživost i produljiti terapijski učinak topikalno primijenjenog melatonina.

Korištenjem Modela II i *ex vivo* modela rožnice svinje ispitan je učinak vrste i koncentracije četiri različita oftalmička neionska površinski aktivna ekscipijensa (nPAE) na barijerna svojstva modela rožnice i permeabilnost četiri oftalmička lijeka različitih fizičko-kemijskih svojstava. Rezultati TEER mjerenja tijekom *in vitro*/*ex vivo* ispitivanja permeabilnosti ukazuju na odsutnost interakcije oftalmičkih nPAE s epitelnom barijerom rožnice. Prisutnost oftalmičkih nPAE uzrokovala je, međutim, smanjenje permeabilnosti pojedinih oftalmičkih lijekova, ovisno o vrsti i koncentraciji oftalmičkih nPAE. Rezultati ispitivanja agregacijskih svojstava oftalmičkih nPAE upućuju na interakciju oftalmičkih lijekova i agregata oftalmičkih nPAE kao mogući mehanizam smanjenja *in vitro*/*ex vivo* permeabilnosti oftalmičkih lijekova. Pri najnižoj ispitanoj koncentraciji oftalmičkih nPAE, pri kojoj je utvrđena odsutnost njihovih agregata, *in vitro*/*ex vivo* permeabilnost oftalmičkih lijekova nije bila promijenjena. Pri višim ispitanim koncentracijama oftalmičkih nPAE, pri kojoj je utvrđena prisutnost njihovih agregata, uočeno je, međutim, smanjenje *in vitro*/*ex vivo* permeabilnosti oftalmičkih lijekova. Najizraženiji učinak smanjenja *in vitro*/*ex vivo* permeabilnosti oftalmičkih lijekova zabilježen je za Cremophor EL, 2% *m*/*V* (za oko 90%), dok je slabiji učinak smanjenja *in vitro*/*ex vivo* permeabilnosti oftalmičkih lijekova uočen za Tiloksapol, 0,1% *m*/*V* (za oko 50%) i Polisorbat 80, 0,2% *m*/*V* (za oko 30%). Pluronic® F68, 1% *m*/*V*, nije imao statistički značajan utjecaj na

in vitro/*ex vivo* permeabilnost oftalmičkih lijekova unutar ispitanog koncentracijskog raspona. Učinak oftalmičkih nPAE na *in vitro*/*ex vivo* permeabilnost oftalmičkih lijekova bio je izraženiji za lipofilne oftalmičke lijekove niže topljivosti. Za timolol maleat, oftalmički lijek bolje topljivosti i slabije lipofilnosti u odnosu na ostale ispitane oftalmičke lijekove, nije uočen utjecaj oftalmičkih nPAE na njegovu *in vitro*/*ex vivo* permeabilnost. Vrlo dobra *in vitro*/*ex vivo* korelacija utjecaja oftalmičkih nPAE na permeabilnost oftalmičkih lijekova upućuje na to da je uočen utjecaj usporedivog opsega i posredovan istim mehanizmom u oba modela rožnice. Rezultati istraživanja daju važan uvid u utjecaj oftalmičkih nPAE na permeabilnost oftalmičkih lijekova što može usmjeriti njihov odabir u budućem razvoju topikalnih oftalmičkih pripravaka s obzirom na potencijal promjene bioraspoloživosti topikalno primijenjenih oftalmičkih lijekova.

Zaključak: Biofarmaceutska ispitivanja provedena u okviru doktorskog rada ukazuju na prikladnost *in vitro* HCE-T modela epitela humane rožnice za ispitivanje permeabilnosti topikalnih oftalmičkih lijekova s ciljem unaprjeđenja procesa optimizacije sastava konvencionalnih i inovativnih oftalmičkih pripravaka u ranim fazama razvoja. Provedena karakterizacija *in vitro* HCE-T modela rožnice u sklopu doktorskog rada ukazuje, međutim, na određenu varijabilnost modela te potrebu za dodatnom karakterizacijom čimbenika uzgoja u smislu utjecaja na barijerna svojstva modela, u okviru njegove daljnje optimizacije i standardizacije. Ispitivanje i klasifikacija pomoćnih tvari s obzirom na potencijal promjene permeabilnosti i bioraspoloživosti topikalno primijenjenih oftalmičkih lijekova ključan su korak za unaprjeđenje ciljanog i isplativog razvoja topikalnih oftalmičkih pripravaka. Rezultati doktorskog rada značajan su doprinos razumijevanju utjecaja oftalmičkih ekscipijensa na permeabilnost oftalmičkih lijekova, što može usmjeriti njihov odabir u budućem razvoju konvencionalnih i inovativnih topikalnih oftalmičkih pripravaka.

KLJUČNE RIJEČI: oftalmički lijek, oftalmički ekscipijensi, površinski aktivni ekscipijensi, nanosustavi, permeabilnost, bioraspoloživost, *in vitro*/*ex vivo* modeli rožnice, HCE-T.

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1. INTRODUCTION

1.1. Topical ophthalmic drug delivery

1.1.1. Pharmacokinetic challenges of topical ophthalmic drug delivery

Topical administration is, due to its ease of use, convenience and non-invasiveness, the most common drug administration route in the treatment of anterior eye segment diseases. Topical ophthalmic drug is applied directly to the ocular surface from which it has to enter and/or permeate the ocular surface tissue barriers to reach the target site. However, different physiological and anatomical barriers which protect the anterior segment of the eye from harmful effects of chemicals, particles and microorganisms, significantly limit the absorption and bioavailability of topically applied ophthalmic drugs. More precisely, anterior eye segment barriers such as tear turnover, reflex blinking, induced lachrimation, nasolacrimal drainage and tight corneal barrier (Figure 1), successfully eliminate topically applied ophthalmic product from the ocular surface and restrict the ophthalmic drug absorption, which results in low ophthalmic drug eye-related bioavailability and limited therapeutic activity. Overall, topically applied ophthalmic product is eliminated from the ocular surface in less than 5 minutes and less than 5% of the topical ophthalmic drug dose is absorbed and available at the target site (Järvinen et al., 1995).

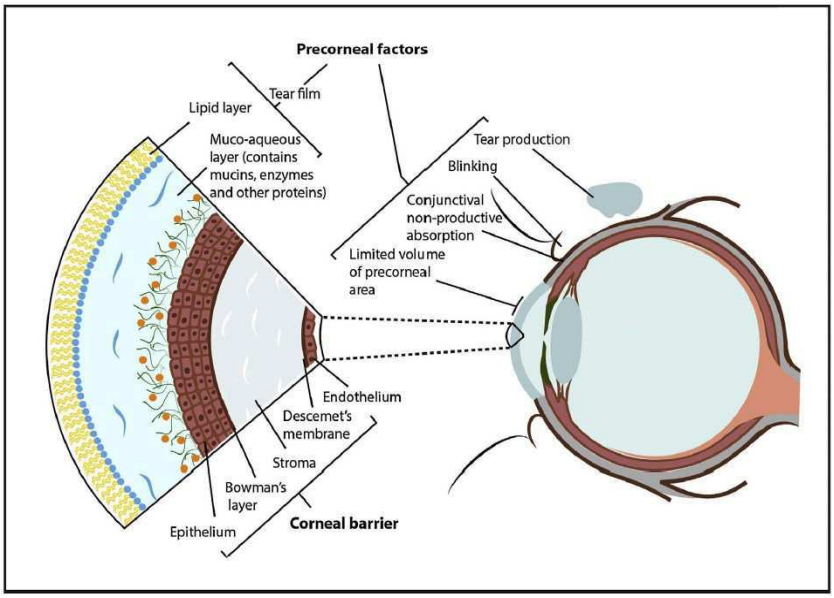

Figure 1. Schematic presentation of the main anterior eye segment barriers which a topically applied ophthalmic drug must overcome to reach the target site (reprinted from Alvarez-Trabado et al., 2017, with permission from Elsevier).

The anterior ocular surface is covered with a thin layer of tears which are produced by the lachrymal gland at a rate of 0.5-2.2 μL/min (approximately 16%/min turnover rate) and are distributed over the ocular surface by blinking. The tear film is composed of an outer lipid

layer which prevents the tear evaporation and stabilizes the tear film, intermediate aqueous layer containing electrolytes, small molecules (urea, glucose), proteins, enzymes and soluble mucins, and the inner layer of mucins anchored to the corneal and conjunctival epithelium. A portion of the tears is lost through evaporation and the rest is drained through the lacrimal punctum and nasolacrimal duct to nasal cavity (Tsubota, 1998). A conventional ophthalmic eye drop dispensing bottle delivers a volume of 25-56 μL (1-2 drops) which mixes with the normal tear volume of 7 μL, however, maximal volume which human conjunctival sac can retain is approximately 30 μL. Larger instillation volumes result in partial ophthalmic product loss due to spillage over the anterior lid border to cheek skin. Furthermore, reflex blinking and lacrimation upon application of larger instillation volumes is induced, which enhances ophthalmic product elimination through nasolacrimal drainage system (Troy and Beringer, 2006). Moreover, proteins and enzymes present in the tears can reduce the fraction of a topically applied ophthalmic drug available for absorption through anterior eye segment tissue barriers by forming drug-protein complexes or inducing drug degradation, respectively (Alvarez-Trabado et al., 2017).

Additional substantial drug loss occurs by systemic absorption through conjunctiva. The conjunctiva is a highly vascularised mucous membrane composed of multi-layered epithelium and connective tissue, characterised by approximately 17 times higher surface area compared to cornea (Watsky et al., 1988). Tight junctions encircling conjunctival epithelial cells are much leakier than the tight junctions in the cornea which makes the conjunctiva 2 to 30 times more permeable to both small and large molecules (Hämäläinen et al., 1997; Huang et al., 1989). Topically applied ophthalmic drug diffuses through conjunctival epithelial membrane and via conjunctival blood vessels enters systemic circulation. As a result, a significant portion of the ophthalmic drug is lost and unavailable for exerting therapeutic activity at the target site. However, due to wider paracellular space and higher permeability the conjunctiva represents an alternative route for the delivery of therapeutic hydrophilic macromolecules by direct absorption to vitreous through conjunctival-scleral-uveal tract pathway (Ahmed and Patton, 1985).

In order to reach the target site located in inner ocular structures a drug must permeate through corneal barrier. However, cornea is a very tight multi-layered barrier which restricts the passage of small molecules and is almost impermeable to large molecules. Highly lipophilic small molecules easily permeate the outer lipophilic multi-layered epithelium, however, the hydrophilic stroma posterior to the epithelium strongly limits their further diffusion. The diffusion of small hydrophilic molecules through corneal epithelium is

confined to intercellular hydrophilic pores, however, tight junctions encircling epithelial cells strongly limit their diffusion, which results in their low corneal permeability. A monolayer of corneal endothelial cells located posterior to the stroma has a marginal barrier role in the permeation of both lipophilic and hydrophilic small molecules (Schoenwald and Huang, 1983; Shih and Lee, 1990).

Once a drug permeates the cornea, it enters the aqueous humour compartment where it undergoes further distribution to intraocular tissues and elimination through local blood circulation. The aqueous humour volume of 300 μL is continuously produced and eliminated at a rate of 2-3 μL/min. The aqueous humour turnover, however, has a minor role in drug elimination from aqueous humour compartment. The ocular drug clearance value of 4.7-15 μL/min indicates that drugs are primarily eliminated through blood circulation of anterior uvea or/and metabolic pathways. Moreover, the estimated drug half-life of 0.8-3.0 h, a lot larger compared to the half-life of aqueous humour (0.77 h), suggests that the drug elimination might be slowed down due to drug binding to intraocular tissues and back-diffusion from tissue reservoirs (Schoenwald, 1990).

1.1.2. Corneal anatomy

Cornea is an optically transparent avascular tissue, providing about three-fourths of the refractive capacity of the eye. The corneal diameter is about 11.5 mm horizontally and 10.5 mm vertically. The corneal thickness is about 0.5-0.7 mm, with central part of the cornea being thicker than the limbus. Five different layers constitute the corneal tissue, namely, the epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium (Eghrari et al., 2015).

The epithelium, the anterior-most layer of the cornea, is composed of 4-6 layers of nonkeratinized stratified squamous cells, measuring approximately 50 μm in thickness. The superficial 2-3 layers of corneal epithelium are comprised of polygonally shaped flat cells with apical microvilli, covered by a charged glycocalyx layer (Mochizuki et al., 2010). Intercellular tight junctions surround the most superficial cells creating a watertight seal which acts as a selective barrier for small molecules and prevents the diffusion of macromolecules (Leong and Tong, 2015). The central cross section of the corneal epithelium consists of 2-3 layers of wing or suprabasal cells, which also demonstrate tight-junctional complexes. The posterior-most epithelial layer is composed of basal columnar cells, which are responsible for corneal cell division and differentiation. Regular apoptosis and desquamation occurs in the corneal epithelium, during which the lost cells are replaced by the division and

differentiation of perilimbal basal epithelial cells, resulting in epithelial cells lifespan of 7-10 days (Hanna et al., 1961). The basal epithelial cells with their hemidesmosomes adhere to the epithelial basement membrane underneath, which consists of different structural proteins and glycoproteins (Torricelli et al., 2013). Posterior to the epithelial basement membrane lies 8-12 μm thick acellular nonregenerating Bowman's layer, composed mainly of collagen fibrils (Jacobsen et al., 1984).

Corneal stroma, situated underneath the Bowman's membrane, is around 500 μm thick highly innervated structure, which comprises around 90% of the corneal thickness. Water comprises approximately 85% of the corneal stroma. Collagen fibres, the main structural component of the stroma, are of a uniform diameter of around 25 nm and run parallel to each other to form specifically arranged collagen lamellae, ensuring thereby both structural support and optical transparency. These collagen lamellae of varying width and thickness are embedded in an extracellular matrix composed mainly of water, inorganic salts, proteoglycans and glycoproteins. The principal stromal cells keratocytes produce extracellular matrix components maintaining thereby the structure of the stroma (Torricelli and Wilson, 2014). Descemet's membrane is the basement membrane of the corneal endothelium, located right beneath the stroma. Similar to Bowman's mebrane, it is a two-layer structure, around 10 μm thick, composed mainly of collagen, as well as other structural proteins and proteoglycans (Johnson et al., 1982).

Endothelium, an approximately 5 μm thick structure, consists of a single layer of flat polygonal cells distributed in a hexagonal pattern. The ionic pumps, which are located at the basolateral membrane of endothelial cells, play a key role in maintaining the normal corneal hydration (Barry et al., 1995). In contrast to the epithelial cells, endothelial cells do not undergo mitosis. In the event of damage or death of endothelial cells, lost endothelial cell function is restored by migration and reorganisation of adjacent endothelial cells (Joyce, 2003).

1.2. Preclinical *in vitro*/*ex vivo* corneal models for the prediction of eye-related bioavailability

The animal studies *in vivo* represent an important step in the ophthalmic drug product development during the selection of the ophthalmic formulations proceeding into the clinical studies. However, ethical and economical concerns, as well as questionable translation of the animal results to humans, have focused researchers on the development of preclinical *in vitro* cell-based and *ex vivo* tissue-based corneal models for ophthalmic drug permeability testing.

For small moderately lipophilic molecules, which still comprise the majority of commercially available topical ophthalmic drugs, the corneal barrier represents the main route to target sites located in inner intraocular structures. The evaluation of the ophthalmic drug permeability using the *in vitro* and *ex vivo* corneal models, with the aim of predicting the ophthalmic drug eye-related bioavailability *in vivo* in the early stages of ophthalmic product development, might improve the process of selection of an ophthalmic drug/formulation reaching the market.

Bioequivalence testing of generic topical ophthalmic products poses a significant challenge, since the measurements of the drug concentration in the ocular tissues and fluids, due to their invasiveness and complexity, most often cannot be performed. For products with systemic effect intended for oral and non-oral administration routes, the determination of the drug plasma concentration-time profile represents the basis for regulatory approval of generic products. For topical ophthalmic products, however, the drug concentration in the systemic circulation does not reflect the fate of a drug in intraocular tissues. It is rather a measure of the systemic exposure to topically applied ophthalmic drug and should be considered in the evaluation of the topical ophthalmic product safety. Current guidelines for bioequivalence testing of topical ophthalmic products include clinical end-point studies, pharmacokinetic studies in aqueous humour or *in vitro* studies (microbial kill-rate studies, physico-chemical characterization, *in vitro* release studies), depending on the dosage form type, active pharmaceutical ingredient, indication, site of action and mechanism of action (Choi and Lionberger, 2016). Obviously, *in vitro* and *ex vivo* corneal permeability models predicting the topical ophthalmic drug bioavailability *in vivo* have the potential to be in the future included in the regulatory guidelines for the topical ophthalmic product bioequivalence testing (Pepić et al., 2014).

However, in order to be implemented in pharmaceutical industry the *in vitro* and *ex vivo* models must be in detail characterised regarding the structural barrier properties, paracellular and transcellular drug transport route and the expression and functionality of membrane transporters and metabolic enzymes, all of which influence the drug transcorneal permeability. Moreover, standardization of the experimental conditions is a prerequisite for establishing reliable *in vitro*/*ex vivo* corneal models yielding reproducible and highly predictive results. Especially challenging aspect of the *in vitro*/*ex vivo* corneal model development is the simulation of the dynamic eye environment *in vivo* (tear fluid composition and dynamics) which determines the residence time of the ophthalmic formulation at the corneal surface. The optimisation of the structural and physiological parameters of the *in*

vitro/*ex vivo* corneal models critical for ocular absorption and bioavailability *in vivo* may ensure the establishment of the correlation between the drug permeability *in vitro*/*ex vivo* and the drug ocular bioavailability *in vivo*. The established *in vitro*/*ex vivo*/*in vivo* correlation can provide the basis for regulatory approval of both innovative and generic topical ophthalmic products (Pepić et al., 2014).

1.2.1. *In vitro* cell-based corneal permeability models

In vitro corneal permeability models range from simple models representing the corneal epithelium to more complex models comprising different types of corneal cells and reflecting the complex anatomical structure of the entire cornea. Significant advancement in the field of the *in vitro* corneal model development has been made, however, it still remains difficult to mimic completely the intricate structure of the cornea *in vivo*. The *in vitro* corneal models, therefore, represent a less predictive tool for the ocular bioavailability prediction compared to *ex vivo* corneal models. However, the possibility of miniaturization and automation makes the *in vitro* models a high capacity tool which would be valuable in the primary high-throughput permeability screening of a large number of ophthalmic drug candidates (Pepić et al., 2014).

For the corneal epithelial model the epithelial cells are cultivated on a semipermeable membrane coated with extracellular matrix proteins (Becker et al., 2008; Goskonda et al., 1999; Hahne and Reichl, 2011; Toropainen et al., 2001). For the cultivation of complex organotypic corneal constructs different types of corneal cells are co-cultivated in layers on top of a semipermeable membrane, to simulate the structure of the cornea *in vivo*, composed of multi-layered epithelium, collagenous stroma containing fibroblasts and mono-layered endothelium (Hahne et al., 2012; Minami et al., 1993; Reichl et al., 2004; Tegtmeyer et al., 2001). Initially, the cells are cultivated submerged in the medium, followed by the exposure of the epithelial cells to the air-liquid interface (ALI), which has been shown to be critical for the induction of epithelial cell proliferation and differentiation leading to the formation of a multi-layered epithelial structure (Ban et al., 2003; Chang et al., 2000; Hahne and Reichl, 2011).

Different *in vitro* corneal permeability models have been developed, differing in cell type and origin (animal/human), semipermeable membrane material, coating protocols, culture medium composition, the duration of the period of submerged cultivation and ALI exposure (Ban et al., 2003; Chang et al., 2000; Hahne et al., 2012; Hahne and Reichl, 2011; Mohan et al., 2003; Reichl and Müller-Goymann, 2003; Tegtmeyer et al., 2001; Toropainen et

al., 2001). Regarding the cell type, both primary cells and immortalized cell lines have been used in the *in vitro* corneal model development. Primary cells are isolated from the tissue either by cell migration from tissue fragments in culture or mechanical/enzymatical tissue disaggregation. Primary cells reflect better biochemical and structural characteristics of the tissue *in vivo*, however, the number of cell division they undergo in culture is finite due to senescence process which eventually leads to cell death. Their short lifespan in culture, which requires repeated laborious cell isolation from excised tissue, limits their use in the *in vitro* corneal model development. Moreover, it has been shown that primary cells may undergo phenotypic changes in culture losing some of their original structural and biochemical properties (Vellonen et al., 2014).

Immortalised cell lines are characterised by almost infinite lifespan due to a mutation in the cell genome which enables the evasion of senescence process. Immortalised cell lines are derived from tumour cells or can be produced from primary cells by spontaneous transformation in culture or by transfection with viral genes interfering with the cell senescence process. In contrast to primary cells, immortalized cell lines have almost infinite replicative capacity and can be stored in liquid nitrogen for a prolonged period and thawed as needed, which makes them more appropriate for the routine use of *in vitro* corneal models. However, genomic and phenotypic changes of immortalized cell lines in culture have been reported, leading to the loss of structural and biochemical properties of the parental tissue (Vellonen et al., 2014).

Of all the *in vitro* corneal epithelial models which have been developed, HCE-T-based and commercially available Clonetics™ (Lonza) are the only models that have been shown to demonstrate sufficiently tight corneal epithelial barrier (Becker et al., 2008; Reichl, 2008). HCE-T immortalised cell line has been the most extensively characterised cell line regarding the porosity and pore size of the formed epithelial barrier *in vitro*, passive paracellular and transcellular transport, expression and functionality of membrane transporters and metabolic enzymes (Becker et al., 2007; Hahne and Reichl, 2011; Kölln and Reichl, 2016a, 2016b, 2012, Toropainen et al., 2003, 2001; Vellonen et al., 2010). The most significant advancement in the field of the *in vitro* corneal model development has been made by Reichl's research group, which has successfully developed the *in vitro* HCE-T cell-based Hemicornea construct cultivated in serum-free medium, resembling native human corneal barrier. The results of the prevalidation studies using seven compounds of different physicochemical properties show that Hemicornea model exhibits permeation behaviour comparable to human cornea (Hahne et al., 2012). Recently, a novel microfluidic cell culture platform, called Dynamic Micro Tissue

Engineering System (DynaMiTES), was designed, which enables the analysis of the drug permeation through Hemicornea model under dynamic conditions, simulating thereby the dynamic precorneal environment *in vivo* (Beiβner et al., 2017).

1.2.2. *Ex vivo* tissue-based corneal permeability models

Ex vivo corneal permeability models, compared to *in vitro* models, are of lower capacity, with regard to the number of tested samples per time unit, but higher predictability. *Ex vivo* models have been shown to be less sensitive to toxic effects of high concentrations of excipients, most probably due to the higher tissue complexity (Ekelund et al., 2005). It is, therefore, expected that *ex vivo* corneal models should be more robust in terms of resilience to potential toxic effects of high concentrations of the ophthalmic ingredients, which makes them more appropriate for testing a complex mixture of ophthalmic drugs and excipients during the later phase of the ophthalmic formulation optimization process.

Due to limited availability of human corneas, which are generally reserved for transplantation purposes, corneas of animal origin are mostly used for *ex vivo* permeability testing. Freshly excised rabbit, porcine and bovine cornea have been most commonly utilised in *ex vivo* permeability testing. The use of rabbit corneas in *ex vivo* studies may facilitate the establishment of *ex vivo*-*in vivo* correlations, since rabbits are most often used for studies of ocular bioavailability *in vivo*. However, the permeability through rabbit corneas has been shown to be higher compared to human corneas, due to specific structural differences, such as the lack of Bowman's layer and lower thickness of the cornea. The bovine cornea, on the other hand, has twice as thick the epithelium as the human cornea, which is presumed to be responsible for lower ophthalmic drug permeability compared to rabbit and porcine corneas. The porcine cornea has been shown to be structurally the most similar to the human cornea, in terms of the globe size, corneal thickness, globe diameter to cornea length ratio and the presence of Bowman's layer (Loch et al., 2012; Myung et al., 2006). Moreover, porcine corneas are highly available at the local abattoirs, where they represent by-products in the meat production, which eliminates ethical issues related to the use of laboratory animals.

For the purpose of *ex vivo* permeability testing freshly enucleated eyeballs are transported in moist conditions at 4°C in sterile containers from an abattoir to a laboratory, where cornea excision is performed. Specialised diffusion system, such as the Franz diffusion cells and vertical/horizontal Ussing diffusion chambers, are used in the *ex vivo* permeability experiment. The excised corneas are mounted onto the diffusion system, with epithelial side of the cornea facing the donor compartment, which represents the precorneal area, and the

endothelial side facing the receptor compartment, which represents the aqueous humour compartment. Tissue viability loss is observed from the moment of its excision. Nutritional buffers containing different salts, glucose, antioxidants, of physiological pH 7.4, as well as oxygenation of the medium, are thus applied to preserve the corneal tissue viability during the 4-6 hours of *ex vivo* permeability testing. Modifications of commercial diffusion system have also been applied by, for example, circulating the fluid in the donor and receptor compartment or adjusting the pressure in the receptor chamber, with the aim of replicating more closely the dynamic eye environment *in vivo* (Abdulrazik, 1996; Bonferoni et al., 1999). Another interesting approach in studying corneal permeability/uptake *ex vivo* is the application of a whole-eye model, in which a small volume of nanoparticle solution is applied to the corneal surface and the uptake of nanoparticles is measured in subsequently extracted ocular tissue layers and fluids. In addition to corneal permeability, the impact of conjunctival and scleral permeability on ophthalmic drug/delivery system distribution to different ocular tissues can be as well evaluated. However, fast onset of tissue decay limits the use of the whole-eye model to a relatively short period of time (Kompella et al., 2006; Luschmann et al., 2014).

1.3. Conventional eye drops

Conventional eye drops comprise majority of marketed topical ophthalmic dosage forms. Most commonly conventional eye drops are formulated as solutions due to dose uniformity, clarity and ease of manufacturability. Their main disadvantage, however, is rapid clearance and short residence time at the ocular surface, as well as limited shelf-life for hydrolytically labile drugs. Polymers which undergo a phase transition from liquid to semisolid as a consequence of temperature, pH or ionic strength changes upon instillation into the eye, may be used in the production of ophthalmic solution which transforms into gel upon contact with the ocular surface. This type of ophthalmic dosage form utilizes the advantages of both the solution (ease of administration) and gel (prolonged residence time). For drugs of low solubility, suspension, which consists of insoluble solid drug particles dispersed in a suitable aqueous vehicle, represents a pharmaceutically acceptable alternative. Physical instability, which may lead to inhomogeneity and problem with dosage uniformity, is the main disadvantage of suspensions, requiring strict control of particle size, sedimentation rate and resuspendabillity (Gibson, 2009).

As for dosage forms for any other administration route, essential requirements such as stability, manufacturability, safety and bioavailability must be fulfilled for topical ophthalmic dosage forms. However, due to direct contact of topical ophthalmic dosage forms with the

corneal absorption surface, formulation factors (pH, buffer capacity, osmolarity, viscosity) and formulation composition (the type and concentration of excipients) largely influence their tolerability and bioavailability. Additionally, specific requirements, such as sterility, limited endotoxin content and particulate matter, must be met, which moreover increases the complexity of the topical ophthalmic dosage form development (Ghosh and Ahmed, 2014).

Physicochemical properties of the drug (solubility, lipophilicity, pK_a, compatibility with excipients and packaging material) represent the most important factor in the selection of a type and composition of the topical ophthalmic dosage form. For ionisable drugs, the pH of the ophthalmic formulation, as well as the buffer type and capacity, are some of the critical parameters which determine the topical ophthalmic dosage form performance, since they may largely influence drug solubility, stability and corneal permeability. For acidic pH adjustment, acetate buffer and citrate buffer are most commonly used, while for alkaline pH adjustment phosphate buffer or borate buffer are most frequently utilised (Gibson, 2009). The pH range which eye can tolerate is 4-8, however, the pH as close to the pH of tear fluid of 7.4 or slight alkaline pH is recommended to avoid the risk of possible irritation and induced lachrimation effect (Dale and James, 1991).

Different tonicity agents (e.g., sodium chloride, glycerol, glucose, mannitol) are added to topical ophthalmic formulation to ensure optimal osmolality. Although a wider range of osmolality is tolerated (160-480 mOsm/kg), isotonic formulations are preferable to minimize the risk of potential discomfort during use.

In order to increase the topical ophthalmic dosage form residence time at the ocular surface, different viscosity-increasing agents may be employed. These mostly include different synthetic polymers (e.g., cellulose derivatives, povidone, polyvinyl alcohol, carbomer), some of which are characterised by mucoadhesive properties, which additionally contribute to prolonging the precorneal residence time. However, high-viscosity formulations are not well-tolerated, causing lachrimation, blinking and discomfort. The viscosity of most commercial topical ophthalmic dosage forms is, therefore, adjusted to 10-25 mPa×s by using appropriate viscosity-increasing agents (Gibson, 2009).

To ensure drug stability, topical ophthalmic dosage forms may additionally include different antioxidants, which protect the drug from degradation, or chelating agents, which bind metal ions which catalize oxidative reactions (Gibson, 2009). Special attention must be given to the sterility of the ophthalmic dosage forms. The ophthalmic dosage forms are sterilized during production with a suitable sterilization technique, however, to maintain the sterility of the formulation after opening and during its use, antimicrobial preservatives are

utilised. A limited number of antimicrobial preservatives is approved for the ophthalmic application, among which benzalkonium chloride still remains one of the most commonly used, due to its antimicrobial effectiveness over a wide pH range and stability in solution and during autoclave sterilization process. Nonetheless, due to increasing evidence of benzalkonium chloride toxicity during chronic use, new antimicrobial agents based on stabilized chloride and oxygen compounds and sodium perborate, determined to be devoid of irritant effects, have been developed and are included in commercial topical ophthalmic dosage forms (Kaur et al., 2009).

With the purpose of enhancing drug solubility to ensure administration of a higher drug dose, different so-solvents (e.g., polyethylene glycols, propylene glycol, polyvinyl alcohol) may be employed (Gibson, 2009). Moreover, surfactants are another group of ophthalmic excipients commonly used for enhancing drug solubility. Due to compatibility, stability and toxicity issues encountered with the use of cationic, anionic and amphoteric surfactants, nonionic surfactants are preferred for the ophthalmic use. Owing to the amphiphilic nature of their structure, non-ionic surfactants possess a broad range of interfacial activity. In addition to their role of solubilisation agents, non-ionic surfactants are also used as emulsifiers to increase the ophthalmic emulsion stability and as wetting agents in ophthalmic suspensions to improve particle wetting, suspension stability and resuspendability (Jiao, 2008).

1.4. Innovative eye drops

In order to overcome both dynamic and static anterior eye segment barriers and more efficiently deliver the drug to its target site, scientific research has been oriented towards the development of nano-sized structures as drug carriers. Nanosystems (of size ranging from few to 1000 nanometers) have been shown to incorporate a wide variety of drugs, protect the drug from potential degradation in the tear fluid, prolong the drug residence time at the ocular surface and enhance the drug corneal permeability (Alvarez-Trabado et al., 2017; Bravo-Osuna et al., 2016; Reimondez-Troitiño et al., 2015; Suresh and Sah, 2014).

The research in the field of nanosystems for topical ophthalmic drug delivery began in the 1980's, while over the last decade the number of published papers in this field has substantially grown (Reimondez-Troitiño et al., 2015). Initially, the researchers were focused on the development of liposomes, spherical vesicles composed of an aqueous core (with the affinity to hydrophilic drugs) encircled by concentric phospholipid bilayers (with the affinity to lipophilic drugs) (Stratford et al., 1983, 1982). Nowadays, the scientific focus has shifted to the development of nanoparticles, solid nanocarriers composed of biocompatible and

biodegradable polymers, such as chitosan (De Campos et al., 2001), hyaluronic acid (de la Fuente et al., 2008), poly(lactic-co-glycolic acid) (PLGA) (Gupta et al., 2010), Carbopol® and Eudragit® (RS100 and RL100) (Pignatello et al., 2002). However, other types of nanosystems have been intensively investigated as well, such as oil-in-water nanoemulsions (composed of nanosized oil droplets dispersed in aqueous phase) (Ammar et al., 2009), niosomes (vesicles structurally similar to liposomes, but composed of nonionic surfactants instead of phospholipids) (Aggarwal et al., 2007), nanocapsules (consisting of an oily core enclosed by a poly-ε-caprolactone (PCL) wall) (Losa et al., 1993), polymeric micelles (formed by diblock or multiblock amphiphilic copolymers that self-assemble into core-shell nanostructures) (Pepić et al., 2004a), dendrimers (symmetric structures containing a central core surrounded by repetitive branched molecules) (Kambhampati and Kannan, 2013), nanocrystals (consisting of a nanosized drug molecule surrounded by stabilizing excipients) (Tuomela et al., 2014) and solid lipid nanoparticles (consisting of a solid lipid core stabilized by surfactants) (Sánchez-López et al., 2017).

Surface properties of the nanosystems have been shown to be critical in the efficient delivery of topical ophthalmic drugs to their site of action, since they determine the interaction of nanosystems with the corneal epithelial surface. More precisely, it has been shown that surface cationic charge increases the adhesion of nanosystems to ocular surface, due to their interaction with the negatively charged mucin layer overlying the conjunctival and corneal epithelial cells (Abdelbary, 2011; Jain et al., 2013; Klang et al., 2000). Moreover, the interaction of nanosystems with the mucin layer can also be enhanced by the addition of mucoadhesive polymers (Fujisawa et al., 2012) or reactive groups (Khutoryanskiy, 2011) to nanosystems surface. Furthermore, some of the nanosystems have been assumed to interact with the lipid layer of the tear film, which results in prolonged residence time of the nanosystems in the conjunctival sac where they act as a drug depot (Alany et al., 2006). In addition to prolonging the drug residence time on the ocular surface, nanosystems have been shown to increase the drug permeation through corneal tissue by either the interaction of surfactants, located at the surface of nanoparticles, with the corneal epithelial cell membranes (Jiao, 2008) or by endocytic mechanisms (de la Fuente et al., 2008).

Different nanosystems have been tested using *in vitro*/*ex vivo* corneal models and *in vivo* animal models, in which they have been shown to increase drug *in vitro*/*ex vivo* corneal permeability and *in vivo* ocular bioavailability, compared to conventional ophthalmic formulations. Moreover, in *in vivo* animal disease models nanosystems have shown superior therapeutic activity in comparison to marketed ophthalmic formulations in the treatment of

different anterior eye segment diseases, with the concomitant reduction in the drug systemic absorption and side-effects. Several nanosystem-based formulations have been marketed in the USA and Europe, most of which are registered for the treatment of dry eye syndrome. However, other therapeutic indications (glaucoma, vernal keratoconjunctivitis, ocular inflammation) have been receiving increasing attention, with a few nanosystem-based formulations currently undergoing clinical trials (Reimondez-Troitiño et al., 2015).

2. HCE-T cell-based permeability model: A well-maintained or a highly variable barrier phenotype?

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HCE-T cell-based permeability model: A well-maintained or a highly variable barrier phenotype?

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ABSTRACT

The most extensively characterized human-derived cell line used in transcorneal permeability studies, in terms of passive transcellular and paracellular transport, transporter expression and metabolic enzymes, is the immortalized human corneal epithelial cell line (HCE-T). The purpose of this study is to describe the changes in the HCE-T barrier phenotype *in vitro* when valid cultivation conditions, in accordance with the standardized HCE-T cell-based model protocol, were employed. Evaluation of the structural and functional barrier properties revealed two different HCE-T barrier phenotypes, depending on the polycarbonate membrane pore size. Model I (pore size 0.4 μm) was characterized by a multilayered HCE-T epithelium at the apical side and a weak barrier function (70–115 $\Omega \times cm^2$), whereas Model II (pore size 3 μm) consisted of an apical lipophilic HCE-T monolayer and a basolateral lipophilic monolayer of migrated HCE-T cells that showed improved barrier properties (1700–2600 $\Omega \times cm^2$) compared with Model I. Considering the permeation of ophthalmic compounds and *in vitro*/*ex vivo* correlation, Model II was better able to predict transcorneal drug permeation. This study highlights the important aspects of HCE-T barrier phenotype variability that should be continuously monitored in the routine application of HCE-T cell-based models across both academic and pharmaceutical industry research laboratories.

1. Introduction

The experimental techniques used in preclinical studies on eye-related drug bioavailability generally focus on the cell and tissue models of ocular drug permeability. The cell-based models that have the correct balance between their predictability and throughput have the potential to be routinely used in the pharmaceutical industry for the permeability testing and prediction of eye-related bioavailability (Pepic et al., 2014). The most extensively characterized human-derived cell line in transcorneal permeability studies in terms of passive transcellular and paracellular transport (Toropainen et al., 2003), transporter expression (Becker et al., 2007; Mannermaa et al., 2006; Reichl et al., 2011; Vellonen et al., 2010; Verstraelen and Reichl, 2013, 2014) and metabolic enzymes (Kölln and Reichl, 2012, 2016a, 2016b) is the immortalized human corneal epithelial cell line (HCE-T), which was established by Araki-Sasaki et al. (1995) and is commercially available from the RIKEN cell bank (Japan) or the ATCC (USA). The HCE-T cells exhibit a typical epithelial cobblestone morphology and optimal growth characteristics. Using this HCE-T cell line, Reichl's research group constructed a human cornea equivalent (Hemicornea) by co-culturing the HCE-T cell line with stromal cells in which all cells can interact *in vitro* in a similar way as they do *in vivo*. Thus, Hemicornea strongly reflects the unique tissue characteristics and barrier properties of the human cornea *in vivo* (Hahne et al., 2012).

Although HCE-T cell-based models are well-defined and optimized for drug permeability studies compared with other immortalized human (CEPI) or rabbit (SIRC) cell lines, it has been reported that HCE-T cell-based models have some obstacles that limit their implementation and routine use across both academic and pharmaceutical industry research laboratories (Becker et al., 2008; Hahne and Reichl, 2011; Reichl, 2008; Toropainen et al., 2001; Toropainen et al., 2003). For instance, the variability in HCE-T cell barrier phenotype and the lack of a standard quality control protocol complicate the interpretation and comparison of permeability data from different laboratories. It is known to be very important to follow a standardized model setup and culture conditions (*e.g.*, growth medium, growth conditions, culture time and lifting to the air-liquid interface) to maintain the HCE-T barrier phenotype *in vitro*. However, rather unstable HCE-T barrier properties have been reported depending on the culture conditions (McCanna et al., 2008; Nagai et al., 2008; Reichl, 2008; Takezawa et al., 2011;

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Toropainen et al., 2001). Thus, further studies should be performed to clarify the key factors responsible for the variability in the HCE-T cell-based model barrier phenotype *in vitro*. The adequate standardization of these factors should help reduce both the inter- and intra-laboratory variability in the HCE-T cell-based model barrier phenotype and, consequently, the variability in reported permeability values (Pepic et al., 2014; Reichl et al., 2011).

The aim of this study is to describe the changes in the HCE-T barrier phenotype *in vitro*, where valid cultivation conditions, in accordance with the standardized HCE-T cell-based model protocol, were followed (Hahne and Reichl, 2011). To obtain deep insight into the morphological features of HCE-T cells from the HCE-T cell-based models, fluorescence microscopy imaging was used. The barrier properties of HCE-T cell-based models and the permeation of ophthalmic compounds (timolol, diclofenac, chloramphenicol, dexamethasone and fluorescein) were determined and compared with those obtained using freshly excised porcine cornea. This study highlights important aspects that should be carefully considered in the routine application of HCE-T cell-based models in both academic and pharmaceutical industry research laboratories.

2. Materials and methods

2.1. Reagents and chemicals

The following compounds were used as received: timolol maleate (Sicor S.R.L., Milan, Italy), diclofenac sodium (Pharma Greven GmbH, Greven, Germany), chloramphenicol (AppliChem, Darmstadt, Germany), dexamethasone (Sanofi, Paris, France) and fluorescein sodium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Krebs-Ringer buffer (KRB) pH 7.4 was prepared by dissolving the following substances in double-distilled water: KCl (0.4 mg/ml), NaCl (6.8 mg/ml), $NaHCO_3$ (2.1 mg/ml), $MgSO_4 \times 7H_2O$ (0.4 mg/ml), D-glucose monohydrate (1.1 mg/ml) (all purchased from Kemig, Zagreb, Croatia), $CaCl_2 \times 2H_2O$ (0.52 mg/ml) (Sigma-Aldrich), $NaH_2PO_4 \times 2H_2O$ (0.158 mg/ml) (Kemika, Zagreb, Croatia) and HEPES (3.575 mg/ml) (AppliChem).

2.2. Cell culture conditions

HCE-T cells (RIKEN Cell Bank, Tsukuba, Japan) were used for the cultivation of the HCE-T cell-based models. Cells were cultivated in DMEM/F12 medium (Lonza, Basel, Switzerland) supplemented with fetal bovine serum (5%, Biosera, Boussens, France), insulin (5 μg/ml, Sigma-Aldrich), dimethyl sulfoxide (0.5%, Applichem), epidermal growth factor (10 ng/ml, Sigma-Aldrich) and penicillin/streptomycin/amphotericin B (Lonza) in a humidified atmosphere containing 5% CO_2 at 37 °C (Hafner et al., 2015). The cells were subcultured at 80–90% confluence. The culture medium was changed every 48 h.

2.3. Cultivation of the HCE-T cell-based model

The HCE-T cell-based models were cultivated according to the modified protocol by Hahne and Reichl (2011). Transwell® polycarbonate membrane cell culture inserts (0.4 μm pore size (1×10^8 pores/cm^2) and 3.0 μm pore size (2×10^6 pores/cm^2), 12 mm diameter, surface area 1.12 cm^2, Corning B.V. Life Sciences, Amsterdam, The Netherlands) were coated with rat tail type I collagen (225 μg/well; Sigma-Aldrich) and human fibronectin (4 μg/well; Sigma-Aldrich). HCE-T cells suspended in the culture medium (10^5 cells in 0.5 ml) were seeded onto the coated polycarbonate filter, and 1.5 ml of culture medium was added to the basolateral side. The cells were cultivated submerged in the medium until a sharp increase in TEER was observed (from 4 to 7 days), after which they were exposed to the air-liquid interface (ALI) for the following 3 days. The culture medium was changed every 2 days during the submerged conditions and every day during exposure to the ALI. During the ALI exposure, the inserts were lifted on a metal plate to increase the basolateral volume to 2 ml.

2.4. Histomorphological characterization

For the histomorphological characterization of the HCE-T cell-based models, the cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4 °C. The cells were then washed with phosphate-buffered saline (PBS; Lonza), and the entire filter membrane was carefully cut out with a scalpel. The membrane was gently dried on filter paper and embedded in Kilik (cryostate embedding medium, Bio-Optica, Milan, Italy), after which 5-μm-thick cross sections were cut using a cryostat (CM1950, Leica, Eisfeld, Germany) (Lovric et al., 2012). The sections were stored at −20 °C until staining. The slides were mounted with VECTASHIELD® Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA), and the cover slips were sealed around the perimeter with nail polish. For a more thorough description of the formed multilayer, HCS CellMask™ red stain (Invitrogen™, Eugene, OR, United States), which labels the entire cell (*i.e.*, the cytoplasm and the nucleus), was used before mounting the slides. The cells were permeabilized using a 0.1% Triton® X-100 (Sigma-Aldrich) solution and incubated with the HCS CellMask™ staining solution for 30 min at room temperature according to the manufacturer's protocol. Cross sections were imaged using an ImageXpress Micro automated high-content screening microscope (Molecular Devices, Sunnyvale, CA, USA) equipped with a 4× objective using fluorescent and transmitted light.

2.5. In vitro permeability assay

The permeability assay using the HCE-T cell-based models was performed directly in the Transwell plates on a horizontal orbital shaker (34 °C; 50 rpm) in KRB. The cells were first rinsed with KRB and incubated for 30 min with 0.4 and 2 ml of KRB in the donor and receptor compartment, respectively. Then, the KRB was removed from the donor compartment, and 0.4 ml of the solution containing a test compound was added (Hafner et al., 2015; Hahne and Reichl, 2011). The concentration of the test compounds in KRB was 100 μg/ml for timolol maleate, 150 μg/ml for diclofenac sodium, 150 μg/ml for chloramphenicol, 60 μg/ml for dexamethasone and 250 μg/ml for fluorescein sodium. A volume of 400 μl was sampled from the receptor compartment at regular time intervals over 2 h and replaced at each time point with the same volume of fresh warmed KRB (34 °C). The donor solution was also sampled at the end of the permeability experiment to calculate the mass balance (%). Finally, the cell surface was rinsed with KRB and exposed to ALI with 2 ml of culture medium in the basolateral compartment for the following 24 h.

2.6. MTT assay

MTT assay was performed on the HCE-T cell-based models according to the protocol by Pauly et al. (2009). The culture medium was aspirated, and the wells were transferred to a clean 12-well cell culture plate (Corning B.V. Life Sciences). Seven hundred microliters of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich) solution in culture medium (0.5 mg/ml) was applied to both the apical and basolateral compartments, and the cells were incubated for 4 h at 37 °C. Subsequently, the MTT solution was removed and the formazan was dissolved by the addition of 700 μl of isopropanol (Kemig) to both compartments. The absorbance was measured at 570 nm by using microplate reader (1420 Multilabel counter VICTOR3, Perkin Elmer, Waltham, MA, USA).

2.7. Ex vivo permeability assay

Fresh porcine eyes were obtained from the local slaughterhouse. After enucleation, each eyeball was rinsed with an isotonic saline solution (NaCl 0.9%; B. Braun, Melsungen, Germany) and transported immersed in cold KRB in a container held on ice. Each container contained gauze on which the eyeball was placed so the cornea was facing upwards and thus remained intact during transport. The transport of porcine eyeballs and the cornea excision were carried out within 2 h of the animal's death. *Ex vivo* permeability assay was performed according to the modified protocol by Hahne and Reichl (2011) and Hahne et al. (2012). The corneas were excised as corneo-scleral buttons and mounted on vertical diffusion chambers (Standard Vertical Ussing/Diffusion Chambers, surface area 0.64 cm^2, Harvard Apparatus, Holliston, MA, USA), with the epithelial side facing the donor compartment. The volume of the donor and receptor compartment was 3.5 ml each. After 30 min of incubation in KRB at 34 °C, KRB from the donor compartment was removed, and 3.5 ml of the solution containing a test compound was added. The concentration of the test compounds in KRB was 250 μg/ml for timolol maleate, 400 μg/ml for diclofenac sodium, 400 μg/ml for chloramphenicol, 60 μg/ml for dexamethasone and 500 μg/ml for fluorescein sodium. The receptor compartment was sampled (0.4 ml for dexamethasone and fluorescein sodium, 1 ml for timolol maleate, diclofenac sodium and chloramphenicol) at regular time intervals over 5 h and replaced at each time point with the same volume of fresh warmed KRB. The donor solution was sampled at the end of the permeability experiment. The medium in both the donor and receptor compartments was continuously oxygenated.

2.8. Transepithelial electrical resistance measurement

Transepithelial electrical resistance (TEER) was measured regularly to monitor barrier integrity during both the cultivation of the HCE-T cell-based models and the *in vitro* and *ex vivo* permeability experiments. An STX-2 electrode and EVOM (WPI Inc., Sarasota, FL, USA) were used for the TEER measurement of the HCE-T cell-based models, and Ag/AgCl electrodes in conjunction with an EC-825A Epithelial Voltage Clamp (Warner Instruments, Hamden, CT, USA) were used for the excised cornea. The blank resistance was also measured and subsequently subtracted to obtain the TEER of the HCE-T cell-based model and excised cornea.

2.9. Data processing

The apparent permeability coefficients (P_{app} in cm/s) of all compounds were calculated by plotting the amount of permeated compound per unit area *versus* time. The P_{app} was calculated from the linear portion of the permeation curve according to the following equation (Hahne et al., 2012; Toropainen et al., 2003):

$$P_{app} = \frac{\partial Q}{\partial t} \times \frac{1}{AC_o} \quad (1)$$

where $\frac{\partial Q}{\partial t}$ is the permeation rate, A is the surface area of the permeation barrier and C_o is the initial concentration of a test compound in the donor compartment (Eq. (1)).

The mass balance (recovery) (%) was calculated according to the following equation (Hubatsch et al., 2007):

$$\text{Mass balance } (\%) = \frac{(C_{r(f)} \times V_r + \sum C_{r(t)} \times V_s) + C_{d(f)} \times V_d)}{C_{d(0)} \times V_d} \times 100 \quad (2)$$

where C_d and C_r are the concentrations in the donor (d) and receptor (r) compartment, respectively, at the start (0) or end (f) of the permeability assay, $Cr(t)$ is the concentration of the samples withdrawn from the receptor compartment at different time points, Vr and Vd are volumes of the receptor and donor compartment, respectively, and Vs is the volume of the sample withdrawn from the receptor compartment (Eq. (2)).

2.10. Quantification

The concentration of fluorescein sodium in samples was determined using a microplate reader at excitation and emission wavelengths of 485 and 535 nm.

The quantitative determination of ophthalmic drugs was performed by Ultra-Performance Liquid Chromatography (UPLC) using an Agilent Infinity 1290 (Agilent, Santa Clara, CA, USA). The Acquity UPLC BEH Shield RP18 Column (1.7 μm, 2.1 mm × 50 mm) (Waters, Milford, MA, USA) was used, and the elution was isocratic. Sodium acetate buffer was prepared by dissolving $CH_3COONa \times 3H_2O$ (Kemika) in Milli-Q™ water (0.3 mg/ml) (Merck-Millipore, Billerica, MA, USA). The pH was adjusted to 4.5 by acetic acid (glacial) (Merck-Millipore). Sodium dihydrogen phosphate buffer was prepared by dissolving $NaH_2PO_4 \times H_2O$ (Merck-Millipore) in Milli-Q™ water (1.2 mg/ml) (Merck-Millipore). The pH was adjusted to 2.5 by phosphoric acid (85%) (Merck-Millipore). Both buffers were filtered through a regenerated cellulose membrane filter (0.2 μm; Whatman, Freiburg, Germany) before use. A mixture of buffer/acetonitrile (Merck-Millipore) was used as the mobile phase. The details of each UPLC method, developed specifically for the permeability assay purposes in this study, are described in Table 1.

The stock solutions of ophthalmic drugs were prepared in methanol (Merck-Millipore) and were further diluted with water for the preparation of standard solutions for the donor compartment samples or KRB for the preparation of standard solutions for receptor compartment samples. The volume of injection was 20 μl for the receptor compartment samples. For the donor compartment samples the volume of injection was 5 μl for timolol maleat, diclofenac sodium, chloramphenicol and 10 μl for dexamethasone. The receptor compartment samples were injected directly or were appropriately diluted with KRB, while the donor compartment samples were diluted with water prior analysis. All samples and standard solutions were filtrated through 0.2 μm Spartan™ regenerated cellulose filters (Whatman, United Kingdom) prior analysis. For each sequence standard solutions were prepared in duplicate and injected alternately. At least five standard solution injections were done in each injection sequence. System suitability was evaluated according to the following criteria: relative standard deviation (RSD) of the detector response factor for all standard solution injections in the sequence is not >3.0%, and tailing factor of ophthalmic drug peak is not >2.0.

All UPLC methods were validated in terms of linearity, accuracy and repeatability. The methods were found to be linear ($R^2 \geq 0.9996$), accurate (recovery values 96.4–107.2%) and repeatable (relative standard deviation of peak area (RSD) 0.1–3.0%) in the following concentration ranges: 0.10–3.13 μg/ml for the receptor compartment samples and 2.50–15.00 μg/ml for the donor compartment samples for timolol maleate, 0.05–2.50 μg/ml for the receptor compartment samples and 5.00–15.00 μg/ml for the donor compartment samples for diclofenac sodium, 0.05–1.50 μg/ml for the receptor compartment samples and 2.50–15.00 μg/ml for the donor compartment samples for chloramphenicol, 0.01–2.00 μg/ml for the receptor compartment samples and 0.60–6.00 μg/ml for the donor compartment samples for dexamethasone.

2.11. Statistical analysis

GraphPad Prism program 5.0 (GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com) was used for the statistical analysis. The correlation between the *in vitro* and *ex vivo* log P_{app} values was analyzed by the Pearson correlation, with $P = 0.05$ as the minimal level of statistical significance.

Table 1
UPLC experimental conditions.

Ophthalmic drug	Mobile phase	Flow (ml/min)	Temperature (°C)	Detection wavelength (nm)	Retention time (min)
Timolol maleate	CH_3COONa buffer pH 4.5/acetonitrile (85/15%, *V/V*)	0.6	55	296	0.73
Diclofenac sodium	NaH_2PO_4 buffer pH 2.5/acetonitrile (65/35%, *V/V*)	0.6	55	276	3.41
Chloramphenicol	CH_3COONa buffer pH 4.5/acetonitrile (80/20%, *V/V*)	0.4	50	275	1.84
Dexamethasone	CH_3COONa buffer pH 4.5/acetonitrile (67/33%, *V/V*)	0.4	50	254	1.18

3. Results and discussion

3.1. Histomorphological features of HCE-T cell-based permeability models

Two HCE-T cell-based models investigated in this study were cultivated according to the protocol by Hahne and Reichl, using their conditions for the culture medium, support-coating materials, cell-seeding density, volumes of the media overlying the apical and basolateral sides of the epithelial barrier and duration of ALI exposure (Hahne and Reichl, 2011). However, the relationship between membrane pore size and HCE-T barrier tightness is not fully understood, as is evident from the conflicting reports on the optimal pore size found within the literature; *i.e.*, 0.4 μm (Toropainen et al., 2003) or 3 μm (Hahne and Reichl, 2011). To investigate the influence of membrane pore size on barrier tightness and the structure of the HCE-T cell-based model, the polycarbonate membranes (Transwell) of two different pore sizes, *i.e.*, 0.4 μm (Model I) and 3 μm (Model II), were used. The HCE-T cells migrated through the membrane with the pore size of 3 μm, as observed by fluorescence imaging, whereas migration was not observed in the case of Model I with the pore size of 0.4 μm (Fig. 1). Thus, Model II consisted of an apical lipophilic HCE-T epithelial monolayer and a basolateral lipophilic monolayer of migrated HCE-T cells. In contrast, Model I had a significantly smaller membrane pore size, and the HCE-T cells could not migrate towards the basolateral side of the membrane. Therefore, the HCE-T cells formed a stratified apical epithelial structure on the underlying polycarbonate matrix characteristic of corneal epithelium. However, both models had a number of structural differences compared with the characteristics of their *in vivo* counterparts. One of the structural and functional differences of Model I compared with the cornea is the lack of the hydrophilic stroma and the innermost lipophilic surface of the cornea. Model II did not form a multilayered epithelial structure, which is well-defined for native corneal epithelium. However, Model II had a unique characteristic, *i.e.*, a basolateral lipophilic layer formed from the migrated HCE-T cells, which could serve as additional barrier to drug transport, thereby mimicking the innermost lipophilic surface of the corneal barrier.

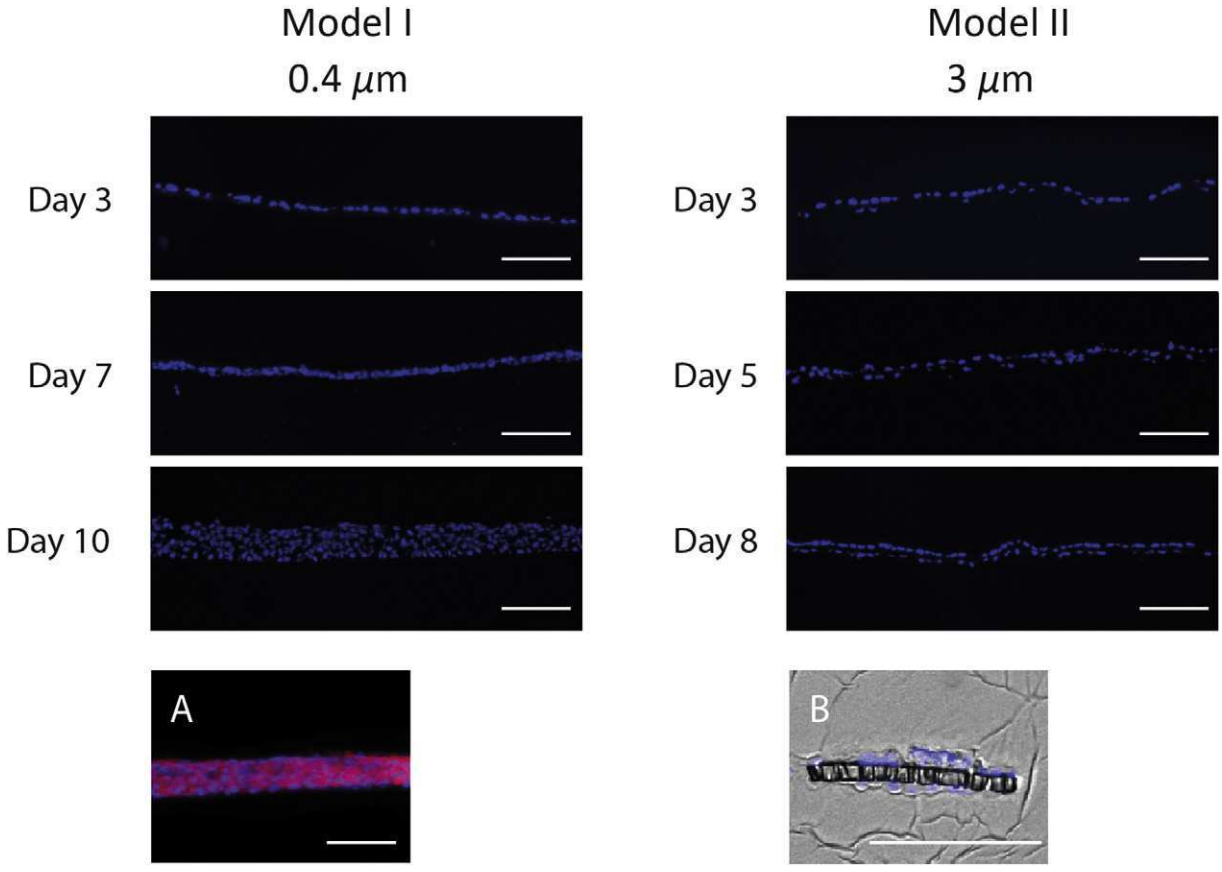

Fig. 1. Cross-sections of Model I and Model II during the initial period of submerged conditions (7 days for Model I, 5 days for Model II) and additional exposure to air-liquid interface (ALI) (3 days). The cross-sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy (Bar: 100 μm). Additionally, cross sections of Model I after 3 days of ALI cultivation were labeled by HCS CellMask™ red stain, which labels the entire cell (A). Cross-sections of Model II after 3 days of ALI cultivation stained by DAPI were subsequently imaged using transmitted and fluorescent light in order to visualize the polycarbonate membrane (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Qualitative measure of the HCE-T cell-based model barrier properties

The barrier function of the HCE-T cell-based models investigated in our laboratory was assessed by measurements of TEER. The measurement of TEER is a well-established method for analyzing the overall electrical resistance of a cell layer as a measure of the tight connections between cells (tight junctions), which encircle the cells just below their apical surface. The electrical resistance of a cell layer consists of paracellular and transcellular electrical resistance, but the clear distinction between these different electrical resistances will be possible with the further development of the impedance spectroscopy technique (Saaber et al., 2014). Ideally, electrical resistance of the HCE-T cell-based models should be comparable to the electrical resistance of freshly excised cornea. Although it is well known that the lifting of HCE-T cells to the ALI is of crucial importance for the development of a tight multilayered epithelium *in vitro* (Ban et al., 2003; Toropainen et al., 2001), the previously reported TEER values were in ranges of 200–800 $\Omega \times cm^2$ (Toropainen et al., 2001), 400 $\Omega \times cm^2$ (Nagai et al., 2008) or 1200 $\Omega \times cm^2$ (Reichl, 2008). Similarly, the two HCE-T cell-based models investigated in our laboratory showed clear differences in TEER.

As a quality control for the integrity of HCE-T epithelial barrier, the TEER was routinely measured during the model cultivation, before the start of the permeability experiment (30 min after replacing the HCE-T cell culture medium with KRB, before a test compound is added to the donor compartment) and both during (at certain sampling time points after the withdrawn volume was replaced by KRB) and at 24 h after finishing the permeability experiments. The confluence of the HCE-T cells layer is determined by a sharp increase in the TEER. The TEER values measured for each filter were averaged and used to calculate the final TEER value for each permeability experiment. In addition, the MTT assay was performed during the cultivation of Model I on the confluent monolayer both before exposing the cells to the ALI and after an additional 3 days at the ALI, when a multilayered structure was formed. The MTT assay measures the activity of the mitochondrial enzyme succinyl dehydrogenase and, as such, reflects the metabolic activity of living cells. The results of the assay showed a considerably higher metabolic activity of a multilayered epithelium compared with the monolayer, in accordance with the higher number of metabolically active cells (data not shown). Even macroscopically, a difference was clearly observed between the light purple cell monolayer in contrast to the dark purple color of the multilayered epithelium. It would be interesting to investigate in further studies whether a correlation between metabolic activity and the histological characteristics of the *in vitro* epithelial model exists. Once this relationship is defined, the use of a simple and fast MTT screening assay, in addition to more complex and time-consuming histological techniques for monitoring the *in vitro* epithelium formation, could be considered. Thus, the MTT assay has the potential to be routinely used as a well discriminating tool for multilayer formation.

TEER experiments revealed weak barrier functions for Model I (69–113 $\Omega \times cm^2$; Table 2). Although the HCE-T cells formed multiple cell layers at the apical side of the membrane, the observed range of TEER values was generally considered insufficient for a tight apical barrier. In tight HCE-T cell culture models, TEER values are expected to exceed 400 $\Omega \times cm^2$ (Toropainen et al., 2001). In this model, HCE-T cells were unable to form a tight barrier, and it is unclear exactly which cultivation conditions should be changed to maintain the HCE-T barrier phenotype *in vitro*. Considering that the total surface area of the filter pores critical for basolateral cell nutrient diffusion in both models was comparable, other factors leading to insufficient barrier properties in Model I could be the cause. In contrast, Toropainen et al. (2001) demonstrated that HCE-T cells achieve a tighter barrier when cultivated on collagen-coated polyester than on a collagen-coated polycarbonate membrane, the latter of which resulted in an epithelial model with lower TEER values of approximately 100 $\Omega \times cm^2$. Aiming to improve the barrier properties of Model I, the culture conditions during ALI exposure were modified. More precisely, the period of ALI cultivation was extended (Toropainen et al., 2003), and moist conditions, which were created by leaving a thin layer of culture medium mimicking the tear fluid apically during ALI exposure, were used (Ban et al., 2003). Moreover, after ALI exposure, the cells were re-submerged in the culture medium for different time periods. Our preliminary results showed that prolonging the ALI exposure to 5 days and re-submerging Model I in culture medium for an additional 2 days led to an improvement of the TEER values to approximately 500 $\Omega \times cm^2$ (data not shown). It is therefore possible that the Model I barrier properties could be enhanced by fine-tuning the time of ALI exposure and by re-submerging the formed multilayer in the culture medium. Recent studies have shown that epithelial barrier assembly requires the coordinated activity of multiple domains of the tight junction proteins (Rodgers et al., 2013), but it is still unclear how the cultivation conditions can be fine-tuned to achieve a well-preserved HCE-T cell-based model phenotype *in vitro*. Until then, the obtained TEER values suggest that Model I is not likely to be suitable for predicting transcorneal permeation for either drugs alone or drugs that are incorporated into different drug delivery (nano)systems. It could be expected that due to the decreased TEER of Model I, the drug permeability coefficient will be increased, which could result in false and misleading drug permeability results compared with other models with appropriate TEER values.

In contrast, Model II showed improved barrier properties (TEER: 1704–2608 $\Omega \times cm^2$; Table 2) in comparison with both the barrier properties of Model I and the excised cornea (TEER: 542–729 $\Omega \times cm^2$; Table 2). One could hypothesize that the extremely high TEER values observed in Model II were a consequence of the presence of the basolateral lipophilic layer formed from the migrated HCE-T cells. More precisely, it has been shown that the migrated cells are polarized, retaining junctional complexes, which could contribute to the overall TEER values (Tucker et al., 1992). Considering such TEER values, it is possible to expect that Model II should more closely reflect the drug transport across the excised cornea. In addition, these extremely high TEER values have the potential to discriminate transcorneal drug permeability well, considering the influence of different ophthalmic excipients (for instance, a permeation-enhancing *vs.* a permeation-retarding effect, depending on the type and concentration of the excipients) in comparative transcorneal permeation studies. However, it is reasonable to expect that this model would be more discriminative for more hydrophilic

Table 2
Apparent permeability coefficients (P_{app}) of ophthalmic compounds and the corresponding transepithelial electrical resistance (TEER) values for Model I, Model II and excised cornea.

	Model I		Model II		Excised cornea	
	P_{app} (10^{-7} cm/s)	TEER[a] ($\Omega \times cm^2$)	P_{app} (10^{-7} cm/s)	TEER[a] ($\Omega \times cm^2$)	P_{app} (10^{-7} cm/s)	TEER[a] ($\Omega \times cm^2$)
Timolol maleate	194.13 ± 7.08	69 ± 21	139.70 ± 16.69	2607 ± 937	89.20 ± 4.68	729 ± 231
Diclofenac sodium	206.17 ± 9.08	109 ± 9	126.13 ± 12.57	2111 ± 1304	17.54 ± 3.13	660 ± 204
Chloramphenicol	74.37 ± 3.79	113 ± 2	18.59 ± 2.27	2608 ± 1284	6.89 ± 1.71	725 ± 382
Dexamethasone	110.83 ± 10.17	85 ± 29	33.84 ± 5.02	1704 ± 783	6.88 ± 2.32	542 ± 130
Fluorescein sodium	26.17 ± 6.59	44 ± 8	1.68 ± 0.81	2393 ± 1655	1.58 ± 0.54	606 ± 199

All values represent the mean ± SD (n = 5–13).
[a] measured at the start of permeability experiments, before a test compound is added to the donor compartment.

than for more lipophilic ophthalmic compounds, for which models with a stroma equivalent would be more appropriate.

3.3. Comparison of drug permeation across the HCE-T cell-based models and the excised cornea

Knowing the P_{app} of the ophthalmic compounds across Model I or Model II compared with their permeation across the excised cornea will allow a more detailed understanding of the models' suitability for predicting transcorneal drug permeability. Using the HCE-T model with the suitable barrier properties in a Transwell set up allows drug permeability studies to be performed with a high correlation to the *in vivo* situation. The relatively wide variation in the HCE-T cell barrier properties between different laboratories (Nagai et al., 2008; Reichl, 2008) appears to be a disadvantage, and it is necessary to standardize the way in which such permeability data are analyzed and interpreted. Thus, in a comparable analysis and interpretation of drug permeability coefficients, the parameters related to barrier characteristics of the HCE-T cell-based model and test conditions (static *vs.* dynamic) need to be carefully considered.

In this study, low molecular weight (M_w values from 295.3 to 392.46) ophthalmic compounds were used. Their lipophilicities (*i.e.*, $\log D_{\text{n-octanol/buffer pH 7.4}}$) varied from −1.5 for fluorescein sodium (Sakai et al., 1997) and −0.2 for timolol maleate (Reichl, 2008) to 1.08 for chloramphenicol (Wu et al., 1996), 1.1 for diclofenac sodium (Chuasuwan et al., 2009) and 1.5 for dexamethasone (Hahne et al., 2012). The selected ophthalmic compounds were characterized by different charge states at the defined permeability assay condition (KRB pH 7.4). Namely, chloramphenicol and dexamethasone were neutral because these molecules do not have a relevant ionizable group. Fluorescein and diclofenac were negatively charged, as these molecules dissociate into dianionic and anionic forms at a pH above the pK_a values of 4.3/6.4 (Sjoback et al., 1995) and 3.8 (Chiarini et al., 1984), respectively. Timolol was positively charged because it accepts a proton below the pK_a value of 9.21.

In both the *in vitro* and *ex vivo* experiments, mass balance was determined to exclude the possible loss of test compound (*e.g.*, due to adsorption to plastic surfaces or drug degradation), which might result in the underestimation of permeability coefficients. The mass balance for all ophthalmic compounds in both HCE-T cell-based models and the excised cornea was in the range of 80–100% (data not shown), which has been shown to result in an acceptable approximation of the P_{app} value (Hubatsch et al., 2007).

Model I had higher P_{app} values for all ophthalmic compounds in comparison with their P_{app} values obtained for both the excised cornea and Model II. More precisely, the P_{app} values for timolol, diclofenac, chloramphenicol, dexamethasone and fluorescein calculated for Model I were approximately 1.39-, 1.6-, 4.00-, 3.26- and 15.57-fold higher than the P_{app} values obtained in Model II, respectively (Table 2). In this comparison, the considerable discrepancy between the calculated P_{app} values was evident for fluorescein. The observed discrepancy was expected because fluorescein is a hydrophilic marker of the paracellular pathway; therefore, its permeation is determined by the HCE-T barrier tightness, which was shown to be very low for Model I (*i.e.*, high fluorescein permeability) compared to the very high barrier tightness for Model II (*i.e.*, low fluorescein permeability). Furthermore, the exact mechanisms by which the selected ophthalmic drugs are transported by the epithelial barrier, both *in vitro* and *in vivo*, have not been fully elucidated. However, it is evident that the Model I showed a higher difference in permeability compared to Model II for neutral (*i.e.*, chloramphenicol and dexamethasone) than for charged (*i.e.*, timolol and diclofenac) ophthalmic drugs. The observed discrepancies were even greater when comparing P_{app} values of the Model I and the excised cornea, *i.e.*, the P_{app} values for timolol, diclofenac, chloramphenicol, dexamethasone and fluorescein calculated for Model I were approximately 2.18-, 11.74-, 10.79-, 16.11- and 16.56-fold higher than the P_{app} values obtained for the excised cornea, respectively (Table 2). Specifically, using these last comparison results, it is possible to conclude that the permeation of all of the investigated ophthalmic compounds was predicted less well by Model I.

A considerably better correlation was achieved in the comparison of the P_{app} values obtained for Model II with those obtained for the excised cornea. More precisely, the P_{app} values for timolol, diclofenac, chloramphenicol, dexamethasone and fluorescein calculated for Model II were approximately 1.57-, 7.19-, 2.70-, 4.92- and 1.06-fold higher in comparison with the P_{app} values obtained for the excised cornea, respectively (Table 2). In this comparison, the considerable discrepancy between the calculated P_{app} values was evident primarily for diclofenac and dexamethasone, whereas the P_{app} of fluorescein fit well. This discrepancy could be explained by the typical disadvantage of pure epithelial models (*i.e.*, lack of hydrophilic stroma), even if the TEER is in the same range as the excised cornea.

Diclofenac and timolol in both *in vitro* models exhibited comparable high P_{app} values, but in the excised cornea, a 5 times lower permeability for diclofenac compared with the highly permeable timolol was observed. Several physico-chemical properties are known to affect passive permeation through biological membranes, namely molecular size, partition coefficient, hydrogen bond formation and permeant charge (Kidron et al., 2010). Although a higher permeation of diclofenac owing to its higher lipophilicity and lower hydrogen bonding capacity (NCBI PubChem Compound Database) might be expected, the negative charge of the diclofenac anionic species at physiological pH might explain the opposite result. Because the native cornea is characterized by an isoelectric point value of 3.2, its net negative charge at physiological pH contributes to the higher permeation of cationic compared with anionic species, as observed by Pescina et al. (2015). Conversely, the permeation of dexamethasone was 1.5- and 1.8-fold higher compared to the permeation of chloramphenicol in Model I and II (Table 2), respectively, whereas in the excised cornea, almost equal permeability coefficients for the two compounds were found. Considering their similar molecular size, the same neutral charge and comparable hydrogen bonding capacity (NCBI PubChem Compound Database), the higher partition coefficient of dexamethasone, which facilitates its diffusion through lipophilic epithelial layers, might explain its higher permeation in Model I and II. However, the difference in the permeation behavior of the four ophthalmic drugs in the excised cornea compared with both *in vitro* models is somewhat more difficult to explain. Both cell models are pure epithelial models, only with a different number of epithelial layers and a lack of a hydrophilic stroma, which certainly influences the passive permeation rate of lipophilic compounds. Moreover, the role of membrane transporters in the observed discrepancy cannot be completely excluded. Both diclofenac and dexamethasone have been shown to interact with influx (Maillefert et al., 2000) and efflux (Kindla et al., 2011) membrane transporters in other cell lines; however, the active transport of these compounds, and of timolol and chloramphenicol, has not been studied in the HCE-T epithelial models or the porcine cornea to date. The expression and functionality of some membrane transporters in the HCE-T cell-based models and excised porcine cornea have already been determined (Becker et al., 2007; Mannermaa et al., 2006; Reichl et al., 2011; Vellonen et al., 2010; Verstraelen and Reichl, 2013, 2014). However, considering the structural alteration of the HCE-T barrier observed in this study, the expression of transporters, which was shown to be functional in simple epithelial and more complex HCE-T cell-based models in other studies, might become questionable in the HCE-T-based Model I and II. In contrast, considering the relatively high concentrations of ophthalmic compounds used in this study, active transport, even if it is present, due to membrane transporters' saturation effect, might contribute to the overall permeation only marginally, thus making passive diffusion a more feasible transport mechanism (Mannermaa et al., 2006). In conclusion, more detailed mechanistic permeability studies regarding the transport mechanism of the studied compounds and the barrier role of particular structures

of the corneal model are definitely required to explain the observed *in vitro* and *ex vivo* permeability differences.

3.4. In vitro/ex vivo correlations

Once *in vivo*-like barrier properties of cell-based models are optimized, some structural and functional differences compared to excised tissue may inevitably remain. Therefore, identical permeability values in these models may not be realistic to expect or necessarily crucial to obtain. More important is the ability of a particular model to discriminate well between different transport mechanisms, which determine the permeation of a wide range of compounds across the corneal barrier *in vivo*. Thus, the correlation of the cell-based and/or tissue-based models with *in vivo* corneal permeability should be established. In this study, Model II was clearly shown to discriminate better between the permeation of timolol and fluorescein, determined to be highly and poorly permeable in the excised cornea, respectively. More precisely, the difference in their P_{app} values in Model II was 83.15-fold, which more closely reflects their 56.46-fold difference in the excised cornea. In contrast, only a 7.42-fold difference was found in the case of Model I. The good predictability of *ex vivo* permeation coefficients for Model II was also confirmed by correlation analyses, the results of which are shown in Fig. 2. Correlation of *in vitro* and *ex vivo* logarithm P_{app} values gave a high and statistically significant Pearson correlation coefficient ($r = 0.90$, $P = 0.036$).

The further improvement of the HCE-T cell-based permeability model may consider additional particularities of a unique corneal environment to ensure the lower variability of barrier phenotype. Thus, the dynamics of the precorneal area are lacking in the investigated static permeability models. Reichl and coworkers observed small ultrastructural differences with regard to surface morphology between the HCET cell-based models and the native corneal epithelium, which was explained by the absence of tear fluid and eyelid wiping during *in vitro* cultivation conditions. In this case, the major aspects of *in vitro* barrier tightness were preserved (Reichl et al., 2004). Recently, Pretor and coworkers studied the permeation of lipophilic compound loaded into lipid nanodispersions across the HCE-T cell-based model under dynamic conditions (*i.e.*, shear stress of 0.1 Pa) to simulate the impact of tear flow and eyelid wiping during the permeability study. However, the influence of applied shear stress on the HCE-T cell barrier tightness was not the target value measured because the lipophilic compounds penetrate the cornea by taking the transcellular route (Pretor et al., 2015).

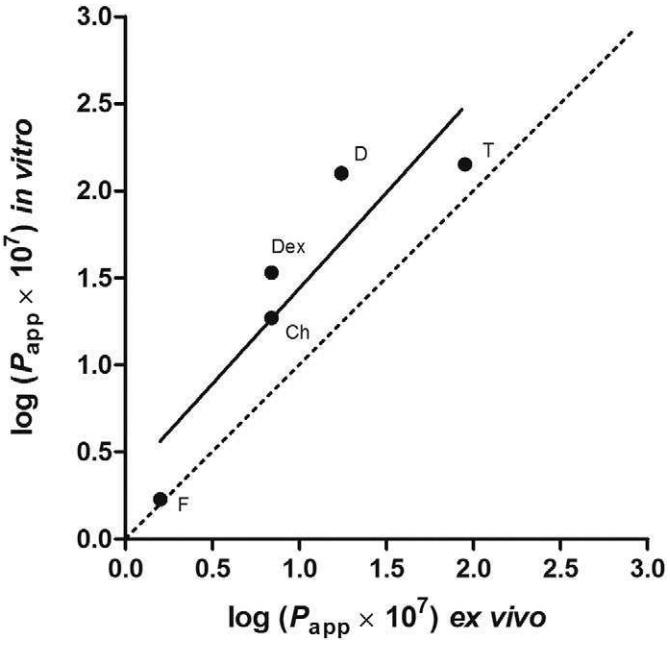

Fig. 2. Correlation between apparent permeability coefficients (P_{app}) of ophthalmic compounds in Model II and excised cornea ($r = 0.90$, $P = 0.036$). The dashed line shows identical P_{app} values in Model II and excised cornea; fluorescein (F), chloramphenicol (Ch), dexamethasone (Dex), diclofenac (D), timolol (T).

4. Conclusion

Cell-based models have gained considerable popularity for drug permeability studies across academic and/or pharmaceutical industry research. As a unique environment, the corneal barrier is difficult to mimic. Considering that a single factor could be enough to change the corneal barrier phenotype *in vitro*, all key factors influencing experimental outcomes by using HCE-T based model should be controlled and standardized. Membrane material, specifically its pore size, was shown to be an important factor in the HCE-T barrier phenotype. A polycarbonate membrane with a 3 μm pore size (Model II) proved to be better than a polycarbonate membrane with a 0.4 μm pore size (Model I) for tight barrier formation with HCE-T cells, although a unique structure for Model II was observed. Model II, which consists of apical lipophilic HCE-T monolayer and basolateral lipophilic monolayer of migrated HCE-T cells, generated more predictable permeability results when compared with the excised cornea.

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3. Evaluation of cationic nanosystems with melatonin using an eye-related bioavailability prediction model

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Evaluation of cationic nanosystems with melatonin using an eye-related bioavailability prediction model

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ABSTRACT

In this study, two types of nanosystems, namely lecithin/chitosan nanoparticles and Pluronic® F127/chitosan micelles, have been prepared and evaluated for their potential for the ocular delivery of melatonin, which is known to exert an ocular hypotensive effect. The melatonin content, particle size, zeta potential and *in vitro* drug release properties were studied as a function of the presence of chitosan in the nanosystem. Lecithin/chitosan nanoparticles were evaluated in terms of the mucoadhesive properties by a newly established method based on HCE-T cells, also used in *in vitro* biocompatibility and permeability studies.

Lecithin/chitosan nanoparticles were significantly larger than the corresponding F127/chitosan micelles (mean diameter of 241.8 *vs.* 20.7 nm, respectively) and characterised by a higher surface charge (22.7 *vs.* 4.3 mV, respectively). The HCE-T cell viability assay did not show significant toxic effects of nanosystems investigated at the (relevant) chitosan concentration tested. The permeability study results confirmed the permeation enhancing effect of F127, which was hindered in the presence of chitosan. Lecithin/chitosan nanoparticles were characterised by prominent mucoadhesive properties and prolonged melatonin release, which was shown to control melatonin permeation across an *in vitro* corneal epithelial model. Such properties demonstrate the potential for nanoparticles to provide an extended pre-corneal residence time of melatonin, ensuring higher eye-related bioavailability and extended intraocular pressure reduction compared to melatonin in both aqueous and micelle solutions.

1. Introduction

Ocular drug delivery is one of the most challenging issues faced by pharmaceutical researchers because of the specific anatomy, physiology and biochemistry of the eye that make it practically inaccessible to drugs. Current research is focused on the development of biopharmaceutically improved and safe delivery nanosystems that have high patient compliance and the ability to surpass ocular barriers and maintain the drug levels in tissues, improving therapeutic outcomes (Diebold and Calonge, 2010).

Innovative delivery nanosystems are designed to ensure a high precorneal residence time and sustained drug release, resulting in enhanced eye-related bioavailability of ophthalmic agents. The physicochemical properties of nanosystems, such as their size, surface charge, and drug release, determine their biopharmaceutical properties, including stability in the precorneal area, mucoadhesivity, biocompatibility and permeability, and therefore their *in vivo* performance. However, to direct the development of nanosystems toward a final ophthalmic formulation with the best therapeutic performance, reliable and convenient *in vitro* models are necessary to study the mechanisms and/or interactions of interest (Pepic et al., 2014). Therefore, concomitant with nanosystem development, it is important to establish *in vitro* models of eye barriers, enabling safe and efficacious screening of formulation candidates.

Melatonin (*N*-acetyl-5-methoxytryptamine), a neurohormone secreted by the pineal gland, has pleiotropic bioactivities, including

Abbreviations: ALI, air–liquid interface; F127, Pluronic® F127 triblock copolymer; IOP, intraocular pressure; HBSS, Hank's balanced salt solution; HCE-T, human corneal epithelial cells; PPO, polypropylene oxide; PEO, polyethylene oxide; TEER, transepithelial electrical resistance.

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the ability to modulate the intraocular pressure (IOP) (Bucolo et al., 2013). Elevated IOP is one of the main risk factors in the development of glaucoma, an optic nerve neuropathy that can result in blindness (Mediero et al., 2009). Melatonin and certain melatonin analogues were demonstrated to reduce the IOP in several species (Agorastos and Huber, 2011; Alcantara-Contreras et al., 2011).

Chitosan is a biocompatible and biodegradable polycationic polymer (Garcia-Fuentes and Alonso, 2012). At a pH below 6.5, it is positively charged and electrostatically interacts with negatively charged epithelial surfaces, resulting in mucoadhesion. This process provides a longer contact time for drug transport across the corneal barrier before the drug is cleared due to the rapid and extensive precorneal elimination. Moreover, chitosan can act as a permeation enhancer, increasing the paracellular drug permeation both *in vitro* and *in vivo*. Chitosan increases the transepithelial permeability due to its ability to reversibly disrupt epithelial tight junctional complexes up to a pH of approximately 6 (Basaran and Yazan, 2012).

Lecithin is a natural lipid mixture of phospholipids that has frequently been used for the preparation of various delivery nanosystems and is considered as a safe and biocompatible excipient (Hafner et al., 2009). Due to its lipophilic nature, it is expected to ensure a relatively high loading of lipophilic drugs as well as sustained drug release (Bhatta et al., 2012).

Pluronic® F127, a polyoxyethylated nonionic surfactant, has been proposed to increase the drug permeability through the corneal epithelial barrier (Pepic et al., 2013). F127 self-aggregates in aqueous solutions, forming micelles with a core that consists of a hydrophobic middle polypropylene oxide (PPO) block surrounded by an outer shell of hydrated hydrophilic polyethylene oxide (PEO) end blocks (Pepic et al., 2008, 2009). When formulated as eye drops, F127 micelle solutions enhance the ocular bioavailability of indomethacin (Chetoni et al., 2000), pilocarpine (Pepic et al., 2004) and plasmid DNA (Liaw et al., 2001; Tong et al., 2007) in rabbits. Our group developed F127/chitosan micelles with dexamethasone as a model drug and demonstrated their potential to solubilise the drug, enhance its precorneal retention and release it in a very effective manner, reaching high bioavailability levels (Pepic et al., 2010). However, no further studies have been performed to elucidate the exact mechanism(s) by which F127/chitosan micelles enhance dexamethasone transport across the corneal barrier.

The aim of this study was to develop a colloidal melatonin nanocarrier with sufficient melatonin entrapment and prominent mucoadhesive properties to protect embedded photosensitive melatonin, facilitate its passage through corneal barriers and/or extend its intraocular activity. For such purposes, two different colloidal nanosystems were investigated, namely lecithin/chitosan nanoparticles and Pluronic® F127/chitosan micelles. For both types of nanosystems investigated, the melatonin content, size and polydispersity, zeta potential and *in vitro* drug release properties were studied as a function of the presence of chitosan in the system. Lecithin/chitosan nanoparticles were also evaluated in terms of the mucoadhesive properties with a newly established method based on the HCE-T cells used in the biocompatibility and permeability *in vitro* studies. To gain insight into the influence of nanosystems on melatonin permeation across the *in vitro* corneal epithelial barrier, the correlation between melatonin *in vitro* release and permeability was evaluated.

2. Materials and methods

2.1. Reagents and chemicals

The following materials were used as received: chitosan in the form of hydrochloride salt (Protasan®UP CL 113, deacetylation degree 86%, NovaMatrix, Sandvika Norway); soybean lecithin Lipoid S45 (fat-free soybean lecithin with 45% phosphatidylcholine; a kind gift from Lipoid GmbH, Ludwigshafen, Germany); Pluronic® F127 triblock copolymer (F127) (molecular weight: 12,600, Sigma–Aldrich Chemie GmbH, Steinheim, Germany); and melatonin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). The substances used for the preparation of Hank's balanced salt solution (HBSS, pH 6) were prepared by dissolving the following substances in double-distilled water: $MgSO_4 \times 7H_2O$ (100 mg l^{-1}), KCl (400 mg l^{-1}), $NaHCO_3$ (350 mg l^{-1}), NaCl (8000 mg l^{-1}), D-glucose monohydrate (1100 mg l^{-1}) (all purchased from Kemig, Zagreb, Croatia), $CaCl_2 \times 2H_2O$ (185 mg l^{-1}) (Sigma–Aldrich), $MgCl_2 \times 6H_2O$ (100 mg l^{-1}) (Merck KGaA, Darmstadt, Germany), KH_2PO_4 (60 mg l^{-1}) (Kemika, Zagreb, Croatia), $Na_2HPO_4 \times 2H_2O$ (60 mgl^{-1}) (Fluka Chemie AG, Buchs, Switzerland), and HEPES (7150 mg l^{-1}) (Applichem, Darmstadt, Germany). Fluorescein sodium and rhodamine B were purchased from Sigma–Aldrich. All other chemicals or solvents were of analytical grade and purchased from Kemika (Zagreb, Croatia) and Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. HPLC assay of melatonin

The quantitative determination of melatonin was performed by HPLC. The system consisted of an Agilent 1100 Series instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector set at 224 nm. The mobile phase, consisting of purified water and acetonitrile in the ratio of 52:48, was applied at a flow rate of 1 ml/min. The column (Kinetex C18 column 50 × 4.6 mm, particle size 5 μm, Phenomenex, USA) was equipped with an inline filter (KrudKatcher Ultra HPLC, 0.5 μm Depth Filter × 0.004 in., Phenomenex, USA) and operated at 30 °C. The sample injection volume was 10 μl. The elution was isocratic, and the run time was 1 min. Melatonin peaks were separated with a retention time of 0.6 min.

2.2.2. Preparation of melatonin-loaded lecithin/chitosan nanoparticles

Melatonin-loaded lecithin/chitosan nanoparticles (MLC) were prepared as previously reported (Hafner et al., 2009). Lecithin was dissolved in 96% ethanol at concentration of 25 mg ml^{-1}. Melatonin was dissolved in the ethanolic solution of lecithin at concentration of 5 mg ml^{-1}, obtaining a lecithin-to-melatonin weight ratio of 5:1. Chitosan was solubilised in distilled water at concentration of 10 mg ml^{-1}. The chitosan solution (0.5 ml) was further diluted with distilled water to a volume of 46 ml. MLC nanoparticles were obtained with the injection of 4 ml of ethanolic lecithin/melatonin solution (syringe inner diameter of 0.75 mm) into 46 ml of diluted chitosan solution with magnetic stirring (900 rpm). A final chitosan concentration of 100 μg ml^{-1} and lecithin-to-chitosan weight ratio of 20:1 was obtained in the prepared nanoparticle suspensions. For comparison, melatonin-loaded lecithin nanoparticles (MLs) were prepared by the injection of ethanolic melatonin/lecithin solutions into distilled water. Non-entrapped melatonin was separated from MLC or ML using the dialysis technique, as described in the Section 2.2.4. Melatonin-free nanoparticles were prepared following the same procedure as for melatonin-loaded nanoparticles (L and LC nanoparticles).

2.2.3. Preparation of melatonin-loaded F127 and F127/chitosan micelles

A stock solution of F127 (100 mg ml^{-1}) was prepared by dissolving F127 in HBSS at pH 6.0 for 48 h at 4 °C. A chitosan stock solution (10 mg ml^{-1}) in water was prepared by stirring for 30 min at room temperature. The F127 micelles (F; 25 mg ml^{-1}) were prepared by diluting the F127 stock solution with HBSS at

pH 6.0. The F127/chitosan micelle solutions (FC; 25 mg ml^{-1} and 0.1 mg ml^{-1}, respectively) were prepared by mixing the F127 and chitosan stock solutions followed by dilution with HBSS at pH 6.0.

Melatonin-loaded F127 (MF) and F127/chitosan micelles (MFC) were prepared by the direct dissolution method. Melatonin (15 mg) was added to the F or FC micelle system (100 ml) and gently stirred for 24 h at room temperature. The non-solubilised melatonin was separated by filtration through 0.22-μm membrane filters (Millipore®, Zug, Switzerland).

2.2.4. Determination of the melatonin content

For nanoparticles (MLC and ML), melatonin content refers to the amount of entrapped melatonin, which is separated from non-entrapped melatonin by dialysis. For micelles (MFC and MF), melatonin content refers to the amount of melatonin left in the micelle solution after filtration. However, in addition to entrapped melatonin, it includes a certain amount of non-entrapped melatonin, which is determined by its solubility in water (0.1 mg ml^{-1}; room temperature).

The dialysis of nanoparticle suspensions (MLC and ML) was performed as previously reported (Hafner et al., 2009). Briefly, a 4 ml aliquot of melatonin-loaded nanoparticle suspensions was placed in a cellulose acetate dialysis bag and sealed (Spectra/Por® 4 Dialysis Tubing, MWCO 12–14 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, California, USA). The samples were dialysed against 100 ml of distilled water (receiver phase), under continuous magnetic stirring at 30 rpm and sink conditions. At scheduled time intervals, the 2 ml samples were withdrawn from the receiver phase and replaced with the same amount of distilled water. The dialysis was stopped when constant drug concentration values were detected in subsequent withdrawals from the receiver phase, taking into account the progressive dilution of the receiver phase. Withdrawn samples were assayed for the melatonin content by HPLC after they were diluted with acetonitrile to obtain a water-to-acetonitrile volume ratio of 52:48. The dialysed nanoparticle suspension samples were used in all further studies.

Filtered micelle solutions were disrupted by dissolving the micelles and melatonin with equal amounts of ethanol. The melatonin content was determined by HPLC. The filtered micelle solutions were used in all further studies.

2.2.5. Physical characterisation of the particle size and surface charge

The size and zeta potential of nanoparticles and micelles were measured by Photon Correlation Spectroscopy (PCS) using Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). The nanoparticle samples were diluted with 0.45 μm filtered distilled water and 10 mM NaCl solution prior to the measurement of the size and zeta potential, respectively. The pH of diluted samples ranged from 5.8 to 6.0. Zeta-potential measurements were performed at 25 °C. Samples were placed in the electrophoretic cell, where a potential of 150 mV was established.

2.2.6. In vitro release studies

The release of melatonin from nanosystems was assessed by the dialysis technique under sink conditions over 5 h. The appropriate volume of melatonin-loaded nanoparticle suspension or micelle solution containing 250 μg of melatonin was placed into a cellulose acetate dialysis bag (Spectra/Por® 4 Dialysis Tubing, MWCO 12–14 kDa or Dialysis Tubing Visking, MWCO 3.5 kDa, Medicell International Ltd., London, UK, respectively), which was sealed from both sides and immersed into 30 ml of pH 5.8 acetate buffer solution at 37 °C that was magnetically stirred at 30 rpm. At scheduled time intervals, agitation was stopped and the aliquots (1 ml) were withdrawn and replaced with equal amounts of fresh buffer solution. Withdrawn samples were diluted with acetonitrile, resulting in a water to acetonitrile volume ratio of 52:48, and we checked for the melatonin content by HPLC. All experiments were performed in triplicate.

2.2.7. Cell culture conditions

In this study, we used human corneal epithelial cells (HCE-T; RIKEN Cell Bank, Tsukuba, Japan). This cell line is the most extensively characterised immortalized human corneal epithelial cell line in terms of passive transcellular and paracellular transport, transporter expression and metabolic enzyme expression (Hahne and Reichl, 2011; Pepic et al., 2014). Cells were cultivated using DMEM/F12 (Lonza, Basel, Switzerland) supplemented with foetal bovine serum (5%, Biosera, Boussens, France), insulin (5 μg ml^{-1}, Applichem, Darmstadt, Germany), dimethyl sulfoxide (0.5%, Applichem), epidermal growth factor (10 ng ml^{-1}, Applichem), and Pen/Strep/Fungizone (Lonza).

2.2.8. Cell viability study

To assess the cytotoxicity of nanoparticles and micelles, HCE-T cells were seeded onto 24-well plates at a density of 2×10^4 -cells/well and allowed to reach confluence over 2 days. The suspension of nanoparticles or micelles was mixed with HBSS, resulting in a (relevant) chitosan concentration of 2.5, 10 or 20 μg ml^{-1}. Prior to the nanosystem treatment, the cell culture medium was withdrawn, and the cells were washed with HBSS. The cells were then exposed to the nanosystem suspensions for 30 min. Cells incubated in HBSS were used as a negative control. After 30 min of nanosystem treatment, the cells were washed twice with HBSS and incubated with fresh medium (500 μl/well) for 24 h. Cell viability was determined with the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma) assay. A total of 50 μl of MTT stock solution (5 mg ml^{-1}) was added to each well, and the cells were then incubated for 2 h at 37 °C. Subsequently, the medium was removed, cells were lysed and formazan dissolved with acidic isopropanol. The amount of formazan was quantified spectrophotometrically at 570 nm (1420 Multilabel counter VICTOR3, Perkin Elmer, Waltham, Massachusetts, USA).

2.2.9. Permeability assay using an in vitro corneal epithelial model

An *in vitro* corneal epithelial model was cultivated on Transwell® polycarbonate filter inserts (3.0-μm pore size, 12 mm diameter, Corning B.V. Life Sciences, Amsterdam, The Netherlands) coated with type I rat-tail collagen (Sigma–Aldrich) and fibronectin (Sigma–Aldrich). HCE-T cells suspended in the culture medium were seeded onto the coated filter (10^5 cells per well) and cultivated submerged for seven days. Subsequently, they were exposed to the air–liquid interface (ALI) during the following three days to induce cell differentiation and multilayer growth (Hahne and Reichl, 2011). The applicability of an *in vitro* corneal epithelial model in permeability studies, especially for assessing the interaction of chitosan-based nanosystems with tight junctions, was evaluated by determining the interdependence between the temperature, transepithelial electrical resistance (TEER) and apparent permeability coefficient of transcellular and paracellular markers. Specifically, the apparent permeability coefficients of rhodamine B and fluorescein sodium were correlated with the TEER values obtained at two different temperatures (room temperature and 37 °C).

The permeability experiments were performed directly in the Transwell® plate on a horizontal orbital shaker (37 °C; 50 rpm) in HBSS (pH 6.0). To determine the barrier integrity and interaction of nanosystems with tight junctions, TEER was measured during the experiments (EVOM, WPI Inc., Sarasota, Florida, USA). Test samples were prepared by diluting melatonin solution (400 μM) and melatonin-loaded nanosystems with HBSS to a melatonin concentration of 50 μM. Prior to the experiment, the cells were

washed with HBSS and incubated with 400 µl and 2000 µl of HBSS in the donor and receptor compartments, respectively, during the subsequent 30 min. At the start of the experiment, HBSS was removed from the donor compartment, and 400 µl of the test sample was added. Samples (400 µl) were taken from the receptor compartment at regular time intervals over 120 min and replaced with the same volume of fresh buffer. At the end of the experiment, the solution remaining in the donor compartment was also collected. The cell surface was rinsed with HBSS and exposed to the air–liquid interface during the subsequent 24 h; then, the final TEER measurement was performed.

Samples were diluted with acetonitrile to a water:acetonitrile volume ratio of 52:48; they were then analysed for the melatonin content by HPLC. The apparent permeability coefficients (P_{app} in cm/s) were calculated according to the following equation:

$$P_{app} = \frac{\partial Q}{\partial t} \times \frac{1}{AC_0}$$

where $\frac{\partial Q}{\partial t}$ is the permeation rate, A is the diffusion area of the epithelial barrier, and C_0 is the initial concentration of melatonin in the donor compartment (Sadeghi et al., 2008).

2.2.10. Mucoadhesive properties of lecithin/chitosan nanoparticles

To determine the mucoadhesive properties of MLC, we developed a method based on the HCE-T cells used in biocompatibility and permeability *in vitro* studies. The extent of the particle bioadhesion was evaluated by measuring the decrease in melatonin content in a nanoparticle suspension following the exposure of the cell monolayer to a melatonin-loaded nanoparticle suspension. HCE-T cells were seeded onto 24-well plates (TPP, Trasadingen, Switzerland) at a density of 2×10^4 cells/well and allowed to reach confluence over 2 days. Cells were treated with 200 µl of MLC, ML or the corresponding melatonin aqueous solution after the removal of culture medium. At scheduled time intervals (6 time points over 90 min), samples (20 µl) were collected for the analysis of the melatonin content. The remaining content of the suspension was then transferred to the next well upon the removal of cell culture medium. Samples were diluted with acetonitrile to a water:acetonitrile volume ratio of 52:48; the melatonin content was then determined by HPLC. All experiments were performed in triplicate.

2.2.11. Statistical analysis

The statistical software package JMP 10.0.2. (SAS Institute, Cary, North Carolina, USA) was used to perform 1-way ANOVA, followed by multiparametric Tukey's post hoc test. Multivariate linear models were calculated and plots are shown as provided by the general linear model function of the JMP software package. A P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Characterisation of the nanosystems

Two types of nanosystems, namely lecithin/chitosan nanoparticles (MLC) and F127/chitosan micelles (MFC), which have equal chitosan and similar melatonin content but are significantly different in size (241.8 *vs.* 20.7 nm, respectively), were prepared and evaluated for their potential for melatonin ocular delivery. Chitosan-free and/or melatonin-free nanosystems were also prepared and were used as controls when appropriate. The composition and main characteristics of the nanosystems prepared are given in Table 1.

Lecithin/chitosan nanoparticles were formed based on the electrostatic interaction between the negatively charged fraction of the lipid material and the positively charged chitosan, as already described in the literature (Gerelli et al., 2008; Hafner et al., 2009; Sonvico et al., 2006). Nanoparticles were separated from non-entrapped melatonin using the dialysis method. The nanoparticle size and surface charge were determined using dialysed systems diluted with water and 10 mM NaCl, respectively. The pH values in the diluted dialysed systems were between 5.8 and 6.0. The lecithin/chitosan nanoparticles (MLC) obtained were significantly larger than the corresponding lecithin nanoparticles (ML) (241.8 ± 0.8 *vs.* 91.8 ± 5.1 nm, respectively). The zeta potential was inverted from negative values for ML (−35.9 ± 4.2 mV) to positive values for MLC (22.7 ± 0.7 mV), which can be ascribed to the presence of chitosan at the nanoparticle surface. Compared to ML, MLC were characterised by higher melatonin loading. The data obtained for melatonin-loaded and melatonin free nanoparticles indicated that melatonin loading did not influence the average particle size or zeta potential (Table 1).

The main characteristics of the nanoparticles are consistent with those reported in our previous studies (Hafner et al., 2011, 2009).

F127 micelle systems are well established and known to enhance ocular drug delivery (Pepic et al., 2012). Our group has proposed the use of F127/chitosan micelle systems and their potential to solubilise the drug and enhance its precorneal retention, reaching high bioavailability, was demonstrated *in vivo* (Pepic et al., 2008, 2009; Pepic et al., 2010). In such systems, pH-related electrostatic interactions between the electronegative oxygen atoms of PEO segments and electropositive chitosan chains, as well as hydrogen bonds, between the oxygen atoms of PEO segments and the OH groups of chitosan have been proposed.

Melatonin-loaded micelle systems were prepared by the direct dissolution method. The undissolved melatonin was separated by filtration. The micelle size and surface charge were determined in the filtrates with no previous dilution. The average hydrodynamic diameter of the micelles ranged between 20.7 and 24.5 nm. The addition of chitosan did not influence the average micelle size. However, melatonin loading resulted in a slight reduction of the average micelle size in both the presence and in the absence of chitosan (Table 1), which is probably due to an interaction with F127. At the same time, an increase in the zeta potential due to melatonin loading was observed in the presence of chitosan (MFC *vs.* FC; 4.3 ± 0.9 *vs.* 1.5 ± 1.1 mV, respectively).

The zeta potential of MF micelles was close to 0 mV. This value was expected due to the electroneutral nature of F127. In the case of MFC micelles, an increase in the zeta potential compared to that of MF micelles was observed and assigned to the presence of chitosan on the micelle surface. As the parameters of the chitosan concentration (100 µg ml^{-1}) and pH (6.0) were kept constant for both types of nanosystems prepared, a significantly higher zeta potential of MLC compared to the zeta potential of MFC can be assigned to the stronger electrostatic interaction of chitosan with lecithin than with F127 and the consequently higher charge density at the particle surface.

For both types of nanosystems prepared, the melatonin content (109.3–142.4 µg ml^{-1}; Table 1) is several times higher than the melatonin concentration in the solution (100 µM (23.2 µg ml^{-1}), instillation volume of 10 µl) previously reported to ensure the reduction of the elevated intraocular pressure in rabbits (Martinez-Aguila et al., 2013).

3.2. In vitro release

The dialysis technique is considered the most suitable method for determining the drug release profile from drug delivery nanosystems (Modi and Anderson, 2013; Zambito et al., 2012). However, the increase in drug concentration in the receiver phase

Table 1
The composition and the main characteristics of melatonin-loaded nanosystems (nanoparticles and micelles).

The main nanosystem constituent	Sample	Chitosan conc. (μg/ml)	Melatonin content (μg/ml)	Size (nm)	PDI	Zeta-potential (mV)
Lecithin (2 mg/ml)	MLC	100	137.3 ± 10.1	241.8 ± 0.8	0.207 ± 0.003	22.7 ± 0.7
	(LC)			(238.0 ± 1.5)	(0.260 ± 0.015)	(22.1 ± 0.8)
	ML	0	109.3 ± 7.2	91.8 ± 5.1	0.397 ± 0.002	−35.9 ± 4.2
	(L)			(79.1 ± 4.5)	(0.405 ± 0.009)	(−34.4 ± 4.7)
Pluronic F127 (25 mg/ml)	MFC	100	138.2 ± 6.9	20.7 ± 0.3	0.176 ± 0.031	4.3 ± 0.9
	(FC)			(23.3 ± 0.1)	(0.340 ± 0.029)	(1.5 ± 1.1)
	MF	0	142.4 ± 8.5	20.0 ± 0.1	0.213 ± 0.017	0.2 ± 0.6
	(F)			(24.5 ± 1.0)	(0.315 ± 0.054)	(0.2 ± 1.6)

Values are mean ± SD (n = 3).
Values in brackets refer to melatonin-free nanosystems.
PDI, polydispersity index.

is a consequence of two processes, namely, drug release to the continuous phase inside the dialysis bag and drug diffusion from the donor to the receiver phase through the dialysis membrane. The membrane provides some resistance to drug diffusion, affecting its release rate. Therefore, an *in vitro* release study was also performed with melatonin aqueous solution to determine the resistance of the dialysis membrane and its influence on the overall drug release rate.

All nanosystems were characterised by prolonged melatonin release (Fig. 1), which is a desirable property for achieving extended IOP reduction (Musumeci et al., 2013). The melatonin release rate from nanoparticles decreased with the presence of chitosan in the system ($t_{50\%}$ values for MLC and ML were 1.75 and 0.75 h, respectively), which is consistent with our previous reports (Hafner et al., 2009).

For micelles, the opposite effect of chitosan on the melatonin release rate was observed. Therefore, the melatonin release from MFC was faster ($t_{50\%}$ 0.75 h) than the melatonin release from MF ($t_{50\%}$ = 1.5 h). The influence of chitosan on the melatonin release rate is probably due to its slight thermodynamic destabilization of micelle aggregates at pH 6.0, as reported in our previous work (Pepic et al., 2008, 2009), accompanied by the intermediate position of melatonin within micelle compartments (Torchilin, 2001).

The diffusion of melatonin through the diffusion membrane for melatonin aqueous solution was significantly faster ($t_{50\%}$ of 0.25 h) than for the investigated nanosystems. Therefore, melatonin release from nanosystems to the continuous phase inside the dialysis bag is the rate-limiting factor that determines the release profile obtained for nanoparticles and micelles.

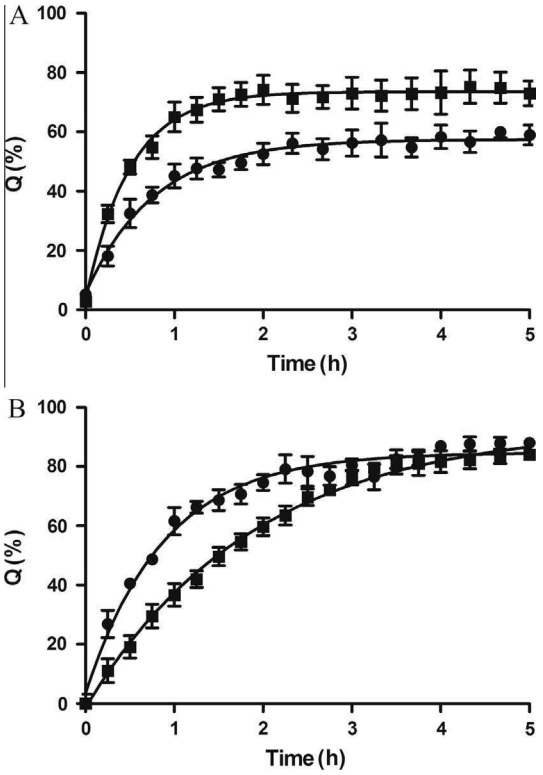

Fig. 1. Release profiles of melatonin from MLC (●) and ML (■) nanoparticles (A) and MFC (●) and MF (■) micelles (B) in acetate buffer at pH 5.8. Data are expressed as the mean ± SD (n = 3).

3.3. Mucoadhesive properties of lecithin/chitosan nanoparticles

Evaluation of mucoadhesion is necessary for characterising the delivery systems aimed to prolong the drug residence time at the eye surface (Musumeci et al., 2013). In this work, the incorporation of melatonin into the nanoparticles served as an indicator of nanoparticle mucoadhesion upon the exposure of the HCE-T cell monolayer to a melatonin-loaded nanoparticle suspension. Melatonin aqueous solution was used as a control to distinguish the decrease in the melatonin content due to the melatonin absorption from its decrease due to mucoadhesion. Our results confirmed the improved mucoadhesive properties of MLC compared to ML (Fig. 2). This finding is in line with the difference in the surface charge of the compared systems. The MLC nanoparticles were characterised by a positive charge originating from chitosan at their surface, whereas the ML nanoparticles were negatively charged (Table 1). The results obtained with this method confirmed the findings reported in our previous study using a standard mucus glycoprotein assay to determine nanoparticle mucoadhesion (Hafner et al., 2009).

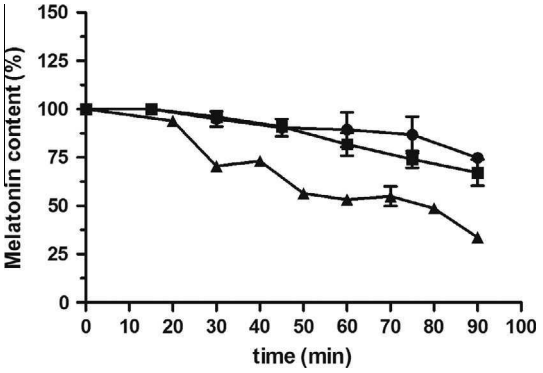

Fig. 2. Evaluation of the mucoadhesive properties of MLC (▲) and ML (■) nanoparticles by measuring the decrease in the melatonin content in a nanoparticle suspension following the treatment of a HCE-T cell monolayer. Melatonin aqueous solution (●) was used as a control. Data are expressed as the mean ± SD (n = 3).

The mucoadhesive properties of micelles cannot be evaluated due to their inherent dynamic properties. However, micelle nanosystems are expected to be superior to melatonin solution because the surface tension values of F127 (38.1–39.2 mN m^{-1}) and F127/chitosan (37.5–38.8 mN m^{-1}) micelle solutions (Pepic et al., 2008) are relatively close to the surface tension of human tears (42–46 mN m^{-1}) (Nagyova and Tiffany, 1999). This improves the spreading properties over the ocular surface immediately after the instillation as well as decreases the contact angle of micelle nanosystems, and the ocular surface forms a thin layer, minimizing the possibility for immediate loss due to spillage (overflow), as observed in our previous *in vivo* study (Pepic et al., 2010).

3.4. Evaluation of cell viability

Ocular biocompatibility and toxicity should always be considered when designing ocular drug delivery systems, particularly if they are intended for chronic administration. Based on the results observed in rabbits, chitosan in solution has been identified as a well-tolerated constituent of topical ophthalmic formulations (Di Colo et al., 2004; Felt et al., 1999). As for the chitosan-based nanosystems, a number of studies, both *in vitro* and *in vivo*, aimed to assess their biocompatibility and tolerance (de la Fuente et al., 2010). *In vitro* tests were performed to determine the toxicological profile of chitosan-based nanoparticles on immortalized cell lines obtained from the ocular epithelia, i.e., cornea (HCE) and conjunctiva (Chang and NHC-IOBA cells) (de la Fuente et al., 2008; Diebold et al., 2007). Initial reports on the cytotoxicity of chitosan nanoparticles indicated acceptable toxicological profiles *in vitro*; also, the levels of cell death did not exceed 5% for a polymer concentration of 2 mg ml^{-1} (de Campos et al., 2004; Enriquez de Salamanca et al., 2006). In the case of more complex structures, such as chitosan/hyaluronic acid nanoparticles and complexes of chitosan nanoparticles with liposomes, the cytotoxicity was lower than for chitosan nanoparticles because of the lower net surface charge (de la Fuente et al., 2008; Diebold et al., 2007). Pluronics are generally recognized as safe for ophthalmic administration, and they are used as formulation components of several commercial topical ocular products (Jiao, 2008). Those with a high content of PEO are particularly safe. Core-forming blocks, such as polyesters and poly(L-amino acids), undergo hydrolysis and/or enzymatic degradation *in vivo*, resulting in biocompatible monomers that are readily eliminated by the kidneys (Kumari et al., 2010).

Here, *in vitro* cytotoxicity studies have been performed to not only evaluate the biocompatibility of nanosystems with ocular epithelial cells, but also define the concentration of nanosystems that can safely be applied on the *in vitro* corneal epithelial model in permeability studies.

A cytotoxicity study of the nanosystems prepared was performed on HCE-T cells at a (relevant) chitosan concentration ranging from 2.5 to 20 μg ml^{-1} and a 0.5 h incubation time. Chitosan was noted as a key component due to its mucoadhesive properties and permeation enhancing properties, which are known to depend on its pH-related net charge. Therefore, it is important to determine the nanosystem biocompatibility under defined experimental conditions because the potential cytotoxicity may be a consequence of the high binding affinity for cellular membranes.

The cell viability assay did not show significant toxic effects of the nanosystems investigated at the (relevant) chitosan concentration tested (Fig. 3, $P > 0.05$). By contrast, concentration-dependent chitosan cytotoxicity was observed when the cells were treated with chitosan solution, indicating that chitosan in a free soluble form is much more cytotoxic than when it is incorporated in the nanosystem.

The biocompatibility of lecithin/chitosan nanoparticles was previously investigated in Caco-2, HaCaT and BJ fibroblasts (Hafner et al., 2011, 2009). Nanoparticles differed in their chitosan content and the negative charge of lecithin used in the preparation. The differences between the cytotoxicity of the nanoparticles have been ascribed to the differences in the charge density of nanoparticles and the chitosan content in its free-soluble form in nanoparticle suspension, both of which may affect the interaction of the systems with the cell membranes and, therefore, their toxicity. However, for lecithin S45/chitosan nanoparticles with lecithin to a chitosan weight ratio of 20:1 (MLC in this study), no influence on cell viability was ever observed.

Pluronic F127 solutions at concentrations ranging from 2.5 to 5000 μg ml^{-1}, were nontoxic and biocompatible with mouse fibroblasts *in vitro*, even at 48 h cell exposure (Arranja et al., 2014). Accordingly, there was no significant decrease in the viability of HCE-T cells treated with micelle systems at F127 concentrations up to 5000 μg ml^{-1}. The increase in the F127 concentration did not decrease the cell viability decrease because F127 unimers are aggregated into micelles at concentrations above the critical micelle concentration, and these micelles are potentially less toxic than the non-aggregate unimer forms.

3.5. Permeability assay using an in vitro corneal epithelial model

The influence of the nanosystems on the melatonin permeability was investigated using an *in vitro* corneal epithelial model based on HCE-T cells.

Considering the interaction of the chitosan-based nanosystems with the tight junctions of the epithelial barrier, the applicability of an *in vitro* corneal epithelial model in permeability studies was evaluated by determining the interdependence between the temperature, TEER and apparent permeability coefficient (P_{app}). The relationship between the TEER and temperature has been established in the literature (Shen et al., 2008) (Gonzalez-Mariscal et al., 1984). The TEER of MDCK cell monolayer was studied as a function of the temperature, from 3 to 37 °C. The change in the TEER elicited by a temperature increase was large (for this temperature interval 306%), fast (less than 2 s) and reversible (Gonzalez-Mariscal et al., 1984).

The permeabilities of fluorescein sodium, as a paracellular marker, and rhodamine B, as a transcellular marker (Hahne and Reichl, 2011; Toropainen et al., 2001), were investigated at 20 and 37 °C using an *in vitro* corneal epithelial model that was characterised by a different initial TEER. To investigate the effect of temperature on the TEER, corneal epithelial models with initial TEER values of 800–1000 Ω × cm^2, measured in culture medium at 37 °C, were selected. After 30 min of incubation in HBSS at 37 °C, the TEER was reduced to 50.0 ± 4.7% of the initial value ($n = 24$). When the incubation in HBSS was performed at 20 °C, a slight reduction in the TEER compared to the initial values was observed (93.0 ± 11.0%). These results confirmed the influence of temperature on the TEER during incubation in HBSS.

The correlation of the apparent fluorescein sodium permeability coefficients (P_{app}) across *in vitro* corneal epithelial models with TEER has been established ($r = 0.9723$ and 0.9519 at 20 and 37 °C, respectively). The increase in the P_{app} of fluorescein sodium with the reduction of the TEER is expected because fluorescein sodium is a marker of the paracellular pathway. No influence of the TEER on the P_{app} of rhodamine B was observed, indicating the transcellular pathway of rhodamine B transport across the *in vitro* corneal epithelial model. However, the P_{app} values at 37 °C were slightly higher than at 20 °C for a similar TEER, owing to the faster diffusion from the increase in temperature.

The relationship between the fluorescein sodium and rhodamine B P_{app} and TEER established here confirms the reliability and convenience of the corneal epithelial model for *in vitro*

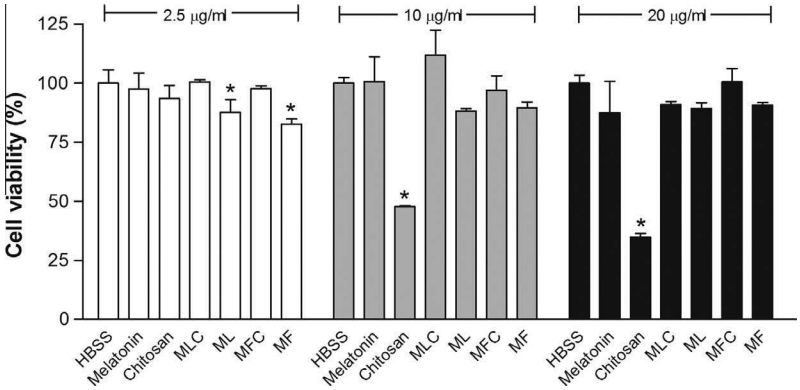

Fig. 3. Influence of melatonin-loaded nanoparticles (MLC and ML) and micelles (MFC and MF) on the viability of HCE-T cells (MTT assay). The suspension of nanoparticles or micelles was mixed with HBSS, obtaining a (relevant) chitosan concentration of 2.5, 10 or 20 μg ml^{-1}. HBSS, melatonin (2.5, 10 and 20 μg ml^{-1}) and chitosan solution (2.5, 10 and 20 μg ml^{-1}) in HBSS were used as controls. The incubation time was 30 min. Data are expressed as the mean ± SD (n = 3). *Differs from untreated cells incubated in HBSS (P < 0.05).

permeability studies and bioavailability prediction. Moreover, it can serve as a basis for the prediction of nanosystems' transport pathways and their modes of interaction with epithelial barriers.

Permeability studies were performed using test samples that were prepared by diluting melatonin-loaded nanosystems (MLC, ML, MFC, and MF) and melatonin stock solution with HBSS buffer (pH 6.0), up to an equal melatonin concentration (50 μM). The ocular administration of 20 μl of melatonin-loaded nanosystem suspension with a melatonin concentration of 50 μM is expected to ensure a reduction in the elevated intraocular pressure. This conclusion is based on the previously reported effective dose of melatonin in solution (Martinez-Aguila et al., 2013). The selected melatonin concentration refers to the chitosan concentration in the range of 8.1–10.6 μg ml^{-1} (depending on the content of melatonin in nanosystems), which has been covered in the biocompatibility studies (Fig. 3).

The effect of nanosystems on tight junction opening and cell viability was investigated by monitoring the TEER across the HCE-T epithelial barrier for 24 h, starting from the application of test samples. For all test samples used, a decrease in the TEER was observed, resulting in TEER values that were approximately 60% of the initial value at the end of the experiment. Despite the known effect of chitosan on tight junction opening (Hafner et al., 2009), there was no difference in TEER decrease between chitosan-based (MLC and MFC) and chitosan-free (ML and MF) nanosystems, suggesting the involvement of other TEER-decreasing factors and model limitations. However, 22 h after removing the test samples from the model surface and its exposure to ALI, the recovery of the initial TEER values was observed, confirming that the investigated nanosystems lacked toxic effects.

The permeation of melatonin from aqueous solution and nanosystem formulations across the *in vitro* corneal epithelial model is demonstrated with the P_{app} (Fig. 4). The results indicated that, except for MF, the incorporation of melatonin into nanosystems significantly decreased its overall permeability compared to the melatonin solution ($1.40 \pm 0.11 \times 10^{-5}$ cm/s). Aside from the type (size) of the investigated nanosystems, the presence of chitosan seems to influence the melatonin permeability. Therefore, MLC is characterised by lower melatonin permeability than ML ($0.59 \pm 0.04 \times 10^{-5}$ cm/s *vs.* $0.84 \pm 0.09 \times 10^{-5}$ cm/s, respectively). As already stated, the presence of chitosan in such a system resulted in slower melatonin release, leading to a lower melatonin permeation rate. In addition, a relatively strong correlation between melatonin *in vitro* release and permeation rate was observed for the nanoparticles, as shown in Fig. 5, suggesting that permeation was

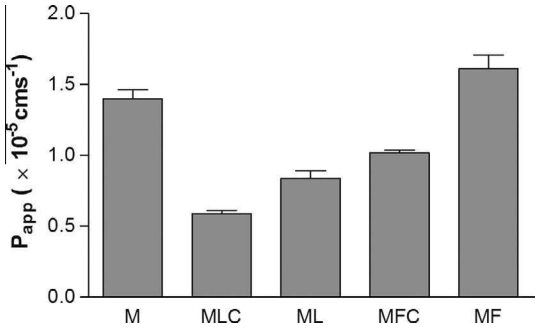

Fig. 4. Permeation of melatonin across the corneal epithelial model based on HCE-T cells from aqueous solution (M), nanoparticle suspensions (MLC and ML), and micelles (MFC and MF) presented by apparent permeability coefficients (P_{app}). Test samples were prepared by diluting MLC, ML, MFC, MF and melatonin stock solution with HBSS buffer (pH 6.0) up to an equal melatonin concentration (50 μM). Selected melatonin concentration refers to chitosan concentration in range of 8.1–10.6 μg ml^{-1}. Data are expressed as the mean ± SD (n = 3). Statistically significant differences between each nanosystem and melatonin aqueous solution were observed (P < 0.05), except for MF.

controlled by the melatonin release profile. Higher melatonin permeability was observed for micelles compared to nanoparticles. Generally, the influence of nanocarriers on the drug permeability is related to particle size; therefore, micelles (approximately 20 nm in size) are expected to provide better melatonin permeation than nanoparticles (91.8 and 241.8 nm). A higher permeability of melatonin from micelle systems is also partially due to the presence of a certain amount of non-entrapped melatonin (determined by its solubility in water; 0.1 mg ml^{-1}) that is readily absorbed across the *in vitro* corneal epithelial barrier. Moreover, improved melatonin permeability from micelle systems can be ascribed to the well-established enhancing effect of amphiphilic copolymers, such as F127, on transcellular permeability (Pepic et al., 2012; Tian and Mao, 2012). Unimers that are dissociated from micelles may perturb the plasma membrane by their incorporation into the lipid bilayer, resulting in acceleration of drug permeation through cell membranes (Kim et al., 2010). When the lipid bilayer is saturated, mixed micelles are formed, resulting in the removal of phospholipids from the cell membranes and increased transcellular permeability (Jiao, 2008). As the melatonin permeability from MFC micelles was significantly lower than that from MF micelles, it seems that chitosan, through its electrostatic interaction

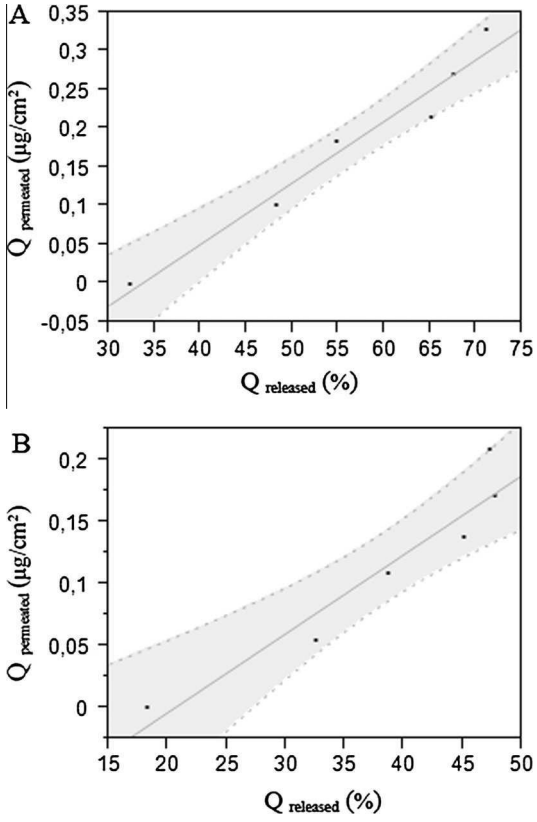

Fig. 5. Correlation between the *in vitro* melatonin release and permeation from ML (A; $r = 0.9677$) and MLC (B; $r = 0.9382$).

with F127, diminished its enhancing effect on the melatonin transcellular permeability ($1.02 \pm 0.03 \times 10^{-5}$ cm/s *vs.* $1.61 \pm 0.17 \times 10^{-5}$ cm/s).

4. Conclusions

Melatonin is readily absorbed across corneal epithelial barriers, as confirmed by the P_{app} of melatonin in solution. However, rapid and extensive precorneal elimination, owing to tear dynamics, nasolachrymal drainage and nonproductive adsorption/absorption, results in a limited eye-related bioavailability of melatonin from solution and a brief pharmacological effect. The enhancing effect of F127 on melatonin permeation demonstrated in this study is expected to provide higher eye-related melatonin bioavailability. However, the permeation-enhancing effect of F127 was hindered in the presence of chitosan, indicating that the addition of chitosan in F127 micelle formulation was not beneficial. Lecithin/chitosan nanoparticles were characterised by prominent mucoadhesive properties and prolonged melatonin release, which was shown to control melatonin permeation across an *in vitro* corneal epithelial model. Therefore, such a nanosystem shows the potential for providing an extended precorneal residence time of melatonin, ensuring both higher eye-related bioavailability and extended IOP reduction compared to melatonin aqueous solution.

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4. Biopharmaceutical evaluation of surface active ophthalmic excipients using *in vitro* and *ex vivo* corneal models

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Biopharmaceutical evaluation of surface active ophthalmic excipients using *in vitro* and *ex vivo* corneal models

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ABSTRACT

The objective of this study was to systematically investigate the effects of surface active ophthalmic excipients on the corneal permeation of ophthalmic drugs using *in vitro* (HCE-T cell-based model) and *ex vivo* (freshly excised porcine cornea) models. The permeation of four ophthalmic drugs (*i.e.*, timolol maleate, chloramphenicol, diclofenac sodium and dexamethasone) across *in vitro* and *ex vivo* corneal models was evaluated in the absence and presence of four commonly used surface active ophthalmic excipients (*i.e.*, Polysorbate 80, Tyloxapol, Cremophor® EL and Pluronic® F68). The concentration and self-aggregation-dependent effects of surface active ophthalmic excipients on ophthalmic drug permeability were studied from the concentration region where only dissolved monomer molecules of surface active ophthalmic excipients exist, as well as the concentration region in which aggregates of variable size and dispersion are spontaneously formed. Neither the surface active ophthalmic excipients nor the ophthalmic drugs at all concentrations that were tested significantly affected the barrier properties of both corneal models, as assessed by transepithelial electrical resistance (TEER) monitoring during the permeability experiments. The lowest concentration of all investigated surface active ophthalmic excipients did not significantly affect the ophthalmic drug permeability across both of the corneal models that were used. For three ophthalmic drugs (*i.e.*, chloramphenicol, diclofenac sodium and dexamethasone), depressed *in vitro* and *ex vivo* permeability were observed in the concentration range of either Polysorbate 80, Tyloxapol, Cremophor® EL or Pluronic® F68, at which self-aggregation is detected. The effect was the most pronounced for Cremophor® EL (1 and 2%, w/V) and was the least pronounced for Pluronic® F68 (1%, w/V). However, all surface active ophthalmic excipients over the entire concentration range that was tested did not significantly affect the *in vitro* and *ex vivo* permeability of timolol maleate, which is the most hydrophilic ophthalmic drug that was investigated. The results of the dynamic light scattering measurements point to the association of ophthalmic drugs with self-aggregates of surface active ophthalmic excipients as the potential mechanism of the observed permeability-depressing effect of surface active ophthalmic excipients. A strong and statistically significant correlation was observed between *in vitro* and *ex vivo* permeability of ophthalmic drugs in the presence of surface active ophthalmic excipients, which indicates that the observed permeability-altering effects of surface active ophthalmic excipients were comparable and were mediated by the same mechanism in both corneal models.

1. Introduction

The eye-related bioavailability of topically applied drugs is affected by a complex interplay of biological factors (*e.g.*, the dynamics of the precorneal area and a tight corneal barrier), formulation factors (pH, buffer, tonicity, viscosity, preservatives and stabilizers) and drug characteristics (*e.g.*, lipophilicity, charge, size and shape of the drug molecule). The experimental techniques used in preclinical studies on eye-related drug bioavailability generally focus on the cell- and tissue-based models of ocular drug permeability (Pepic et al., 2014). Formulation factors affecting ophthalmic drug permeability across corneal barriers are very complex and depend on the total composition of the ingredients in ophthalmic formulation, as well as on their interaction and supramolecular organization (Davies, 2000). The use of surfactants as excipients in ophthalmic formulations has long been a preferred approach for enhancing drug solubility, improving solution clarity and

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stabilizing suspensions of drugs with poor solubility (Jiao, 2008). However, there is still a lack of systematic investigations regarding the effects of surface active ophthalmic excipients on drug permeability using standard conditions and testing protocols.

For other routes of application (*i.e.*, oral), the *in vitro* or *ex vivo* drug permeability results have shown that the use of surfactants may lead to decreased, increased or unchanged drug permeability. For example, Caco-2 permeability of ketoprofen and nadolol were found to be decreased by a non-ionic surfactant Pluronic® F68 due to drug-micelle association in a concentration-dependent manner (Fischer et al., 2011a). Furthermore, *ex vivo* jejunal permeability of digoxin as a P-gp substrate was increased in the presence of a non-ionic surfactant Cremophor® EL due to its potential P-gp inhibitory effects, while the permeability of digoxin in the presence of Polysorbate 80 remained unchanged due to the absence of P-gp inhibition over the concentration range that was tested (Katneni et al., 2007). Moreover, slightly increased *in vitro* corneal permeability of melatonin from Pluronic® F127 micelle systems compared to melatonin solution has been reported (Hafner et al., 2015). In addition to the *in vitro/ex vivo* drug permeability studies, significant work has been done on studying the *in vivo* effect of drug-loaded nanocarriers containing surface active ophthalmic excipients on eye-related bioavailability. Specifically, Pluronic® F127 micelle formulation has been shown to enhance the ocular bioavailability of indomethacin (Chetoni et al., 2000) and pilocarpine (Pepic et al., 2004) in a rabbit eye model. Equally, plasmid DNA/Pluronic® F68 micelle formulation provided efficient and stable intraocular transfer of the functional gene in rabbit and mouse eye models (Liaw et al., 2001). Furthermore, Pluronic®F127/chitosan micelle formulation with dexamethasone demonstrated their potential to solubilize the drug, enhance its precorneal retention and release it in a very effective manner while also reaching high eye-related bioavailability levels (Pepic et al., 2010). Additionally, glucocorticoid nanosuspension formulation stabilized with Pluronic® F68 has shown the enhanced rate and extent of ophthalmic drug absorption, as well as the intensity of drug action in a rabbit eye model (Kassem et al., 2007). The above-mentioned results from the *in vitro/ex vivo* and *in vivo* studies may seem contradictory because a particular non-ionic surfactant (*e.g.*, Pluronic® F68) has been shown to decrease *in vitro* permeability and increase *in vivo* eye-related bioavailability. Even though different models were employed to test transcorneal drug transport, the *in vivo* studies have been performed with the final ophthalmic formulation in which the solubility-permeability balance was optimized during formulation development. Nonetheless, without a systematic and standardized approach allowing comparison of permeability/bioavailability data from different laboratories, it remains difficult to precisely identify the effect of surface active ophthalmic excipients on transcorneal drug permeability.

The aim of the present study is to systematically investigate the effects of type and concentration of surface active ophthalmic excipients on the corneal permeation of ophthalmic drugs using *in vitro* and *ex vivo* corneal permeability models. For these purposes, four different types of surface active ophthalmic excipients at three different concentrations were investigated. Because non-ionic surfactants are preferred for ophthalmic use, Polysorbate 80 (0.0005, 0.1 and 0.2%, w/V), Tyloxapol (0.0005, 0.05 and 0.1%, w/V), Cremophor® EL (0.01, 1 and 2%, w/V) and Pluronic® F68 (0.025, 0.1 and 1%, w/V) were selected and evaluated over the concentration range approved for ophthalmic use (FDA database: Inactive Ingredients). Additionally, a higher concentration of Pluronic® F68 outside the approved concentration range was tested since the *in vivo* ocular tolerability of Pluronic® F68 at higher concentrations up to 20% was demonstrated (Al Khateb et al., 2016; Asasutjarit et al., 2011). To obtain the information on the surface active ophthalmic excipient self-organization over the investigated concentration range and determine its influence on ophthalmic drug permeation across *in vitro* and *ex vivo* corneal models, the concentration-dependent formation of self-aggregates was monitored.

2. Materials and methods

2.1. Reagents and chemicals

The following compounds were used as received: timolol maleate (Sicor S.R.L., Milan, Italy), diclofenac sodium (Pharma Greven GmbH, Greven, Germany), chloramphenicol (AppliChem, Darmstadt, Germany), dexamethasone (Sanofi, Paris, France), Polysorbate 80 (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany), Tyloxapol (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany), Cremophor® EL (BASF, Ludwigshafen, Germany) and Pluronic® F68 (Sigma-Aldrich).

For the preparation of Krebs-Ringer buffer (KRB), pH 7.4, the following substances were dissolved in double-distilled water: KCl (0.4 mg/ml), NaCl (6.8 mg/ml), $NaHCO_3$ (2.1 mg/ml), $MgSO_4 \times 7H_2O$ (0.4 mg/ml), D-glucose monohydrate (1.1 mg/ml) (all purchased from Kemig, Zagreb, Croatia), $CaCl_2 \times 2H_2O$ (0.52 mg/ml) (Sigma-Aldrich), $NaH_2PO_4 \times 2H_2O$ (0.158 mg/ml) (Kemika, Zagreb, Croatia) and HEPES (3.575 mg/ml) (AppliChem).

The following substances were used for preparing the mobile phases for UPLC analyses: $NaH_2PO_4 \times H_2O$ (Merck-Millipore, Billerica, MA, USA), sodium acetate trihydrate (Kemika), and acetonitrile (Merck-Millipore).

2.2. Preparation of test samples

Stock solutions of Polysorbate 80 (2%, w/V), Tyloxapol (2%, w/V) and Cremophor® EL (5%, w/V) were prepared by dissolving surface active ophthalmic excipients in KRB at room temperature. A stock solution of Pluronic® F68 (2%, w/V) was prepared by dissolving Pluronic® F68 in KRB at 4 °C overnight. Test samples of surface active ophthalmic excipients without the addition of ophthalmic drugs were prepared by dilution of stock solutions with KRB to obtain the following concentrations: 0.0005, 0.1 and 0.2%, (w/V) for Polysorbate 80, 0.0005, 0.05 and 0.1, (w/V) for Tyloxapol, 0.01, 1.0 and 2.0%, (w/V) for Cremophor® EL and 0.025, 0.1 and 1.0%, (w/V) for Pluronic® F68. Similarly, test samples of ophthalmic drugs without the addition of ophthalmic excipients were prepared by dissolving a drug in KRB to obtain the following concentrations: 100 μg/ml for timolol maleate, 150 μg/ml for diclofenac sodium, 150 μg/ml for chloramphenicol, 60 μg/ml for dexamethasone for *in vitro* permeability testing; 250 μg/ml for timolol maleate, 400 μg/ml for diclofenac sodium, 400 μg/ml for chloramphenicol and 60 μg/ml for dexamethasone for *ex vivo* permeability testing. Test samples of ophthalmic drugs with the addition of surface active ophthalmic excipients were prepared from surface active ophthalmic excipient stock solutions with the final constant ophthalmic drug concentration (as defined above) and variable surface active ophthalmic excipient concentrations (as defined above) by diluting samples with KRB.

2.3. Hydrodynamic diameter measurements

The hydrodynamic diameter (d_h) and polydispersity index (PDI) of the surface active ophthalmic excipient solutions in the absence and in the presence of ophthalmic drugs were determined by dynamic light scattering (DLS) (Zetasizer 3000 HSA, Malvern Instruments, Malvern, UK). The size measurements were performed at a scattering angle of 90° and a temperature of 25 °C. The hydrodynamic diameter and PDI were calculated from the autocorrelation function of the intensity of light scattered from particles. The samples were filtered through a 0.45-μm membrane filter (Isolab, Wertheim, Germany) before measurement.

2.4. Cell culture conditions

HCE-T cells (RIKEN Cell Bank, Tsukuba, Japan) were cultivated in DMEM/F12 medium (Lonza, Basel, Switzerland) supplemented with fetal bovine serum (5%, Biosera, Boussens, France), insulin (5 μg/ml,

Sigma-Aldrich), dimethyl sulfoxide (0.5%, Applichem), epidermal growth factor (10 ng/ml, Sigma-Aldrich) and penicillin/streptomycin/amphotericin B (Lonza). The cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C (Hafner et al., 2015). The culture medium was changed every 48 h, and the cells were subcultured at 80%–90% confluence.

2.5. Cultivation of the HCE-T cell-based model

The HCE-T cell-based model was cultivated according to the protocol described by Juretić and coworkers (Juretic et al., 2017), as well as Hahne and Reichl (Hahne and Reichl, 2011). Briefly, Transwell® polycarbonate membrane cell culture inserts (3.0 µm pore size, 12-mm diameter, surface area 1.12 cm^2, Corning B.V. Life Sciences, Amsterdam, The Netherlands) were coated with rat tail type I collagen (Sigma-Aldrich) and human fibronectin (Sigma-Aldrich). HCE-T cells (10^5 cells in 0.5 ml of culture medium) were seeded onto the coated polycarbonate filter, and 1.5 ml of culture medium were added to the basolateral side. The cultivation of cells submerged in the medium was conducted in the initial 4 to 7 days (until a sharp increase in the transepithelial electrical resistance (TEER) was observed). Afterwards, the exposure of cells to the air-liquid interface (ALI), shown to be critical for inducing the cell proliferation and differentiation into a multilayered epithelial structure, was performed during the following 3 days. The culture medium was changed every 2 days during the submerged conditions and every day during exposure to the ALI. During the ALI exposure, the inserts were lifted on a metal plate to increase the basolateral volume to 2 ml.

2.6. In vitro permeability assay

The *in vitro* permeability assay using the HCE-T cell-based model has been described by Juretić and coworkers (Juretic et al., 2017). Briefly, the assay was performed directly in the Transwell® plates on a horizontal orbital shaker (34 °C; 50 rpm) in KRB. After rinsing the cells with KRB, the cells were incubated in the following 30 min with 0.4 and 2 ml of KRB in the donor and receptor compartment, respectively. The KRB was later removed from the donor compartment, and 0.4 ml of a test solution was added. The receptor compartment was sampled (0.4 ml) at regular time intervals over 2 h and replaced at each time point with the same volume of fresh warmed KRB (34 °C). The donor solution was also sampled at the end of the permeability experiment.

2.7. Ex vivo permeability assay

The *ex vivo* permeability assay using freshly excised porcine corneas has been described by Juretić and coworkers (Juretic et al., 2017). Briefly, for the purpose of the *ex vivo* permeability assay, fresh porcine eyes were obtained from Large White pigs (age 6–7 months, weight 90–115 kg, both female and male animals) from a local slaughterhouse. Porcine eyeballs were enucleated, rinsed with an isotonic saline solution (NaCl 0.9%; B. Braun, Melsungen, Germany) and transported in cold KRB in a container held on ice. The transport of porcine eyeballs and the cornea excision were performed within 2 h of the animal's death. The corneoscleral buttons were mounted on vertical diffusion chambers (Standard Vertical Ussing/Diffusion Chambers, surface area 0.64 cm^2, made from acrylic, Harvard Apparatus, Holliston, MA, USA), with the epithelial side facing the donor compartment. The volume of the donor and receptor compartment was 3.5 ml each. The corneas were first incubated for 30 min in KRB at 34 °C, after which KRB from the donor compartment was removed, and 3.5 ml of a test solution was added. A sample (0.4 ml for dexamethasone, 1 ml for timolol maleate, diclofenac sodium and chloramphenicol) was withdrawn from the receptor compartment at regular time intervals over 5 h and replaced at each time point with the same volume of fresh warmed KRB. The donor solution was sampled at the end of the permeability experiment to calculate the mass balance (%). The medium in both the donor and receptor compartments was continuously oxygenated (2–3 bubbles/s), which provided both the tissue oxygenation and mixing of the medium.

2.8. Transepithelial electrical resistance measurement

To monitor barrier integrity during the *in vitro* and *ex vivo* permeability experiments, transepithelial electrical resistance (TEER) was measured. For the TEER measurement of the HCE-T cell-based model, an STX-2 electrode and EVOM (WPI Inc., Sarasota, FL, USA) were used. Ag/AgCl electrodes in conjunction with an EC-825A Epithelial Voltage Clamp (Warner Instruments, Hamden, CT, USA) were used for the excised cornea. The blank resistance was also measured and subsequently subtracted to obtain the TEER of the HCE-T cell-based model and excised cornea-based model.

2.9. Data processing

The apparent permeability coefficients (P_{app} in cm/s) of all compounds were calculated by plotting the amount of permeated compound per unit area *versus* time. The P_{app} was calculated from the linear portion of the permeation curve according to the following equation (Hahne et al., 2012; Toropainen et al., 2003):

$$P_{app} = \frac{\partial Q}{\partial t} \times \frac{1}{AC_o} \quad (1)$$

where $\frac{\partial Q}{\partial t}$ is the permeation rate, A is the surface area of the permeation barrier and C_o is the initial concentration of a test compound in the donor compartment (Eq. (1)).

For the purpose of examining the influence of the surface active ophthalmic excipients on the ophthalmic drug permeability the P_{app}R value was calculated according to the following formula:

$$P_{app}\ \mathrm{R} = \frac{P_{app}\ (ophthalmic\ drug\ +\ ophthalmic\ excipient)}{P_{app}\ (ophthalmic\ drug)} \quad (2)$$

where P_{app} (*ophthalmic drug + ophthalmic excipient*) is the mean of the P_{app} value of an ophthalmic drug in the presence of a surface active ophthalmic excipient and P_{app} (*ophthalmic drug*) is the mean of the P_{app} value of an ophthalmic drug in the absence of a surface active ophthalmic excipient (Eq. (2)).

The mass balance (recovery) (%) was calculated according to the following equation (Hubatsch et al., 2007):

$$Mass\ balance\ (\%) = \frac{(C_r(\mathrm{f}) \times V_r + \sum C_r(\mathrm{t}) \times V_s) + C_d(\mathrm{f}) \times V_d)}{C_d(0) \times V_d} \times 100 \quad (3)$$

where C_d and C_r are the concentrations in the donor (d) and receptor (r) compartment, respectively, at the start (0) or end (f) of the permeability assay, Cr(t) is the concentration of the samples withdrawn from the receptor compartment at different time points, Vr and Vd are volumes of the receptor and donor compartment, respectively, and Vs is the volume of the sample withdrawn from the receptor compartment (Eq. (2)).

2.10. Quantification

The quantitative determination of ophthalmic drugs in the samples from *in vitro* and *ex vivo* permeability experiments was performed by Ultra-Performance Liquid Chromatography (UPLC) using an *Agilent Infinity 1290* (*Agilent, Santa Clara, CA, USA*). The Acquity UPLC BEH Shield RP18 Column (1.7 µm, 2.1 mm × 50 mm) (Waters, Milford, MA, USA) was used, and the elution was isocratic. For the quantitative analysis of each ophthalmic drug, the following UPLC conditions were applied: timolol maleate (CH_3COONa buffer pH 4.5/acetonitrile (85/15%, V/V), 0.6 ml/min, 55 °C, 296 nm), diclofenac sodium (NaH_2PO_4

buffer pH 2.5/acetonitrile (65/35%, V/V), 0.6 ml/min, 55 °C, 276 nm), chloramphenicol (CH_3COONa buffer pH 4.5/acetonitrile (80/20%, V/V), 0.4 ml/min, 50 °C, 275 nm), and dexamethasone (CH_3COONa buffer pH 4.5/acetonitrile (67/33%, V/V), 0.4 ml/min, 50 °C, 254 nm). The volume of injection was 20 µl for the receptor compartment samples. For the donor compartment samples, the injection volume was 5 µl for timolol maleate, diclofenac sodium, chloramphenicol and 10 µl for dexamethasone. For each sequence, standard solutions were prepared in duplicate and injected alternately. At least five standard solution injections were performed in each injection sequence. System suitability was evaluated according to the following criteria: the relative standard deviation (RSD) of the detector response factor for all standard solution injections in the sequence is no more than 3.0%, and the tailing factor of ophthalmic drug peak is no more than 2.0. All UPLC methods have been previously validated in terms of linearity, accuracy and repeatability (Juretic et al., 2017).

2.11. Statistical analysis

GraphPad Prism program 5.0 (GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com) was used for the statistical analysis. Statistical data analyses were performed on *in vitro* and *ex vivo* permeability data using a one-way ANOVA followed by Dunnet's post-hoc test with $P < 0.05$ set as the minimal level of significance. The correlation between the *in vitro* and *ex vivo* ophthalmic drug permeability in the presence of surface active ophthalmic excipients was analyzed by the Pearson correlation with $P = 0.05$ set as the minimal level of statistical significance.

3. Results and discussion

3.1. In vitro/ex vivo ophthalmic drug permeability

The HCE-T cell-based model as an *in vitro* model for transcorneal permeability studies has been widely accepted and well-characterized (Kölln and Reichl, 2016; Reichl et al., 2011; Toropainen et al., 2003; Verstraelen and Reichl, 2013, 2014). In our previous work, the variability in the barrier phenotype of HCE-T cell-based permeability model has been reported and characterized in detail (Juretic et al., 2017). This variability strongly influences the comparison of data from different laboratories and should be carefully considered in routine application of HCE-T cell-based permeability models across both academic and pharmaceutical industry research laboratories. Specifically, our model consists of an apical lipophilic HCE-T epithelial monolayer and a basolateral lipophilic monolayer of migrated HCE-T cells, which shows suitable barrier properties (1700–2600 Ω × cm^2) and good correlation with the drug permeability results obtained in the *ex vivo* corneal model.

In this study, the permeability of ophthalmic drugs was evaluated in the presence and absence of surface active ophthalmic excipients using *in vitro* and *ex vivo* corneal models. The selected physicochemical parameters of ophthalmic drugs and surface active ophthalmic excipients are shown in Tables 1 and 2, respectively. At the defined permeability assay conditions (KRB pH 7.4), chloramphenicol and dexamethasone were neutral because these molecules do not have a relevant ionizable group, while diclofenac and timolol were negatively and positively charged, respectively. The P_{app} values for all ophthalmic drugs obtained in both *in vitro* and *ex vivo* models (Table 3) in comparison with the P_{app} values obtained in our previous work (Juretic et al., 2017) are reproducible, which indicates acceptable intra-laboratory variability of the models. Similar *ex vivo* P_{app} values have been reported for timolol and dexamethasone (Hahne et al., 2012), as well as the *in vitro* P_{app} value for timolol (Becker et al., 2008). However, Loch and coworkers (Loch et al., 2012) reported around an 8-fold higher *ex vivo* P_{app} value for dexamethasone and 2-fold lower *ex vivo* P_{app} value for timolol. Furthermore, around a 3-fold higher *ex vivo* P_{app} value for diclofenac was obtained in the study conducted by Pescina and colleagues (Pescina et al., 2015). Considering the literature data for chloramphenicol, no studies on its permeability either in the *in vitro* or *ex vivo* corneal model have been conducted to date. The variability in the reported ophthalmic drug P_{app} values *ex vivo* might be ascribed to the differences in the animal species characteristics (breed, age, sex), the tissue collection and handling procedure, as well as the experimental setup. Evidently, the observed inter-laboratory variability provides an opportunity for future collaboration among research laboratories in this area with the aim of overcoming the difficulties in interpreting the results that are possibly leading to wrong conclusions.

Table 1
Physicochemical parameters of ophthalmic drugs used in *in vitro* and *ex vivo* permeability experiments.

Ophthalmic drug	M_w of acid/base moiety (g/mol)	Aqueous solubility (mg/mL)	pK_a[e]	$LogD_{n\text{-}octanol/buffer\ pH7.4}$[e]
Timolol maleate	316.42	30–100[a]	9.21	−0.20
Diclofenac sodium	296.15	5.15[b]	3.80	1.10
Chloramphenicol	323.13	2.5[c]	Without ionisable groups	1.08
Dexamethasone	392.46	0.093[d]	Without ionisable groups	1.50

[a] Brittain (2012).
[b] Chuasuwan et al. (2009).
[c] Anderson et al. (2012).
[d] Colombo et al. (2017).
[e] References for verifying the source of the ophthalmic drug pKa and logD values are provided by Juretic et al. (2017).

Table 2
Selected characteristics of surface active ophthalmic excipients used in *in vitro* and *ex vivo* permeability experiments.

Surface active ophthalmic excipient	M_w (g/mol)[a]	Hydrophilic-lipophilic balance (HLB)[a]	Maximal concentrations in different ophthalmic dosage forms approved by FDA[b]
Polysorbate 80	1310	15.0	Solution; 0.2% Suspension; 0.1% Emulsion; 4.0%
Tyloxapol	4500	12.5	Solution; 0.1% Suspension; 0.1%
Cremophor® EL	2560	12–14	Solution; 5%
Pluronic® F68	8400	29	Solution; 0.1%

[a] Jiao (2008).
[b] FDA Drug Databases (n.d.).

3.2. The influence of surface active ophthalmic excipients on the in vitro/ex vivo barrier properties

To monitor the possible concentration-dependent effects of both ophthalmic drugs and surface active ophthalmic excipients on the barrier properties of *in vitro* and *ex vivo* corneal models, the TEER measurements were performed starting from the application of test samples to the end of the experiments. Prior to the addition of test samples, the corneal models were characterized by different initial TEER values. For the *in vitro* model, the initial TEER values were in the range of 2074–2510 Ω × cm^2, while for *ex vivo* models, they were in the range of 604–829 Ω × cm^2. The obtained initial TEER values for both corneal models indicate suitable barrier properties for permeability experiments (Toropainen et al., 2003). At the end of both *in vitro* and *ex vivo* permeability experiments, both increases and decreases in the TEER value for the ophthalmic drug test samples with or without ophthalmic excipients compared to KRB alone were observed.

Table 3
Apparent permeability coefficients (P_{app}) of ophthalmic drugs and corresponding transepithelial electrical resistance (TEER) values for *in vitro* and *ex vivo* corneal models. All values represent the mean ± SD (n = 11–22).

	In vitro corneal model		*Ex vivo* corneal model	
	P_{app} (10^{-7} cm/s)	TEER[a] (Ω × cm^2)	P_{app} (10^{-7} cm/s)	TEER[a] (Ω × cm^2)
Timolol maleate	134.23 ± 17.79	2274 ± 1058	107.68 ± 21.28	756 ± 259
Diclofenac sodium	129.09 ± 12.36	2279 ± 1372	17.38 ± 3.74	816 ± 346
Chloramphenicol	18.79 ± 3.11	2510 ± 1207	7.62 ± 2.81	829 ± 464
Dexamethasone	32.02 ± 6.09	2074 ± 1085	8.82 ± 2.97	604 ± 273

[a] Measured at the start of permeability experiments.

However, the observed differences in the TEER values of approximately 30% compared to KRB alone were not statistically significant (data not shown), which indicates that neither the test samples without ophthalmic excipients nor the test samples with ophthalmic excipients significantly affected the barrier properties of both models under the investigated experimental conditions.

3.3. The self-aggregation properties of surface active ophthalmic excipients

To obtain the information on the self-aggregation properties of the surface active ophthalmic excipients in a complex KRB solution in the presence of ophthalmic drugs, the dynamic light scattering measurements were performed (Table 4). At the lowest concentration of the tested surface active ophthalmic excipients (*i.e.*, 0.0005%, w/V, for both Polysorbate 80 and Tyloxapol, 0.01%, w/V, for Cremophor® EL, 0.025% as well as 0.1%, w/V, for Pluronic® F68) a poor signal-to-noise ratio was observed, which indicates the absence of self-aggregates in these test samples. However, at higher concentrations of Polysorbate 80 (0.1 and 0.2%, w/V), Tyloxapol (0.05 and 0.1%, w/V) and Pluronic® F68 (1%, w/V), self-aggregates of variable sizes were observed. Nonetheless, the quality control parameters of the measurements suggested the existence of metastable self-aggregates instead of stable micelles. In contrast, for two higher concentrations of Cremophor® EL (1 and 2%, w/V), the quality control parameters of the measurements suggested the presence of stable micelles with suitable dispersity. The comparable results of the dynamic light scattering measurements of the surface active ophthalmic excipients in KRB solution in the absence of ophthalmic drugs (data not shown) suggest that the presence of ophthalmic drugs did not influence the size and dispersity properties of surfactant self-aggregates/micelles.

3.4. The influence of surface active ophthalmic excipients on the in vitro/ex vivo ophthalmic drug permeability

The results of the *in vitro* and *ex vivo* permeability studies of four ophthalmic drugs in the presence of surface active ophthalmic excipients are shown in Table 4 and are presented as the ratio between the P_{app} values of an ophthalmic drug in the presence and absence of surface active ophthalmic excipients (P_{app}R). In order to determine that no significant loss of a drug occurs during the *in vitro* and *ex vivo* permeability studies (due to, *e.g.*, drug adsorption to plastic surfaces, drug degradation/metabolisation, drug accumulation inside corneal barrier) the mass balance was calculated. The mass balance for all ophthalmic drugs in both *in vitro* and *ex vivo* corneal models was in the range of 80% to 100% (data not shown), which was shown to result in acceptable approximation of the ophthalmic drug P_{app} value (Hubatsch et al., 2007). Moreover, no statistical significance was found in the difference between the mass balance for an ophthalmic drug in the absence and the mass balance for an ophthalmic drug in the presence of an ophthalmic excipient. In Fig. 1, the permeation profiles of one of the ophthalmic drug in the absence and in the presence of one of the surface active ophthalmic excipient, both in *in vitro* and *ex vivo* corneal models, are shown. It can be observed that the presence of Cremophor® EL (1%, w/V) had no effect on the shape of the diclofenac permeation curve regarding the linearity of the portion of the curve reflecting the steady-state conditions. However, the slope of the linear portion of the curve in the presence of Cremophor® EL was reduced, yielding lower diclofenac P_{app} value compared to the diclofenac P_{app} value in the absence of Cremophor® EL. The presented permeation curves are representative of all the *in vitro* and *ex vivo* permeation profiles obtained in this study. More precisely, in the presence of a surface active ophthalmic excipient the linearity of the portion of the ophthalmic drug permeation curve characteristic for steady-state conditions has been preserved, however, the slope of the linear portion of the curve has been either decreased, increased or unchanged, which resulted in the calculated lower, higher or equal P_{app} value, respectively, compared to the P_{app} value of an ophthalmic drug in the absence of a surface active ophthalmic excipient.

The permeability of timolol in the presence of all surface active ophthalmic excipients over the whole concentration range showed only slight changes in both *in vitro* and *ex vivo* corneal models. However, these slight changes of the P_{app} value of timolol have not been statistically significant, which demonstrates that the permeability of timolol has not been affected by the presence of surface active ophthalmic excipients. A similar trend of P_{app}R values was observed for chloramphenicol. A statistically significant 41% increase in the chloramphenicol P_{app} value, however, was observed in the presence of 0.2% (w/V) Polysorbate 80 in the *in vitro* but not in the *ex vivo* corneal model. Moreover, at the higher concentrations of Cremophor® EL that were investigated (*i.e.*, 1% and 2%, w/V), a statistically significant decrease of the chloramphenicol P_{app} value up to 50% was detected in the *ex vivo* model. Conversely, no statistically significant influence on the permeability of chloramphenicol was detected for the same concentrations of Cremophor® EL in the *in vitro* corneal model.

As opposed to timolol and chloramphenicol, a strong influence of Polysorbate 80, Tyloxapol and Cremophor® EL on the permeability of diclofenac was established in both the *in vitro* and *ex vivo* corneal models. At the lowest concentration of these surface active ophthalmic excipients, no statistically significant change in the diclofenac P_{app} value was observed. At higher surface active ophthalmic excipient concentrations, however, the diclofenac P_{app} values substantially decreased. Specifically, the observed P_{app} value decrease was approximately 30% in the presence of Polysorbate 80 (0.1 and 0.2%, w/V), approximately 50% in the presence of Tyloxapol (0.05 and 0.1%, w/V) and up to approximately 90% in the presence of Cremophor® EL (1 and 2%, w/V). The extent of the permeation-depressing effect of the three surface active ophthalmic excipients in the *in vitro* and *ex vivo* corneal models was comparable. Pluronic® F68, on the other hand, at a concentration of 1% (w/V) reduced the diclofenac P_{app} value in the *ex vivo*, but not in the *in vitro* corneal model. The 35%-reduction in the diclofenac *ex vivo* P_{app} value, however, was not statistically significant. At the lower concentrations tested in this study, Pluronic® F68 showed no influence on diclofenac permeability in both corneal models.

The permeability of dexamethasone in the presence of surface active ophthalmic excipients was affected in a manner similar to the permeability of diclofenac. At the lowest concentration of Polysorbate 80,

Table 4
The influence of surface active ophthalmic excipients on the permeability of ophthalmic drugs (presented as P_{app}R value) in *in vitro* and *ex vivo* corneal models. The self-aggregation properties of the surface active ophthalmic excipients in the presence of ophthalmic drugs were monitored by dynamic light scattering (DLS) and are presented as hydrodynamic diameter (d_h) and polydispersity index (PDI).

Ophthalmic drug	Surface active ophthalmic excipient	Surface active ophthalmic excipient concentration (%, m/V)	P_{app}R *In vitro*	P_{app}R *Ex vivo*	d_h (nm) (mean ± SD)	PDI (mean ± SD)
Timolol maleate	Polysorbate 80	0.0005	0.97	0.89	nd	nd
		0.1	1.13	0.97	17.1 ± 0.4	0.263 ± 0.01
		0.2	1.33	0.99	14.7 ± 0.3	0.251 ± 0.014
	Tyloxapol	0.0005	0.92	0.80	nd	nd
		0.05	1.12	0.88	12.3 ± 0.2	0.233 ± 0.023
		0.1	1.08	1.23	16.1 ± 1.1	0.239 ± 0.020
	Cremophor® EL	0.01	1,19	1.26	nd	nd
		1	1.04	1.11	14.0 ± 0.5	0.096 ± 0.083
		2	0.95	1.00	13.3 ± 0.1	0.076 ± 0.012
	Pluronic® F68	0.025	0.94	1.00	nd	nd
		0.1	0.95	0.93	nd	nd
		1	0.87	0.94	13.9 ± 1.8	0.220 ± 0.023
Diclofenac sodium	Polysorbate 80	0.0005	0.88	1.17	nd	nd
		0.1	0.66*	0.73	11.6 ± 1.3	0.192 ± 0.005
		0.2	0.68*	0.74	10.5 ± 0.3	0.196 ± 0.065
	Tyloxapol	0.0005	0.98	0.87	nd	nd
		0.05	0.54*	0.45*	15.2 ± 1.3	0.230 ± 0.021
		0.1	0.42*	0.53*	10.2 ± 1.7	0.153 ± 0.023
	Cremophor® EL	0.01	0.92	0.92	nd	nd
		1	0.26*	0.18*	14.4 ± 0.1	0.165 ± 0.004
		2	0.18*	0.09*	13.2 ± 0.3	0.043 ± 0.024
	Pluronic® F68	0.025	0.88	0.78	nd	nd
		0.1	1.02	0.86	nd	nd
		1	1.14	0.65	12.5 ± 3.3	0.184 ± 0.039
Chloramphenicol	Polysorbate 80	0.0005	0.94	1.21	nd	nd
		0.1	0.98	1.15	16.8 ± 0.7	0.252 ± 0.009
		0.2	1.41*	1.09	11.9 ± 0.7	0.227 ± 0.061
	Tyloxapol	0.0005	0.85	0.84	nd	nd
		0.05	0.88	1.01	21.7 ± 1.0	0.272 ± 0.001
		0.1	0.91	0.91	15.7 ± 2.2	0.230 ± 0.035
	Cremophor® EL	0.01	0.94	0.86	nd	nd
		1	0.88	0.62*	14.1 ± 0.0	0.121 ± 0.006
		2	0.81	0.47*	13.2 ± 0.1	0.072 ± 0.033
	Pluronic® F68	0.025	0.92	1.18	nd	nd
		0.1	1.07	1.12	nd	nd
		1	1.09	0.96	12.5 ± 0.7	0.216 ± 0.001
Dexamethasone	Polysorbate 80	0.0005	0.79	1.27	nd	nd
		0.1	0.85	0.72	14.6 ± 0.7	0.207 ± 0.011
		0.2	0.66	0.59	13.3 ± 1.0	0.217 ± 0.016
	Tyloxapol	0.0005	1.14	1.08	nd	nd
		0.05	0.92	1.24	17.09 ± 1.2	0.299 ± 0.019
		0.1	0.59*	0.76	14.3 ± 0.9	0.249 ± 0.021
	Cremophor® EL	0.01	1.08	1.02	nd	nd
		1	0.54*	0.43*	13.7 ± 0.3	0.108 ± 0.048
		2	0.41*	0.27*	13.2 ± 0.1	0.056 ± 0.033
	Pluronic® F68	0.025	0.90	0.80	nd	nd
		0.1	0.96	0.90	nd	nd
		1	1.26	0.80	26.0 ± 2.1	0.383 ± 0.018

P_{app}R = ratio between the mean value of the apparent permeability coefficient of an ophthalmic drug in the presence and the mean value of the apparent permeability coefficient of an ophthalmic drug in the absence of a surface active ophthalmic excipient (n = 3). nd = self-aggregates of surface active ophthalmic excipients not detected.

* Statistically significant difference of the P_{app} value of an ophthalmic drug in the presence of a surface active ophthalmic excipient compared to the P_{app} value of an ophthalmic drug in the absence of a surface active ophthalmic excipient ($P < 0.05$).

Tyloxapol and Cremophor® EL, the slight changes in the dexamethasone P_{app} value were not statistically significant, whereas at higher concentrations, the permeability-decreasing effect of surface active ophthalmic excipients was observed. More precisely, Polysorbate 80 and Tyloxapol at the highest concentrations of 0.2% and 0.1% (w/V) reduced the dexamethasone P_{app} value by approximately 30%–40% in both corneal models. The observed P_{app} reduction, however, was not statistically significant in all cases. As was observed for diclofenac sodium, Cremophor® EL of all of the tested surface active ophthalmic excipients showed the strongest influence on the permeation of dexamethasone. There was up to a 60%–70% decrease in the dexamethasone P_{app} value in the presence of 1% and 2% (w/V) of Cremophor® EL in both corneal models.

The negative effect of surface active excipients on the permeability of drugs with poor water solubility has already been reported and was primarily ascribed to the interaction of surface active excipient micelles and drugs. More precisely, the incorporation of lipophilic drugs into the hydrophobic core of surface active excipient micelles reduces the fraction of free drug available for permeation across a biological barrier, which results in a decrease in the overall drug permeability (Fischer et al., 2011b; Yano et al., 2010). In our study, the lowest concentration of surface active ophthalmic excipients without self-aggregates demonstrated no influence on ophthalmic drug permeability. However, at higher concentrations of surface active ophthalmic excipients, at which dynamic light scattering measurements pointed to the presence of metastable self-aggregates or stable micelles, a strong

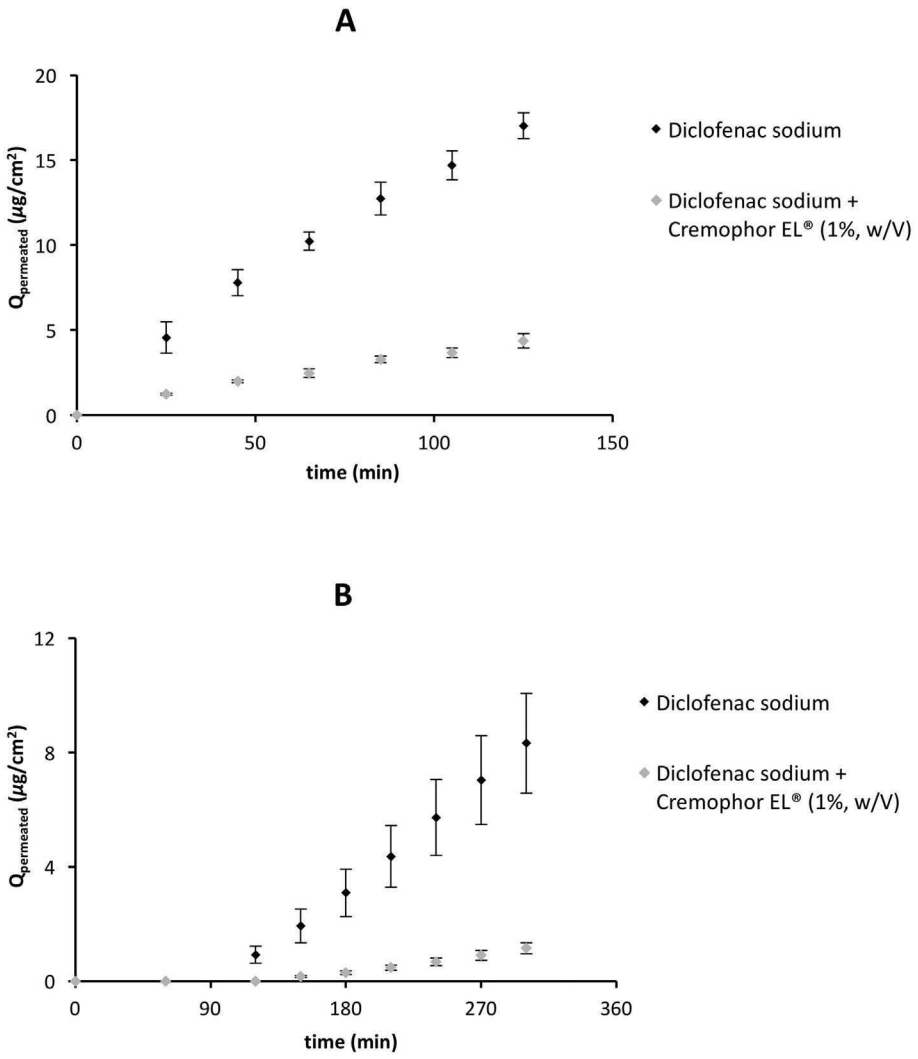

Fig. 1. The permeation profiles of diclofenac sodium in the absence and in the presence of Cremophor® EL (1%, w/V), in *in vitro* (A) and *ex vivo* (B) corneal models. Data are expressed as mean ± SD (n = 3).

permeability-depressing effect for certain surface active ophthalmic excipient-drug combinations was observed. This outcome indicates that the association of drugs with self-aggregates of surface active ophthalmic excipients, which results in reduced drug thermodynamic activity, might be responsible for the observed decrease in drug permeability. Of the four tested surface active ophthalmic excipients, Cremophor® EL exerted the strongest permeability-decreasing effect followed by Polysorbate 80 and Tyloxapol, which both reduced the ophthalmic drug permeation rate to a smaller but comparable extent over the tested concentration range. In contrast, Pluronic® F68 demonstrated no statistically significant influence on the permeability of the tested ophthalmic drugs over the whole concentration range. Even at the highest concentration of 1% (w/V), at which the presence of self-aggregates was established, the P_{app} value changes were not statistically significant and pointed to the absence of interaction between Pluronic® F68 and the ophthalmic drugs. The results are in agreement with the previously published study in which both Polysorbate 80 and Cremophor® EL over the concentration range of 0.01–1% (w/V) reduced the permeation rate of low-soluble drugs in an *ex vivo* rat jejunal model (Katneni et al., 2007). Furthermore, Pluronic® F68 at a concentration of 1% (w/V) was reported to have no influence on the permeability of ketoprofen and nadolol in *in vitro* intestinal epithelial models. However, at higher concentrations of Pluronic® F68 up to 5% (w/V), the interaction between the drugs and Pluronic® F68 micelles resulted in the reduced drug permeation rate (Fischer et al., 2011a; Fischer et al., 2011b).

In this study, the permeability of a weakly basic drug timolol characterized by high solubility and low lipophillicity was not affected by the presence of surface active ophthalmic excipients over the entire concentration range that was tested. On the other hand, for the more lipophilic and low soluble neutral drug dexamethasone, a permeability decrease in the presence of self-aggregates of surface active ophthalmic excipients was observed that was in line with the results of the aforementioned permeability studies. Somewhat unexpectedly, however, the reduction in the permeation rate in the presence of self-aggregates of surface active ophthalmic excipients was even greater for the weakly acidic drug diclofenac, which is characterized by higher solubility and lower lipophilicity compared to dexamethasone. However, the overall results of the *in vitro* and *ex vivo* permeability studies of the neutral compound chloramphenicol, which has solubility and lipophilicity properties comparable to diclofenac, point to the absence of its interaction with self-aggregates of surface active ophthalmic excipients. Although it has been shown that the surfactant micelles preferentially solubilize more lipophilic compounds, the situation might become more complex for acidic and basic drugs at physiological pH when the ionized molecular form is dominant. More precisely, solubilization studies have demonstrated that under certain conditions, the ionized form may exhibit even greater solubility in micelles than its unionized

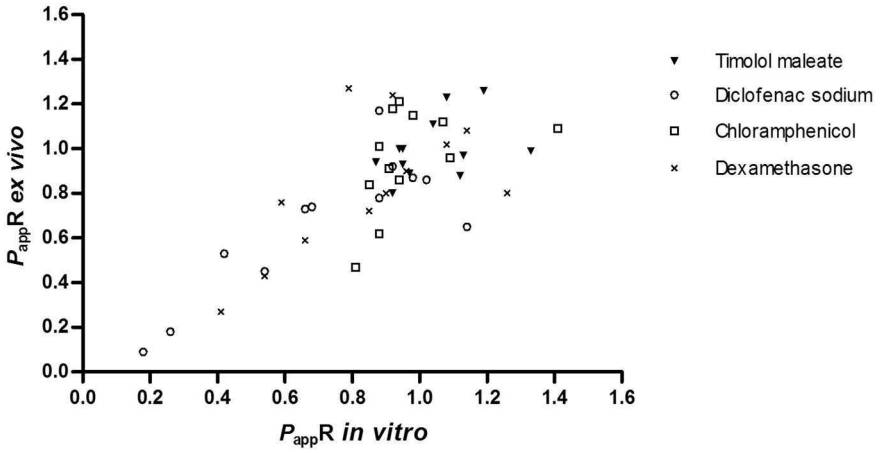

Fig. 2. Correlation between P_{app}R values for four ophthalmic drugs in the presence of three different concentrations of four surface active ophthalmic excipients in *in vitro* (HCE-T cell-based model) and *ex vivo* (freshly excised porcine cornea) models. P_{app}R represents the ratio between the mean value of the apparent permeability coefficient of an ophthalmic drug in the presence and the mean value of the apparent permeability coefficient of an ophthalmic drug in the absence of a surface active ophthalmic excipient (n = 3).

equivalent (Li et al., 1999).

In contrast to the permeability-decreasing effect of Polysorbate 80 in the *in vitro* permeability studies of diclofenac sodium and dexamethasone, a statistically significant increase of the chloramphenicol P_{app} value in the presence of 0.2% (w/V) Polysorbate 80 in the *in vitro* corneal model was detected. The permeability-enhancing effect of surface active excipients, including Polysorbate 80, in the *in vitro* and *ex vivo* models for both hydrophilic and lipophilic compounds has been reported in the literature. The permeability enhancement was either related to the modulation of tight junctions, which increases paracellular transport of a hydrophilic compound (Ujhelyi et al., 2012), or to the interference with the activity of influx and efflux membrane transporters, which increases transcellular transport of more lipophilic compounds (Katneni et al., 2007). To the best of our knowledge, the literature data regarding the *in vitro* and *ex vivo* permeability of chloramphenicol, which includes the mechanism of its transport across corneal or other biological barriers, is lacking. The potential mechanism of the Polysorbate 80-induced increase of chloramphenicol permeability, therefore, has yet to be investigated.

3.5. The in vitro/ex vivo correlation of the influence of surface active ophthalmic excipients on the ophthalmic drug permeability

In Fig. 2, the correlation between the *in vitro* and *ex vivo* P_{app}R values for four ophthalmic drugs in the presence of three different concentrations of four surface active ophthalmic excipients obtained in this study is shown. A statistically significant and strong correlation between the *in vitro* and *ex vivo* P_{app}R values was observed (Pearson's $r = 0.74$, $P < 0.0001$), which additionally confirms the comparability of the *in vitro* and *ex vivo* permeability results obtained in this study. The strong *in vitro* and *ex vivo* P_{app}R value correlation shows that the observed changes of the ophthalmic drug *in vitro* and *ex vivo* permeability in the presence of surface active ophthalmic excipients were detected as a P_{app} value decrease for the majority of surface active ophthalmic excipient-drug combinations, and the decreases had comparable magnitudes. Furthermore, the results indicate that the effects of the tested surface active ophthalmic excipients on the ophthalmic drug permeability might be mediated by the same mechanism in both corneal models. The results of this study demonstrate that with further optimization these *in vitro* and *ex vivo* corneal models have the potential to be used for permeability testing and ocular bioavailability evaluation in the early phases of ophthalmic product development.

4. Conclusions

Ophthalmic product development requires an in-depth understanding of the factors affecting ophthalmic drug permeability across corneal barriers. In this study, four surface active ophthalmic excipients commonly used in ophthalmic formulations have been evaluated for the influence on the permeability of four ophthalmic drugs using *in vitro* and *ex vivo* corneal models. The presence of surface active ophthalmic excipients over the investigated concentration range resulted in either no change or a decrease in ophthalmic drug *in vitro* and *ex vivo* permeability. The observed permeability-depressing effect of surface active ophthalmic excipients might be caused by the association of ophthalmic drugs with self-aggregates of surface active ophthalmic excipients, as indicated by dynamic light scattering measurements. A strong correlation of the influence of surface active ophthalmic excipients on ophthalmic drug permeability was observed between *in vitro* and *ex vivo* corneal models, which indicates that the observed effects had comparable sizes and were mediated by the same mechanism in both corneal models. The structural differences in the *in vitro* and *ex vivo* corneal barrier (*e.g.* the lack of stroma in the *in vitro* model), as well as the potential functional differences (*e.g.* the expression of membrane transporters) may possibly explain the observed *in vitro/ex vivo* correlation discrepancies, however, further more detailed histomorphological, functional and mechanistic permeability studies are required for establishing the exact underlying cause.

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5. GENERAL DISCUSSION

Given the direct application of topical ophthalmic formulations to corneal surface, ophthalmic formulation composition, including the type and concentration of each excipient, their interaction and supramolecular organization, largely impacts the ophthalmic drug permeation through corneal barrier and consequently its eye-related bioavailability. Understanding the influence of the ophthalmic excipients on ocular permeability/bioavailability is, therefore, of crucial importance for the efficient development of topical ophthalmic products. Ideally, the establishment of a biopharmaceutical classification system categorizing the ophthalmic excipients with respect to their effect on ophthalmic drug permeability/bioavailability would enable more prudent selection of the type and concentration of ophthalmic excipients during the early phase of ophthalmic formulation composition optimization, with positive impact on the economical and ethical aspect of the overall topical ophthalmic product development.

In addition to their application in conventional topical ophthalmic formulations, the excipients approved for the ophthalmic use, as well as novel excipients, are increasingly utilized in the development of innovative nanosystem-based topical ophthalmic formulations. Under particular conditions excipients specifically interact with each other to form supramolecular nano-sized structures, which have the potential to circumvent the ocular barriers limiting the efficacy of conventional topical ophthalmic formulations. In the nanosystem development excipients can serve as nanosystem structural components (Mandal et al., 2017), stabilizing agents to stabilize energetically unstable nanosystems by adsorption to nanosystem surface (Ammar et al., 2009; Sharma et al., 2016) or coating agents to direct the nanosystem delivery to the target site (Singh and Lillard, 2009). The main goal of the nanosystem design is to achieve prolonged drug precorneal residence time, sustained drug release and enhanced permeation of a drug across corneal barrier, with the result of improved drug eye-related bioavailability and therapeutic outcome (Reimondez-Troitiño et al., 2015). Knowledge of the ophthalmic excipients influence on the ophthalmic drug permeability would surely contribute to their more efficient selection in the process of the nanosystem composition optimization in the early phase of development. However, for the evaluation of biopharmaceutical properties of the final nano-sized supramolecular structures of excipients, *in vitro* studies of their interactions with the precorneal biological environment are needed.

For small moderately lipophilic molecules, which still comprise the majority of commercially available ophthalmic drugs, the corneal barrier represents the main route to their target site. The evaluation of drug permeability across the corneal barrier represents, therefore, an essential step in the assessment of drug eye-related bioavailability. The *in vitro*

corneal cell-based and *ex vivo* tissue-based permeability models for eye-related bioavailability prediction have the potential to substantially improve the screening process of ophthalmic drug and formulation candidates in the early phases of development and to contribute thereby to higher efficiency of the overall topical ophthalmic product development. However, detailed characterization and standardization of the *in vitro* and *ex vivo* models is a prerequisite for their implementation in the routine use in pharmaceutical industry during preclinical phases of topical ophthalmic product development (Pepić et al., 2014).

Different cell-based models mimicking the corneal epithelium or the entire corneal tissue using different cell lines and culture conditions have been developed and used for the permeability testing of ophthalmic drugs and drug delivery systems (Attama et al., 2008; Ban et al., 2003; Chang et al., 2000; Hahne et al., 2012; Hahne and Reichl, 2011; Mohan et al., 2003; Reichl and Müller-Goymann, 2003; Tegtmeyer et al., 2001; Toropainen et al., 2001). However, cell-based models using the HCE-T cell line have been the most extensively characterized regarding the membrane porosity and intercellular pore size, paracellular and transcellular passive transport, the expression of membrane transporters and metabolic enzymes (Becker et al., 2007; Hahne and Reichl, 2011; Kölln and Reichl, 2016a, 2016b, 2012, Toropainen et al., 2003, 2001; Vellonen et al., 2010). Nonetheless, variability of the barrier properties of the HCE-T cell-based models, most probably related to the use of different culture conditions, has been reported in the literature (Hahne and Reichl, 2011; McCanna et al., 2008; Nagai et al., 2008; Takezawa et al., 2011; Toropainen et al., 2001), which substantially complicates the comparison of the HCE-T cell-based permeability results from different laboratories, potentially resulting in the misleading conclusions. Further studies are, therefore, required to identify and standardize the cultivation factors with critical influence on the HCE-T cell barrier phenotype, with the final goal of establishing a standardized *in vitro* corneal model yielding reliable and reproducible permeability results.

The aim of this thesis was to evaluate the effect of unimers and supramolecular aggregates of the ophthalmic excipients on corneal barrier properties and ophthalmic drug permeability, using *in vitro* (HCE-T cell-based) and/or *ex vivo* (excised porcine cornea) corneal models. For this purpose, further characterization of the barrier phenotype of the HCE-T cell-based model, cultivated according to the previously developed and optimized protocol, was performed (Hahne and Reichl, 2011). The culture medium, support-coating materials, cell-seeding density, volumes of the medium in the apical and basolateral compartment and the duration of ALI exposure, all of which were determined to influence the corneal model barrier properties (Hahne and Reichl, 2011), were used as defined in the

protocol. However, contradictory results on the optimal pore size of the supporting membrane (i.e., 0.4 μm (Toropainen et al., 2001) and 3.0 μm (Hahne and Reichl, 2011) pore size) have been reported in the literature. Polycarbonate membranes of two different pore sizes, 0.4 and 3.0 μm, were thus used to investigate the pore size influence on barrier properties of the HCE-T cell-based model, considering the model histological structure, barrier tightness and the permeability of five ophthalmic compounds (timolol maleate, diclofenac sodium, chloramphenicol, dexamethasone and fluorescein sodium) differing in physicochemical properties. For the barrier tightness evaluation, a well-established method of transepithelial electrical resistance (TEER) monitoring was performed, which analyzes the overall electrical resistance of a cell barrier as a measure of the tight connections between the epithelial cells (tight junctions) (Saaber et al., 2014).

Two different HCE-T barrier phenotypes depending on the pore size were revealed, namely Model I (0.4 μm pore size) and Model II (3.0 μm pore size).

Model I consisted of a multilayered epithelium on top of the supporting polycarbonate membrane. Despite the formation of a multilayered epithelium reflecting well the structural properties of the corneal epithelium *in vivo*, TEER measurements revealed weak barrier function for Model I, which points to the lack of expression or dysfunction of intercellular tight-junctional complexes. As a result of insufficient barrier properties, Model I exhibited substantially higher permeability of all the tested ophthalmic compounds, compared to both Model II and excised porcine cornea, which confirms lack of its suitability in predicting transcorneal drug permeation.

On the other hand, Model II was comprised of an apical lipophilic monolayer of HCE-T cells overlying the supporting membrane and a basolateral lipophilic monolayer of migrated HCE-T cells. Model II was characterized by improved barrier tightness compared to both Model I and excised porcine cornea, which might possibly be related to the presence of a basolateral layer of migrated HCE-T cells, since it was shown that migrated cells exhibit polarized phenotype and tight-junctional complexes (Tucker et al., 1992). Moreover, Model II reflected better the barrier properties of the excised porcine cornea in terms of permeation behavior of ophthalmic compounds. Model II exhibited ophthalmic compounds permeability more similar to the one for excised porcine cornea. Moreover, the ability to discriminate between the permeability of timolol and fluorescein, determined to be highly and poorly permeable compounds in excised porcine cornea, was significantly improved for Model II.

The results of the histomorphological and functional characterization of the HCE-T cell-based model conducted in this thesis demonstrate significant variability of the model

regarding the histological structure and tightness of the corneal barrier formed under particular cultivation conditions (Hahne and Reichl, 2011; Toropainen et al., 2001). It has, however, been demonstrated that, although initially derived from a single clone, the HCE-T cells undergo genomic alterations in culture resulting in a heterogenous cell line composed of a number of cell subpopulations, which were determined to differ in morphology, cell growth kinetics and tight barrier forming properties (Yamasaki et al., 2009). Moreover, it has been shown that migration through filter pores might be selective and limited to a more invasive subpopulation of parental cell line (Tucker et al., 1992), which might additionally explain the observed differences in HCE-T cell line behavior. These findings highlight the obvious need for more thorough investigation of the influence of cultivation conditions on HCE-T cell line and HCE-T cell-based corneal model phenotype, which is of crucial importance for establishing reproducible and reliable HCE-T cell-based permeability model.

Passive permeation across biological membranes has been shown to be influenced by physicochemical properties such as molecular size, partition coefficient, hydrogen bond formation capacity and permeant charge (Kidron et al., 2010). The difference in the partition coefficient and charge of compounds might explain the observed permeability rank order of the ophthalmic compounds in Model II and excised porcine cornea, with higher partition coefficient and positive charge facilitating the permeation through lipophilic and negatively charged corneal barrier. However, some discrepancies in the permeability rank order for Model II and excised porcine cornea were observed, which may be explained by the lack of stroma in Model II, shown to be a rate-limiting permeation barrier for highly lipophilic compounds (Shih and Lee, 1990), and potential functional differences in terms of the expression of membrane transporters. More detailed mechanistic permeability studies regarding the transport mechanism of the tested compounds and the barrier role of particular structures of the corneal models are required to clarify the *in vitro*/*ex vivo* permeability differences. Nonetheless, correlation analysis of the ophthalmic compounds permeability for Model II and excised porcine cornea resulted in very strong and statistically significant correlation. This, in addition to improved barrier tightness, points to the suitability of the Model II for evaluating transcorneal permeation of ophthalmic drugs alone and ophthalmic drugs in the presence of different ophthalmic excipients.

Regarding the potential interaction of excipients with tight-junctional complexes of corneal epithelial barrier and their consequent effect on the drug transcorneal permeability, the suitability of the *in vitro* Model II was further determined by establishing the interdependence of temperature, TEER and the permeability of paracellular and transcellular

compounds. The TEER values were shown to be inversely related to temperature changes, in accordance with the reports in the literature (González-Mariscal et al., 1984), which allowed for permeability testing of the two compounds across corneal barrier of different TEER values, indicating its different tight-junctional integrity and paracellular leakiness. The permeability of a hydrophilic compound fluorescein sodium was shown to be inversely correlated with TEER of the Model II at both temperature studied, which confirms the paracellular transport as the main mechanism of its permeation across corneal epithelial barrier. On the other hand, no influence of TEER on the permeability of a lipophilic compound rhodamine B was found, which points to its transcellular transport across corneal epithelial barrier (Hahne et al., 2012). However, higher permeability was observed at 37°C than at 25°C, most probably due to faster transcellular diffusion of rhodamine B at higher temperature. The established relation between TEER and fluorescein sodium and rhodamine B permeability indicates the suitability of the Model II for the evaluation of the interaction of excipients with corneal epithelial barrier and its related impact on ophthalmic drug permeability. It can, moreover, serve as a basis for studying the mechanism of transport across corneal barrier of both ophthalmic drugs alone and ophthalmic drugs incorporated into drug delivery nanosystems.

For evaluating the influence of excipients on biopharmaceutical properties of different drug delivery nanosystems for melatonin, an intraocular pressure (IOP)-reducing agent, Model II in conjunction with other *in vitro* biopharmaceutical studies was used. Melatonin-loaded lecithin/chitosan nanoparticles (MLC) and Pluronic® F127/chitosan micelles (MFC) were prepared and characterized, with the final aim of developing a nanosystem which would prolong precorneal residence time and promote transcorneal transport of melatonin, with consequent intraocular activity enhancement. The formation of nanosystems arises from electrostatic interaction between the negatively charged lecithin and positively charged chitosan in MLC (Hafner et al., 2009), and both electrostatic and hydrogen bond interaction between the electronegative oxygen atoms of Pluronic® F127 and electropositive chitosan chains in MFC (Pepić et al., 2010). Lecithin, a natural lipid mixture of phospholipids, has been frequently used as excipient in the preparation of various nanosystems, and was, due to lipophilic nature, expected to provide high loading of lipophilic drugs and sustained drug release (Bhatta et al., 2012). Pluronic® F127, a polyoxyethylated nonionic surfactant approved for the ophthalmic use, was suggested to promote the drug permeation across corneal epithelial barrier (Pepić et al., 2010). Formulated as eye drops, Pluronic® F127 micelles were shown to enhance eye-related bioavailability of both small and large drug molecules (Chetoni et al., 2000; Liaw et al., 2001; Pepić et al., 2004b). Chitosan, a biodegradable and

biocompatible excipient, known to exert mucoadhesive and permeability-enhancing properties due to its polycationic and polymeric nature (Garcia-Fuentes and Alonso, 2012), was identified as a critical component regarding the interaction of nanoparticles with the corneal barrier and melatonin ocular bioavailability *in vivo*. Lecithin nanoparticles (ML) and Pluronic® F127 micelles (MF) were, therefore, additionally prepared to examine the chitosan influence on nanoparticles biopharmaceutical properties.

MLC and MFC were shown to significantly differ in size (241.8 ± 0.8 nm and 20.7 ± 0.3 nm, respectively) and surface charge properties (zeta potential of 22.7 ± 0.7 mV and 4.3 ± 0.9 mV, respectively). Both types of nanosystems were shown to possess prolonged-release behavior, determined to be a desirable attribute for accomplishing extended IOP reduction (Musumeci et al., 2013), with MFC exhibiting faster melatonin release ($t_{50\%}$ = 0.75 h) than MLC ($t_{50\%}$ = 1.75 h). Each of the component of the nanosystems investigated have been proved as a biocompatible and safe excipient for topical ophthalmic administration (Garcia-Fuentes and Alonso, 2012; Hafner et al., 2009; Jiao, 2008), however, biocompatibility studies of final nanosystems should always be conducted to ensure no toxic effects are present as a result of the potential interaction of the excipients and their presence in a nano-sized supramolecular form. The HCE-T cell-based viability studies showed all the investigated nanosystems were biocompatible with the corneal epithelial cells, which indicates their safe use for topical ophthalmic administration and confirms the absence of toxicity during both mucoadhesivity and permeability HCE-T cell-based studies.

The mucoadhesivity of nanoparticles is one of the mechanisms of prolonging the precorneal residence time (Musumeci et al., 2013) and was in this study evaluated using a newly-established HCE-T cell monolayer-based mucoadhesivity assay. Chitosan, due to its positive charge at pH below 6.5, has been shown to interact with the negatively-charged epithelial cells, which can attenuate the effect of tear drainage and extend the residence time of nanoparticles at the ocular surface *in vivo* (de la Fuente et al., 2010). Indeed, the results show that MLC exhibit mucoadhesive properties, which are most probably related to their positive surface charge originating from chitosan located at their surface. This confirms the previous findings of the MLC mucoadhesivity obtained by a standard mucus glycoprotein assay (Hafner et al., 2009), which demonstrates the potential of the HCE-T cell-based mucoadhesivity assay to be further optimized for the purpose of nanoparticle mucoadhesivity testing.

Previous studies have shown that chitosan can induce tight-junction opening and promote transcorneal drug transport (Hafner et al., 2009), however, no difference in the

decrease of TEER values was observed for nanosystems with and without chitosan, which points to other mechanisms of TEER decrease. The recovery of the initial TEER values after the permeability study indicates that the interaction of nanosystems with epithelial barrier is reversible, confirming their non-toxic nature observed in biocompatibility studies. The permeability of melatonin incorporated into nanosystems was, except for MF, decreased compared to melatonin in solution, with micelles demonstrating higher overall melatonin permeability compared to nanoparticles. The higher permeability of melatonin incorporated into micelles might be related to the smaller size of these nanosystems, the presence of non-entrapped melatonin in the micellar solution, as well as the transcellular permeability-enhancing effect of Pluronic® F127 (Pepić et al., 2010). In addition, the presence of chitosan in both nanoparticles and micelles seems to be linked to the observed decrease in the melatonin permeability. For MFC the presence of chitosan seems to have diminished the permeability-enhancing effect of Pluronic® F127, which resulted in lower permeability compared to MF. On the other hand, for nanoparticles the permeability-decreasing effect of chitosan seems to reflect its influence on melatonin release rate, which in *in vitro* release studies was determined to be lower for MLC than for ML. Strong correlation between melatonin *in vitro* release and transcorneal permeation rate was established, which indicates that the melatonin permeation across HCE-T epithelial barrier is controlled by the melatonin release profile.

Overall, the conducted HCE-T cell based permeability studies in conjunction with other *in vitro* biopharmaceutical and physicochemical studies revealed chitosan as a key excipient providing the MLC with desirable biopharmaceutical properties. Prominent mucoadhesive properties and prolonged melatonin release, which was shown to control melatonin permeation across *in vitro* corneal barrier, demonstrated the potential of MLC to provide prolonged melatonin precorneal residence time and extended IOP reduction, with overall improved eye-related bioavailability and therapeutic outcome, compared to micelles and aqueous melatonin solution.

Surface active excipients are commonly used in ophthalmic formulations as solubilization agents for increasing drug solubility and improving solution clarity or as wetting agents for stabilizing drug suspensions. A number of *in vitro*/*ex vivo* studies on the influence of surface active excipients on drug permeability have been conducted. The studies demonstrated that the presence of surface active excipients can lead to increased, decreased or unchanged drug permeability, as a result of their interaction with the biological barrier (the intercellular tight-junctional complexes, cell membrane influx/efflux transporters and cell

membrane phospholipid bilayer) or with the drug itself. Majority of these studies were, however, focused on investigating the effect of surface active excipients on the permeability of drugs intended for oral administration route, using *in vitro* and *ex vivo* intestinal permeability models (Fischer et al., 2011a, 2011b; Katneni et al., 2007; Legen et al., 2006; Rege et al., 2002; Saha and Kou, 2000). Additionally, *in vivo* studies on the effect of drug loaded nanosystems containing surface active excipients on eye-related bioavailability have been performed, in which surface active excipients demonstrated the potential to solubilize a drug, prolong the drug precorneal retention time, enhance the rate and extent of drug absorption, reaching thereby high eye-related bioavailability levels (Chetoni et al., 2000; Kassem et al., 2007; Liaw et al., 2001; Pepić et al., 2010, 2004a). However, to identify precisely the effect of surface active ophthalmic excipients on transcorneal drug permeability, systematic, but simpler *in vitro* and *ex vivo* studies, examining the influence of a type and concentration of surface active ophthalmic excipient on the transcorneal permeability of different ophthalmic drugs, are needed.

In this thesis, *in vitro* Model II in conjunction with *ex vivo* corneal model (excised porcine cornea) was used for investigating the effect of the type and concentration of four different nonionic surface active ophthalmic excipients on the transcorneal permeation of ophthalmic drugs. The transcorneal permeability of the four ophthalmic drugs, namely, timolol maleate, diclofenac sodium, chloramphenicol and dexamethasone, was determined in previous work of this thesis and was found to strongly correlate between Model II and excised porcine cornea. Additional studies on self-aggregation properties of surface active ophthalmic excipients, using dynamic light scattering measurement, were conducted to obtain insight into their interaction with ophthalmic drugs, influencing potentially the drug transcorneal permeation rate.

The *in vitro* and *ex vivo* permeability of the four ophthalmic drugs obtained in this study are in agreement with the permeability results from the previous work of this thesis, which points to the acceptable intra-laboratory reproducibility of both corneal models. However, a 2-8-fold difference between the *ex vivo* permeability reported in the literature and *ex vivo* permeability measured in this study is observed (Loch et al., 2012; Pescina et al., 2015), which might be ascribed to the differences in animal species characteristics (breed, age, sex), the tissue collection and handling procedure, as well as the experimental setup. The observed *ex vivo* inter-laboratory variability obviously points to a compelling need for in-depth studies defining the critical parameters of the *ex vivo* corneal model and establishing standardized *ex vivo* transcorneal permeability testing protocols.

In this study no interaction of the surface active ophthalmic excipients with corneal epithelial barrier in both Model II and excised porcine cornea was observed, as indicated by statistically insignificant TEER changes. However, ophthalmic drug permeability was generally either unchanged or decreased, depending on the surface active ophthalmic excipient concentration. Decreasing effect of surface active excipients on the permeability of poorly soluble drugs was reported in the literature and was ascribed to the interaction of drugs and micelles of surface active excipients. More precisely, lipophilic poorly soluble drugs associate with the micelle hydrophobic core which reduces the free drug fraction available for permeation across a biological barrier, resulting in an overall drug permeability decrease (Fischer et al., 2011b; Yano et al., 2010). Accordingly, in our study the association of drugs with the self-aggregates/micelles of surface active ophthalmic excipients might be a potential mechanism of the observed ophthalmic drug permeability decrease, as indicated by the dynamic light scattering measurements. More precisely, at the lowest concentration of surface active ophthalmic excipients, at which absence of self-aggregates was established, no influence of surface active ophthalmic excipients on drug permeability was observed. However, at higher surface active ophthalmic excipients concentrations, a strong permeability depressing effect was observed for certain drug-surface active ophthalmic excipient combinations, which might possibly be linked to the drug interaction with the formed surface active ophthalmic excipients self-aggregates. The strongest permeability-decreasing effect was observed for Cremophor EL, 2.0% *w*/*V* (up to around 90%), followed by Tyloxapol, 0.1% *w*/*V* (up to around 50%) and Polysorbate 80, 0.2% *w*/*V* (up to around 30%). Pluronic® F68, on the other hand, had no statistically significant effect on the ophthalmic drug permeability over the entire investigated concentration range, which is in agreement with the results of the studies on *ex vivo* intestinal permeability models (Fischer et al., 2011a, 2011b; Katneni et al., 2007).

The measured permeability-decreasing effect of surface active ophthalmic excipients was more pronounced for lipophilic ophthalmic drugs. Less lipophilic and highly soluble weakly basic drug timolol exhibited no interaction with surface active ophthalmic excipients over the whole surface active ophthalmic excipients concentration range tested, as indicated by unchanged timolol permeability in both corneal models. In contrast, for more lipophilic and poorly soluble neutral drug such as dexamethasone, up to around 70% decrease in *in vitro*/*ex vivo* permeation rate was measured, most probably related to the drug association with surface active ophthalmic excipient self-aggregates, formed at higher surface active ophthalmic excipient concentrations. On the other hand, for weakly basic drug diclofenac

sodium, characterized by higher solubility and lower lipophilicity compared to dexamethasone, even greater up to around 90% reduction in permeation rate in the presence of surface active excipients in both corneal models was measured, which may be explained by previous solubilization studies demonstrating greater solubility of the ionized species compared to its unionized equivalent (Li et al., 1999).

Strong and statistically significant *in vitro*/*ex vivo* correlation of the influence of surface active ophthalmic excipients on ophthalmic drug permeability was obtained in this study, which indicates that the observed influence is of comparable extent and mediated by the same mechanism in both corneal models. The structural differences and potential functional differences of the Model II and excised porcine cornea might be linked to the observed *in vitro*/*ex vivo* discrepancies, which require additional histological, functional and mechanistic permeability studies to establish the exact underlying cause. Nonetheless, the obtained results give valuable insight into the effect of surface active ophthalmic excipients on the ophthalmic drug transcorneal permeability, influencing potentially ophthalmic drug eye-related bioavailability, which may contribute to their more efficient and directed use in the future topical ophthalmic product development.

6. CONCLUSIONS

In this thesis, the effect of unimers and supramolecular aggregates of the ophthalmic excipients on corneal barrier properties and ophthalmic drug permeability was evaluated, using *in vitro* (HCE-T cell-based) and/or *ex vivo* (excised porcine cornea) corneal models. Histological and functional characterization of the HCE-T cell-based model revealed the membrane pore size to be an important factor influencing HCE-T barrier phenotype. The use of a 3.0 μm pore size resulted in HCE-T corneal epithelial phenotype of improved functional barrier properties, but of a specific histological structure consisting of an apical lipophilic HCE-T monolayer and a basolateral lipophilic layer of migrated HCE-T cells. The increased barrier tightness and very strong *in vitro*/*ex vivo* correlation of the ophthalmic compounds permeability suggests that Model II is suitable for the ophthalmic drug transcorneal permeability evaluation. The established interdependence of temperature, TEER and permeability of a paracellular and transcellular compound indicates furthermore the suitability of Model II for biopharmaceutical evaluation of ophthalmic excipients regarding their interaction with the corneal epithelial barrier and consequent impact on the drug transcorneal permeability.

Model II-based permeability studies were proved valuable in the biopharmaceutical characterization of melatonin-loaded lecithin/chitosan nanoparticles and Pluronic® F127/chitosan micelles, by allowing the evaluation of the contribution of each excipient to the overall impact of nanosystems on the melatonin transcorneal permeability. Significant influence of chitosan on the transcorneal permeability of melatonin incorporated into nanosystems was revealed. In micelles chitosan hindered the Pluronic® F127 permeation-enhancing effect, while for nanoparticles the presence of chitosan ensured prolonged melatonin release rate, which was shown to control melatonin permeation rate across corneal epithelial barrier. HCE-T cell-based mucoadhesivity assay revealed prominent mucoadhesive properties of the lecithin-chitosan nanoparticles, which were ascribed to the positive surface charge originating from chitosan located at the nanoparticles surface. This attribute, together with prolonged release and controlled transcorneal permeation of melatonin, demonstrated the potential of lecithin/chitosan nanoparticles to provide extended melatonin precorneal residence time, ensuring high eye-related bioavailability and extended intraocular pressure reduction, compared to micelle and aqueous melatonin solution.

Using *in vitro* Model II in conjunction with excised porcine cornea the concentration- and self-aggregation-dependent influence of four nonionic surface active ophthalmic excipients on the permeability of four ophthalmic drugs was investigated. None of the nonionic surface active ophthalmic excipients over the entire concentration range

demonstrated interaction with epithelial barrier of the corneal models. The *in vitro*/*ex vivo* permeability of the ophthalmic drugs was, however, affected, depending on the nonionic surface active ophthalmic excipients concentration. At the lowest concentration of all four surface active ophthalmic excipients the permeability of ophthalmic drugs was unchanged, while at higher concentrations a permeability-decreasing effect was observed. The results of the dynamic light scattering studies point to the interaction of the ophthalmic drugs with self-aggregates of surface active ophthalmic excipients as a potential mechanism of the observed ophthalmic drug permeability decrease. Strong *in vitro*/*ex vivo* correlation of the influence of surface active ophthalmic excipients on the ophthalmic drugs permeability suggests that the observed permeability-altering effects of surface active ophthalmic excipients were of comparable magnitude and were mediated by the same mechanism in both corneal models.

In conclusion, the results of this thesis demonstrate the suitability of Model II for drug transcorneal permeability evaluation, considering the influence of excipients in either monomolecular or supramolecular nano-sized form. However, variability of the HCE-T corneal epithelial barrier phenotype was observed which points to a pressing need for identification and standardization of cultivation factors influencing the HCE-T barrier properties in further optimization of the HCE-T cell-based corneal model. Nonetheless, the *in vitro*/*ex vivo* permeability results obtained in this thesis give valuable insight into the influence of ophthalmic excipients on the ophthalmic drug transcorneal permeability, which potentially impacts the topical ophthalmic drug eye-related bioavailability. Biopharmaceutical classification of ophthalmic excipients with respect to their effect on ophthalmic drug transcorneal permeability and eye-related bioavailability is a crucial step towards a more efficient process of optimization of topical ophthalmic formulation composition in the early development phases, with positive impact on the economic aspect of the overall topical ophthalmic product development.

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8. BIOGRAPHY

Marina Juretić was born on 3rd of September 1987 in Zagreb, Croatia. She finished elementary school and the VIIth Gymnasium in Zagreb. She graduated from the Faculty of Pharmacy and Biochemistry, University of Zagreb, in 2011. During her studies she was awarded the Dean's Prize for scientific work "Antitumour and antimicrobial effects of *Epilobium angustifolium* Schreb. and *Epilobium Parviflorum* L." and the "Dean's award for excellence in academic year of 2010/2011". From 2011. to 2012. she worked at Hanžić Pharmacies in Zagreb as a pharmacist-intern. In 2013. she started working as a research assistant at the Department of Pharmaceutical technology, Faculty of Pharmacy and Biochemistry, University of Zagreb, on the project entitled "Development of *in vitro* and *ex vivo* models for permeability testing of new topical ophthalmic formulations" (Partnership in research: 04.01/56, Croatian science foundation and PLIVA Croatia Ltd.), as part of which she enrolled in postgraduate doctoral study "Pharmaceutical-Biochemical Sciences" at the Faculty of Pharmacy and Biochemistry, University of Zagreb. In 2013. she visited Technical University in Braunschweig, Germany, to receive training in the field of *in vitro* cell-based corneal model cultivation and transcorneal permeability testing. In 2014. she made a short stay at the Institute for Applied Ophthalmobiology (IOBA), University of Valladolid, Spain, where she received education in the field of primary porcine corneal/conjunctival cell isolation. From 2015. to 2016. she worked as a research assistant at the Department of Pharmaceutical technology, Faculty of Pharmacy and Biochemistry, University of Zagreb, on the project "Modelling of the Pharmaceutical Spray Drying Process of the Emulsions in Laboratory and Pilot Scale" (industrial partner PLIVA Croatia Ltd.). From 2016. to 2017. she was employed as a teaching assistant at the Department of Pharmaceutical technology, Faculty of Pharmacy and Biochemistry, University of Zagreb. In 2017. she started working as Lead Research Analyst in PLIVA Croatia Ltd. (Research and Development). Marina is a member of Croatian Chamber of Pharmacists. She co-authored 6 peer-reviewed papers and participated in different international scientific conferences.

List of Publications:

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Basic documentation card

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Doctoral dissertation

BIOPHARMACEUTICAL EVALUATION OF OPHTHALMIC EXCIPIENTS USING *IN VITRO* AND *EX VIVO* CORNEAL MODELS

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SUMMARY

Bioavailability of topical ophthalmic products is substantially determined by the type and concentration of excipients. In addition to the use in conventional ophthalmic formulations, excipients are increasingly utilized in the development of innovative nanosystem-based ophthalmic formulations, in which excipients are supramolecularly organized into nano-sized structures, which can specifically interact with the anterior eye segment barriers and enhance the drug eye-related bioavailability. Evaluation of the drug transcorneal permeability is a critical step in predicting eye-related bioavailability of topical ophthalmic drugs. In this thesis, the effect of unimers and supramolecular aggregates of the ophthalmic excipients on corneal barrier properties and ophthalmic drug permeability was evaluated, using *in vitro* (HCE-T cell-based) and/or *ex vivo* (excised porcine cornea) corneal models. Histological and functional characterization of the *in vitro* corneal model revealed variability of the HCE-T barrier phenotype, which highlights the important aspects to be considered in the further model development. HCE-T cell-based model of a specific histological structure, but showing improved barrier tightness and a very strong correlation of ophthalmic compounds permeability with excised porcine cornea, was shown to be suitable for the ophthalmic drug permeability evaluation considering the influence of ophthalmic excipients. Biopharmaceutical evaluation of melatonin-loaded nanosystems using HCE-T cell-based models revealed significant influence of chitosan on nanosystem biopharmaceutical properties. In lecithin/chitosan nanoparticles the presence of chitosan ensured mucoadhesive properties and prolonged melatonin release, controlling melatonin *in vitro* transcorneal permeation, which may provide enhanced melatonin eye-related bioavailability and therapeutic effect. The *in vitro*/*ex vivo* evaluation of the effect of nonionic surface active ophthalmic excipients on the ophthalmic drug permeability revealed their concentration-dependent permeability-decreasing effect, which was potentially related to the association of ophthalmic drugs with self-aggregates of surface active ophthalmic excipients. The results of this thesis provide valuable insight into the effect of ophthalmic excipients on ophthalmic drug transcorneal permeability, which may contribute to more directed and economical development of both conventional and innovative topical ophthalmic products.

The dissertation is deposited in the Central Library of Faculty of Pharmacy and Biochemistry.

Dissertation includes: 70 pages, 10 figures, 7 tables i 109 references. Original is in English language.

Keywords: ophthalmic drugs, ophthalmic excipients, surfactants, nanosystems, drug permeability, eye-related bioavailability, *in vitro* and *ex vivo* corneal models, HCE-T.

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BIOFARMACEUTSKA ISPITIVANJA OFTALMIČKIH EKSCIPIJENSA NA *IN VITRO* I *EX VIVO* MODELIMA ROŽNICE

Marina Juretić

SAŽETAK

Bioraspoloživost topikalnih oftalmičkih pripravaka značajno je određena vrstom i udjelom eskcipijensa. Osim u konvencionalnim oftalmičkim oblicima, ekscipijensi primjenu pronalaze i u izradi inovativnih oftalmičkih oblika temeljenih na nanosustavima za dostavu lijeka. Interakcija ekscipijensa rezultira tvorbom supramolekulskih struktura nanodimenzija, koje mogu povećati bioraspoloživost lijeka u oku prevladavanjem barijera prednjeg segmenta oka. Ispitivanje permeabilnosti lijeka preko barijere rožnice ključan je korak u određivanju bioraspoloživosti topikalnih oftalmičkih lijekova. Cilj ovog doktorskog rada je bio ispitati utjecaj unimera i supramolekulskih agregata oftalmičkih ekscipijensa na barijerna svojstva rožnice i permeabilnost oftalmičkih lijekova korištenjem *in vitro* HCE-T modela epitela humane rožnice i *ex vivo* tkivnog modela rožnice svinje. Histološkom i funkcionalnom karakterizacijom *in vitro* modela utvrđena je određena varijabilnost fenotipa HCE-T barijere, što ukazuje na potrebu za dodatnom karakterizacijom čimbenika uzgoja s utjecajem na barijerna svojstva modela. HCE-T model specifične histološke strukture, ali čvršćih međustaničnih veza te vrlo dobre korelacije permeabilnosti oftalmičkih supstancija s *ex vivo* tkivnim modelom rožnice svinje, pokazao se prikladnim za ispitivanje učinka oftalmičkih ekscipijensa na permeabilnost oftalmičkih lijekova. Biofarmaceutskom karakterizacijom nanosustava za dostavu melatonina korištenjem *in vitro* HCE-T modela utvrđen je značajan utjecaj kitozana na biofarmaceutska svojstva nanočestica. U slučaju lecitinsko-kitozanskih nanočestica kitozan je osigurao mukoadhezivna svojstva, produljeno oslobađanje i kontroliranu permeaciju melatonina preko HCE-T barijere rožnice, što može pospješiti bioraspoloživost i terapijski učinak melatonina. U *in vitro*/*ex vivo* ispitivanjima uočeno je smanjenje permeabilnosti oftalmičkih lijekova ovisno o vrsti i koncentraciji neionskih površinski aktivnih oftalmičkih ekscipijensa, što je potencijalno uzrokovano interakcijom lijeka s agregatima površinski aktivnih oftalmičkih ekscipijensa. Rezultati ovog doktorskog rada značajan su doprinos razumijevanju učinka oftalmičkih ekscipijensa na permeabilnost oftalmičkih lijekova, što može pridonijeti usmjerenijem i ekonomičnijem razvoju konvencionalnih i inovativnih topikalnih oftalmičkih pripravaka.

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