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Topical delivery of a Rho-kinase inhibitor to the cornea via mucoadhesive film

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ABSTRACT

The application of inhibitors of the Rho kinase pathway (ROCK inhibitors) to the surface of the eye in the form of eyedrops has beneficial effects which aid the recovery of diseased or injured endothelial cells that line the inner surface of the cornea. The aim of this study was to test the plausibility of delivering a selective ROCK inhibitor, Y-27632, to the cornea using a thin polymeric film. Mucoadhesive polymeric thin films were prepared incorporating Y-27632 and diffusional release into PBS was determined. Topical ocular delivery from the applied film was investigated using freshly excised porcine eyes and eyedrops of equivalent concentration acted as comparators; after 24 h the formulations were removed and the corneas extracted. Drug-loaded thin polymeric films, with high clarity and pliability were produced. ROCK inhibitor Y-27632 was weakly retained within the film, with release attaining equilibrium after 1 h. This in turn facilitated its rapid ocular delivery, and an approximately three-fold greater penetration of Y-27632 into cryoprobe-treated corneas was observed from the thin film ($p < 0.01$) compared to eyedrops. These findings support the further development of ROCK inhibitor delivery to the cornea via release from thin mucoadhesive films to treat vision loss cause by corneal endothelial dysfunction.

1. Introduction

The cornea of the eye, which is just over 0.5 mm thick in humans, owes its transparency to the characteristic spatial arrangement of its constituent collagens and other extracellular matrix components which make up much of its thickness (Knupp et al., 2009; Meek and Knupp, 2015). Lining the inner surface of the cornea is a single layer of metabolically active endothelial cells which separate the corneal matrix from the adjacent aqueous humor and prevent the ingress of fluid into the cornea (Hodson and Miller, 1976). If the endothelial cell layer is compromised the cornea swells and becomes oedematous, scatters light and loses its transparency. This results in severe vision loss and in most cases the only option is corneal transplant surgery. Typically, corneal endothelial dysfunction occurs because of an inherited disease called Fuch's endothelial corneal dystrophy (FECD) in which corneal endothelial cells deteriorate or are lost over the years. It can also occur as a result of accidental damage to the corneal endothelium during cataract surgery to remove or replace the lens of the eye, and this condition is often referred to as bullous keratopathy. Either way, the endothelial cell damage causes cornea to imbibe excess fluid and swell, making the cornea appear hazy (Fig. 1) clouding vision (Fig. 2) (Heiting, 2015; Royal National Institute of Blind People, 2015).

Techniques to treat vision loss caused by corneal endothelial dysfunction include an anterior corneal micropuncture and laser treatment to puncture the corneal epithelium. Although these procedures can be effective they carry a high risk of rejection and sometimes result in complications including corneal perforation and scarring (Rahman et al., 2008). But, by far the most common treatment for corneal endothelial dysfunction is a corneal graft, and the numbers of surgeries performed indicate the scale of the problem. The prevalence of FECD, generally, is estimated to be around 4% of individuals over the age of 40, but in inbred American, Singaporean and Icelandic populations this rises to 22%, 7% and 9% respectively (Zoega et al., 2006; Eghrari et al., 2012; Kitagawa et al., 2002).

In terms of drug therapy, the ROCK signaling pathway has received recent attention in light of the diverse therapeutic potential of changing cell behaviour in various diseases such as hypertension, vasospasm, and glaucoma (Arnold et al., 2013); and more recently FECD (Koizumi et al., 2013; Okumura et al., 2009, 2011, 2013, 2015; Nakagawa et al., 2015). ROCK inhibitors are protein serine/threonine kinases with various functions throughout the body (Riento and Ridley, 2003). Out of several different ROCK inhibitors with different therapeutic effects (Liao et al., 2007; Wang and Chang, 2014), a selective ROCK inhibitor Y-27632 was reported to have promoted the proliferation of corneal endothelial cells in vitro (Okumura et al., 2009) and the healing of the corneal endothelium in vivo (Okumura et al., 2011). Sufficient corneal endothelial cell density is crucial for the ionic pump and barrier functions (Okumura et al., 2013), which in FECD would lead to an increased overall cell size and cell shape alteration, resulting in endothelial

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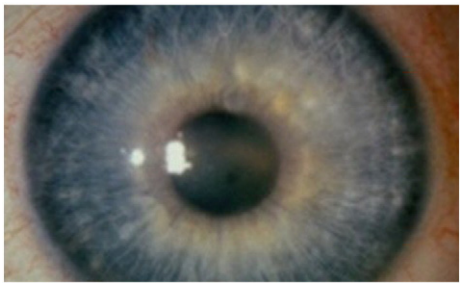

Fig. 1. Corneal haze of Fuchs Dystrophy.

dysfunction, and it was reported that Y-27632 was able to enhance cell density to the normal levels and resume the pump and barrier function, providing a functional recovery of the cornea. Koizumi et al. (2013) reported that some patients with FECD could be treated with ROCK inhibitor Y-27632 eye drops subsequent to transcorneal freezing to destroy damaged corneal endothelial cells.

The delivery of drugs to the eye is basically divided into three routes; topical, systemic and intraocular. The intraocular route involves an injection into the eye or use of implants, which is surgically fairly invasive, carries the risk if infection and is seen undesirable by patients. Systemic delivery is inefficient with potential unwanted side effects. The topical delivery of drugs to the eye is thus judged to be an attractive route. Examples of topical delivery include the use of eye drop solutions, ointments, suspensions and emulsions. Eye drops are widely used, but are easily washed out by blinking and nasolacrimal drainage; this means that only a small amount of the applied dose is likely to penetrate the cornea. Ointment, suspension and emulsion formulations are widely reported to cause ocular adverse effects, which include irritation and visual disturbance (Patel et al., 2013). Studies have been carried out on alternative topical approaches such as the OCUSERT® system, Topical Opthalmic Drug Delivery Device, medicated contact lenses and intraocular lenses (Morrison, 2015). However, none of these approaches are available commercially on the market. In this study, we investigated an alternative approach based upon drug-eluting thin polymeric muacoadhesive films that affix to the cornea, in an effort to improve the drawbacks of current methods. Our approach could conceivably lead to a more efficacious and user-friendly device capable of targeted delivery of ROCK inhibitors to attain improved clinical effect. Such therapy would also minimize wastage of costly ROCK inhibitor and avoid potential toxicity from dosing other tissues unnecessarily, in particular the tear duct and nasal cavity. Therefore, the hypothesis of this study is that topical delivery of ROCK inhibitor Y-27632 from thin mucoadhesive films is a superior alternative to eye drops for trans-corneal drug delivery. This study aimed to test the plausibility of topical delivery to the cornea from polymeric films by the fabrication of appropriate films, determination of drug release and finally the determination of trancorneal drug delivery in vitro.

Fig. 2. The hazy and clouded vision of a Fuchs patient (left) as compared to normal vision.

2. Materials and methods

2.1. Materials

ROCK inhibitor Y-27632 dihydrochloride (MW 247.3) was obtained from ApexBio Technology LLC (Houston, US). Acetonitrile (HPLC grade), water (HPLC grade) and phosphate buffered saline (PBS) tablets were obtained from Fisher Scientific (Loughborough, UK). Methylene blue (MW 319.9), polyethylene glycol 400 (PEG 400) and trifluoroacetic acid (≥99.0%) were from Sigma-Aldrich Company Ltd. (Poole, UK). Carbopol 917 (CP) was a gift from Noveon Inc. (Cleveland, U.S.) and hydroxyproplymethyl cellulose (HPMC) was a gift from Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan). Porcine eyeballs were obtained from a local abattoir by blunt dissection, immediately following slaughter.

2.2. Preparation of thin films

Ingredients were weighed into a 250 mL conical flask as detailed in Table 1 to provide a 1% w/v polymeric solution loaded with either 1.7, 5.2 or 10 mg Y-27632·2HCl; the aqueous solubility of Y-27632·2HCl is reported as 14 mg/mL (Santa Cruz Biotechnology) and that of methylene blue 43.6 mg/L (Pubchem). The mixtures were stirred overnight on a magnetic stirrer at room temperature. The following day, the mixtures were placed in an ultrasonic bath for 2 h to further assist particle comminution, and to degas the solution. Next, 50 mL of the solutions were poured into Petri dishes and left to dry in an oven overnight set at 60 °C. Once completely dry, the clear films were carefully removed from the petri dish and checked for any imperfections – those that had bubbles or crystals were discarded.

2.3. Diffusional release from thin films

Ten 0.5 × 0.5 cm patches were excised from each drug loaded film and each patch was accurately weighed before being immersed in 1 mL of PBS solution in an Eppendorf tube for various durations (10 s, 20 s, 30 s, 1 min, 2 min, 5 min, 30 min, 1 h, 3 h and 6 h). The patches were then carefully removed from the PBS solution with forceps. The remaining solutions were transferred to autosampler vials, prior to analysis by HPLC (Section 2.8). The diffusional release was carried out over the timescale of up to 6 h to broadly simulate an overnight dosage

Table 1
Working formulae to produce methylene blue and ROCK inhibitor Y-27632 films.

Film	Working formula	
Methylene blue	HPMC	0.175 g
	PEG 400	0.25 g
	CP	0.075 g
	Methylene blue	0.01 g
	DI H_2O	to 50 mL
ROCK inhibitor Y-27632 10 mg	HPMC	0.175 g
	PEG 400	0.25 g
	CP	0.075 g
	Y-27632·2HCl	10 mg
	DI H_2O	to 50 mL
ROCK inhibitor Y-27632 5.2 mg	HPMC	0.175 g
	PEG 400	0.25 g
	CP	0.075 g
	Y-27632·2HCl	5.2 mg
	DI H_2O	to 50 mL
ROCK inhibitor Y-27632 1.7 mg	HPMC	0.175 g
	PEG 400	0.25 g
	CP	0.075 g
	Y-27632·2HCl	1.7 mg
	DI H_2O	to 50 mL

application. Preliminary method development experiments were carried out with methylene blue film.

2.4. Transcorneal freezing of porcine eyes

Transcorneal freezing has been used as a therapeutic modality prior to Y-27632 application in the form of eye drops (Koizumi et al., 2013). In the experiments described here, to test if cryoprobe-treated eyeballs achieved a better ingress of drug, transcorneal freezing was conducted (Fig. 3) by gently touching the tip of a newly designed cryoprobe onto the surface of freshly obtained porcine eyeballs for 3 s, which we have shown to be optimal to produce reliable and reproducible areas of corneal endothelial cell death. The cryoprobe tip is 3.4 mm in diameter and reaches −50 °C via the internal expansion of a cryogen (medical grade nitrous oxide), and is made of silver for heightened thermal conductivity. Six of 12 eyeballs used for the experiment underwent transcorneal freezing and six did not.

2.5. Formulation of eye drops

Eyedrops of equal concentration to the methylene blue and ROCK inhibitor Y-27632 film were formulated to act as a control. The working formulae of the eyedrops are detailed in Table 2. The concentration of Y-27632 incorporated was designed to replicate the concentration used previously, which is 10 mM (Koizumi et al., 2013).

2.6. Ocular delivery from applied formulations, in vitro

Freshly excised porcine eyeballs were placed, cornea uppermost, in a 6-well cell culture plate. Circular patches, 1 cm in diameter, were excised from the medicated (methylene blue or Y-27632) mucoadhesive films (n = 3) using a cork borer and applied to the corneas, which had been wetted beforehand with 300 μL PBS, and a combination of capillarity and film deformability ensured contact with the cornea. Eye drops of equal concentration were also applied to porcine eyeballs as a comparator (n = 3 for Y-27632, n = 2 for methylene blue). To simulate blinking and tearing, the eyes were periodically irrigated with 300 μL isotonic PBS. Y-27632 eye drops were used based on the dosing procedure reported by Koizumi et al. (2013), in which 50 μL of 10 mM Y-27632 was applied six times a day. To limit dehydration the eyeballs were placed in PBS, such that the solution reached half way up the eye (not contacting the cornea). The set-up was covered with a plastic lid to retain moisture and was incubated in a water bath at 37 °C overnight. The eyes were periodically bathed with aliquots of PBS in an effort to simulate tears (Chan et al., 2014).

After 24 h, films were carefully removed from the corneas using fine forceps and the corneal surface of eye-drop treated eyes were gently wiped with cotton buds. Corneas were then excised from the eyes and each was cut into small pieces before being comminuted to a fine powder using a mortar and pestle after being frozen in liquid nitrogen. The powders were carefully recovered and extracted into water after placing on a rotating blood tube mixer overnight. Tubes were then centrifuged at 5750 rpm for 30 min, the supernatant decanted and evaporated at 60 °C in an oven. The residue was reconstituted in 1 mL of deionised H_2O, prior to analysis by HPLC.

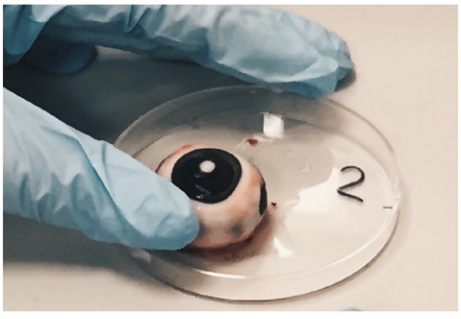

Fig. 3. Thaw forming in the middle of the eyeball, showing transcorneal freezing has taken place.

Table 2
Working formulae of methylene blue and ROCK inhibitor Y-27632 comparator eyedrops.

Eyedrops	Working formula	
Methylene blue	Methylene blue	87.7 mg
	DI H_2O	to 5 mL
ROCK inhibitor Y-27632	Y-27632	16 mg
	DI H_2O	to 5 mL

2.7. HPLC analysis of methylene blue and ROCK inhibitor Y-27632

The concentration of methylene blue and ROCK inhibitor Y-27632 released from each film and extracted from each cornea was quantified by reverse phase HPLC using an Agilent series 1100 HPLC system, fitted with a Phenomenex Kinetex 5μ C18 100 Å 150 × 4.6 mm column, eluted with a mobile phase comprised of H_2O:ACN (50:50 v/v) with 0.5% of TFA. The flow rate was 1 mL min^{-1}. For methylene blue the wavelength was 246 nm and the retention time 3.9 min; for Y-27632 the wavelength was 270 nm and retention time was 1.5 min. Calibration curves of methylene blue and ROCK inhibitor Y-27632 were constructed using standard solutions over the concentration range of 1–3.91 × 10^{-3} mM. The linearity of the curves was as follows: R^2 = 0.9999 for methylene blue; 0.9997 for Y-27632.

2.8. Statistical analysis

All data were analyzed using an InStat 3 statistical package (GraphPad Software Inc., San Diego, CA, U.S.A). Comparisons of concentration released from formulations into native and cryoprobe-treated corneas were determined using ANOVA (Analysis of variance) and Kruskal-Wallis post-tests, where $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Film preparation

Initially, a blank film (i.e. not loaded with any medication) was produced and was found to have good clarity, transparency and pliability, with the entire film weighing approximately 500 mg and having an average thickness of 0.1 mm as determined using a digital micrometer. Recovery (film mass/total mass of additives except water) was 98.3%. Representative images of the films loaded with methylene blue or ROCK inhibitor Y-27632 are shown in Fig. 4(a) and (b), respectively. The films appeared optically clear and were approximately 0.1 mm thick. Both films were pliable, which is important as they are to fit well to the curved cornea. Also, it was encouraging to find no evidence of drug crystals in the films at the levels added. Crystals would be potentially damaging to the cornea and would lead to unpredictable drug release rates.

Homogeneity and recovery were determined by sampling 5 × 1 cm diameter patches from across the film, dissolving in water and assaying for drug by HPLC – no statistically significant differences were found (not shown). The recovery of the methylene blue film was 111.8% while the recovery of the Y-27632 film was higher at 129.3%. Stability was determined by sampling the film stored at room temperature over an 8 week period and assaying, again with no statistically significant differences found. The recovery of the medicated films was higher than that of the blank film probably because the drugs attract a hydration shell, resulting in a higher water content. In the case of Y-27632, which was added as the hydrochloride salt, the positive charge would

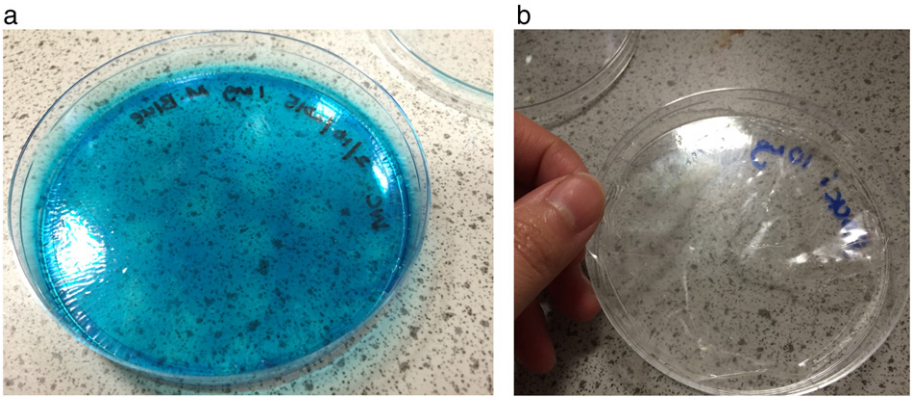

Fig. 4. (a) Methylene blue film. (b) ROCK inhibitor Y-27632 film.

attract even more water molecules. This analysis is supported by the fact that increasing the drying time did not significantly reduce the recovery.

The polymers hydroxyproplymethyl cellulose (HPMC), polyethylene glycol 400 (PEG 400) and Carbopol 917 (CP) were used in the film-forming excipients described here. The suitability of a HPMC film as a drug vehicle considered several factors, such as the mechanical properties, the swelling of films, and in vitro and in vivo bioadhesion evaluations (Peh and Wong, 1999). Compared to SCMC (sodium carboxymethyl cellulose) film, another polymer, HPMC films were shown to possess a greater bioadhesive strength value in vivo, and were reported to be tougher and more elastic. Consequently, we decided to use HPMC as our drug vehicle in this study. Moreover, PEG 400, a commonly used solubilizer which exists in liquid form, was included for its plasticizing properties, giving softness and pliability to the films to their fitting to the curved corneas. In previous experiments, PEG of a lower molecular weight (<300) had produced tough, brittle films that would not be suitable for ophthalmic applications (unpublished data). Our use of PEG 400 is supported by the work of Llabot et al. (2007) who revealed that its addition to films enhanced mucoadhesion as compared to the film without it. This can be explained by the diffusion theory of bioadhesion (Chickering and Mathiowitz, 1999) in which the intra-chain polymeric interaction is reduced in the presence of plasticizers, increasing the flexibility of the chains. This would result in strengthened mucoadhesive interactions by increasing the inter-penetration and entanglement of bioadhesive polymer chains with mucin. Carbopol is another polymer used to develop our polymeric film. It is a high molecular weight polymer containing a high content of carboxylic groups (Rowe et al., 2012), which is believed to contribute to the mucoadhesiveness of the film (Chickering and Mathiowitz, 1999). Carbopol plays an important role in increasing the softness, elasticity and bioadhesive strength of polymeric films (Lubrizol, 2015). Each of the polymers used is an approved excipient. In conclusion, the combination of HPMC, PEG 400 and CP provided homogeneous, stable, soft, pliable and mucoadhesive films, which are crucial properties of a viable product.

3.2. Diffusional release

Diffusional release is the process whereby drug molecules spontaneously migrate from the polymeric system to the exterior of the polymer and then into the release medium (Langer, 1990), which in our case was PBS to provide a basic model for tears. The diffusional release profiles of loaded drug characterize the amount of drug released from the film as a function of time into a given receiving medium. Low release is indicative of low drug loading or interaction and extensive retention of loaded drug within the film matrix; whilst this can reduce the dose delivered, it can also provide zero-order release. Zero-order kinetics is generally desirable in drug delivery systems, where drug levels released would remain constant throughout the delivery period (Ummadi et al., 2013). This is generally considered to be important in improving therapeutic outcomes and patient compliance and it also reduces the frequency of drug administration (Gokhale, 2014), which is discussed in Section 3.5. On the other hand, high release gives rise to 'burst' kinetics, and reflects excess drug added and/or weak retention within the polymer matrix – it is generally considered undesirable in drug delivery devices as it would not provide sustained release; furthermore excess drug is often present as crystals.

3.2.1. Methylene blue film

The purpose of producing a film containing methylene blue was to provide a model solute which was visible to the naked eye and allowed us to develop the film production technique – it is however, also used clinically as a photosensitizer (Tardivo et al., 2005). A diffusional release profile of methylene blue was constructed as cumulative mass and moles released, normalized per amount of film, hence, mass (mg drug mg^{-1} of film) and amount (μMol mg^{-1} of film) released are presented as a double-Y plot in Fig. 5; for further comparison purposes the percentage released (%) is also shown in Fig. 7 over the timescale of 6 h to reflect an overnight application. The intensity of the blue coloration of the receiving PBS solution visibly increased the longer the film was retained in the Eppendorf tube. From this observation it is deduced that an increased concentration of methylene blue diffused into PBS solution with increased contact time. This was verified following quantitative analysis by HPLC analysis as shown in Fig. 5, where the concentration of methylene blue released from film increased with increased immersion time. Fig. 5 shows an initial linear, or 0-order, relationship between cumulative amount and mass within the first 60 min. The rate constant, as determined from the plot gradient, was determined to be 0.0448 mg min^{-1}. The steady state phase was followed by a tailing off due to drug depletion. Methylene blue was detected in the first sample taken at 10 s as 0.22 μMol mg^{-1} (0.07 mg mg^{-1}) and at 6 h was 48.84 μMol mg^{-1} (15.62 mg mg^{-1}).

3.2.2. ROCK inhibitor Y-27632 film

Diffusional release profiles of ROCK inhibitor Y-27632 were constructed as cumulative mass (mg mg^{-1} of film) (Fig. 6a) and percentage released (%) (Fig. 6b). For further comparison purposes the percentage released (%) is also shown in Fig. 7. Drug was detected in the first samples taken at 10s (10 mg film; 1.94 mg mg^{-1}: 5.2 mg film; 1 mg mg^{-1}: 1.7 mg film; 0.99 mg mg^{-1}), with release increasing rapidly until it approaches equilibrium between 40 and 60 min (10 mg film; approximately 11.5 mg mg^{-1}: 5.2 mg film; 9 mg mg^{-1}: 1.7 mg film; 2.5 mg mg^{-1}). Thereafter, there was no major increase, and both amount and mass are proportionate to each other. Fig. 6 shows the cumulative concentration and mass of Y-27632 released over the 6 h test period. It is immediately apparent that the release profile for Y-27632 differed markedly to that of methylene blue, in that there was no

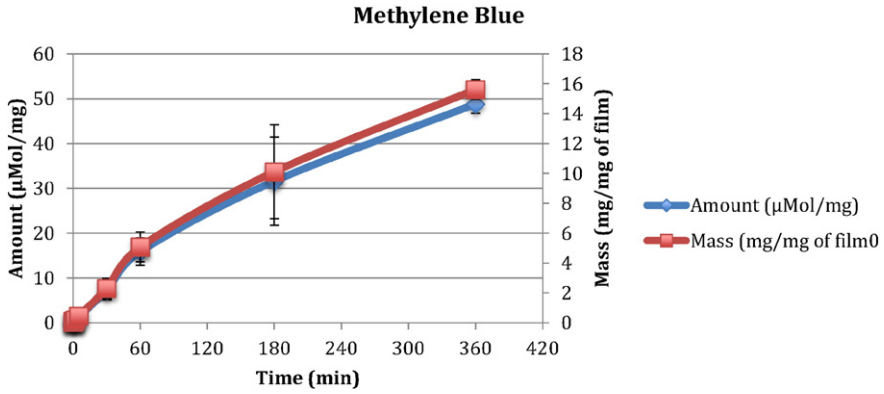

Fig. 5. Double Y-plot of methylene blue cumulative mass and μMol released over the timescale of 6 h, and normalized in terms of the mass of the film sample (n = 3 ± SD).

major steady state phase. Instead, there was a rapid rise or burst within the first 10 min.

The diffusional release of Y-27632 was replicated using three concentrations of Y-27632 films; 10 mg, 5.2 mg and 1.7 mg. The difference in concentrations has resulted in the difference in mass of Y-27632 released (Fig. 6a), so that the higher the concentration of Y-27632 within the film, the higher the mass of drug released. Interestingly, the difference between the 10 mg film and the 5.2 mg film was not large (the difference in mass was mostly kept within 2 mg mg^{-1}). However, the 10 mg film showed a clearer burst release. Mass released from the 1.7 mg film was much lower than the others where the highest mass released was only 2.8 mg mg^{-1}, but the overall release pattern was similar to both 5 mg and 10 mg films. Though concentrations varied, the percentage released was fairly similar (Fig. 6b). All three concentrations

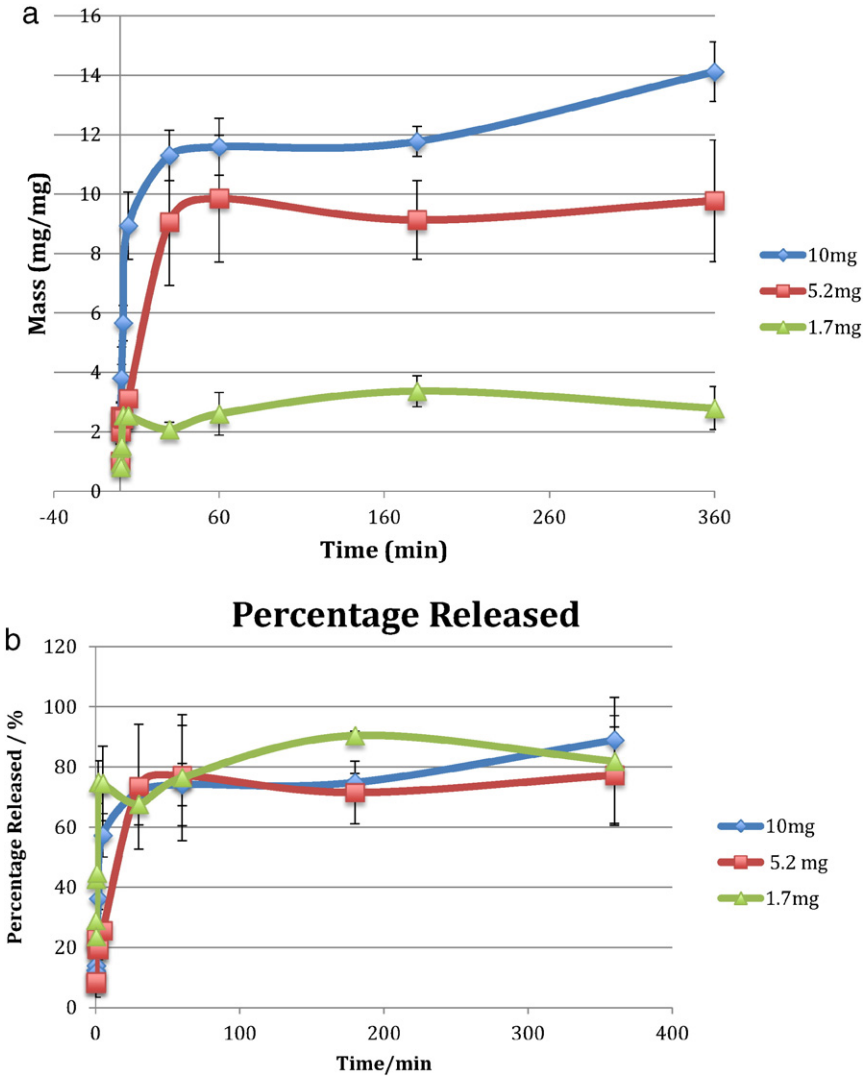

Fig. 6. (a) Cumulative drug release from thin films loaded with 1.7, 5.2 and 10 mg ROCK inhibitor Y-27632 over 6 h and normalized in terms of the mass of the film sample (n = 3 ± SD); (b) percentage of drug released from thin films loaded with 1.7, 5.2 and 10 mg ROCK inhibitor Y-27632 over 6 h (n = 3 ± SD).

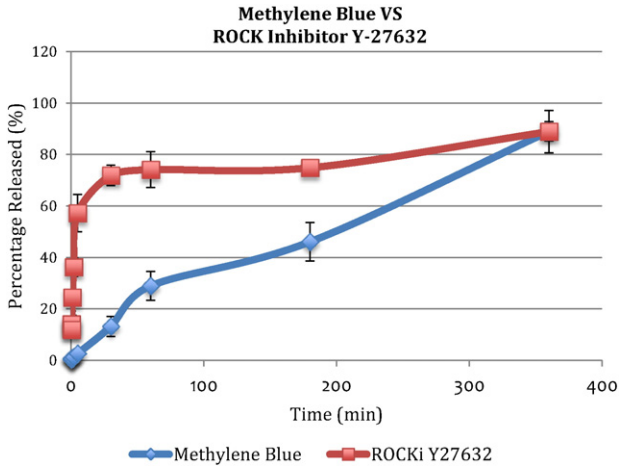

Fig. 7. Diffusional release profiles of methylene blue versus ROCK inhibitor Y-27632 in percentage released (%) (n = 2 or 3 ± SD).

showed rapid release up to approximately 70% within 30 min with a maximum release ≥80%. As the release kinetics were greatest from the 10 mg film, this was chosen to progress to the in vitro ocular delivery experiment.

3.3. Comparison of methylene blue and ROCK inhibitor Y-27632 release

Fig. 7 shows that both methylene blue and ROCK inhibitor Y-27632 were released from respective films up to approximately 90% after 6 h - the pathways appear very different, although further work will be needed to confirm this. High release indicates low retention of drug in the polymeric films, which is due to swelling and would be cost effective by reducing wastage of drugs to a minimal level. Y-27632 was found to be more weakly retained within the polymeric film than methylene blue, meaning it is more easily released, allowing more Y-27632 to penetrate the cornea; hence, a burst release from Y-27632 was observed. It is worth noting that the release of Y-27632 depleted after 40 min (approximately 80% has been released), which could have the potential benefit of getting more drugs into the eyes rapidly. While methylene blue displayed zero-order release kinetics over the 6 h timescale, Y-27632 showed mixed-order release, reaching equilibrium within 50 min. It is deduced that this is due to the lack of interaction of Y-27632 with the film polymers. It is assumed that methylene blue is able to form stronger bonds with the polymers (Chickering and Mathiowitz, 1999) and that this results in more entrapment in the polymeric film. It is also hypothesized that the burst release of Y-27632 was facilitated by its low molecular size and the presence of the charge, which the recovery data (Section 3.1) showed attracted more water. This, in turn, accelerated release of the hydrated complex into the aqueous receiving medium. It should also be borne in mind that such rapid release may be a concern if it gives rise to toxic levels in vivo (Shively et al., 1995; Jeong et al., 2000).

On a side note, methylene blue is used clinically as a visual guide during surgery or endoscopy (Tardivo et al., 2005), and is also a therapeutic agent for various conditions, including ifoasfamide-induced encephalopathy (Patel, 2006) and methemoglobinemia (Sikka et al., 2011) - the current delivery data of methylene blue using the thin film has potential relevance in such procedures.

3.4. Transcorneal freezing

As mentioned, transcorneal freezing achieved by touching the corneal surface with a cold probe can cause corneal endothelial cell death, either by forming intracellular ice that is lethal to the cell or by the formation of extracellular ice, which will create an osmotic imbalance leading to an increase in intracellular electrolytes and the collapse of cellular membranes. It was previously reported that corneal endothelial cells are expected to proliferate at the frozen wound sites after cryotherapy (Buco et al., 1978; Staatz and Van Horn, 1980), and potentially the application of Y-27632 eye drops enhances this recovery (Koizumi et al., 2013). Fig. 8 shows a picture taken upon the excision of a cryoprobe-treated cornea to which a methylene blue film had been applied. From this observation it was evident that penetration of methylene blue occurred predominantly at the centre of the cornea where transcorneal freezing took place.

3.5. Corneal penetration

As the corneal endothelium is the target site for Y-27632 following its surface application, it was important to ensure that the drug is penetrating towards the back of cornea to reach endothelial tissue. Since Y-27632 is colourless, methylene blue film was used as a model. Fig. 9 shows a schematic diagram of the cornea throughout its full thickness alongside a lateral view of the cornea from a cryoprobe-treated eyeball under a microscope. The endothelium is stained blue, depicting that drug is penetrating well through the cornea from the epithelial surface where the patch had been positioned on eye.

3.6. Ocular drug delivery

Koizumi et al. (2013) reported some patients with FECD who were successfully treated with ROCK inhibitor Y-27632 eye drops subsequent to transcorneal freezing to remove diseased and damaged corneal endothelial cells. While the reported results are encouraging, the eye drop application was repeated six times a day for 7 days. Eye drops have long been used to treat ocular pathology and injury, however, it is widely appreciated that upon application an eye drop dose is rapidly eliminated by dilution and washing out by tears and nasolacrimal drainage mechanisms. More often than not the instilled dose that enters ocular tissue is less than 1% (Morrison, 2015). Furthermore, ROCK inhibition is known to have a hypotensive effect and vascular resistance, which would result in potential adverse effects on unwanted systemic exposure (Hahmann and Schroeter, 2010). Another reported adverse effect of ROCK inhibitor eye drop application is ocular hyperemia because ROCK inhibitors are vasodilators, and this has been evident in ongoing clinical trials (Tanihara et al., 2013; Zhang et al., 2012). Drug wasted via eye drop delivery also represents sub-optimal drug therapy for the patient. Hence, the potential benefit of the development of a mucoadhesive film that is capable of sustained delivery of higher doses, specifically targeting the corneal endothelium.

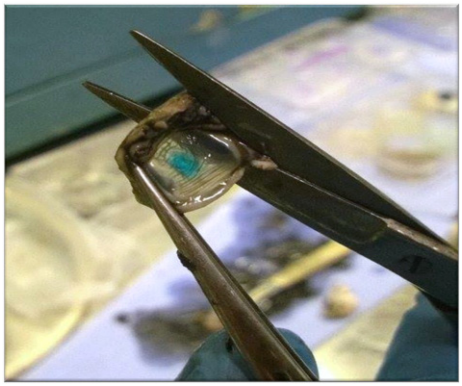

Fig. 8. Delivery of methylene blue to a cryoprobe-treated porcine cornea.

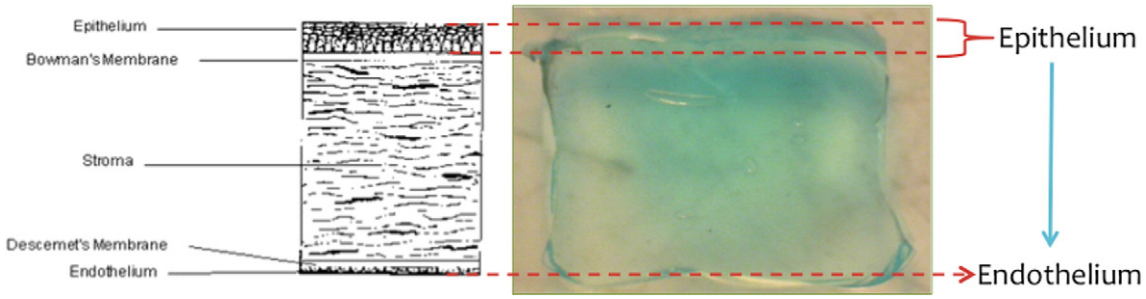

Fig. 9. Schematic diagram of the cornea layers and a microscopy lateral view of the cornea from a cryoprobe-treated porcine eyeball.

3.7. Run off and wash away

Run off of the dose applied as eyedrops is a well-known aspect of this mode of delivery and was vividly depicted in this work involving methylene blue (Fig. 10). In the experimental setup used, it was a challenge to accurately mimic the washing out action of tears and blinking. However, during the experiment the porcine eyeballs were periodically irrigated with 300 μL PBS in an effort to crudely simulate washing out. This revealed that methylene blue was visibly washed away on wetting as the intensity of the blueness decreased (as shown in Fig. 10). Methylene blue in films, on the other hand, remained and the drug did not drain away appreciably even after irrigation. This indicates a major advantage delivery via mucoadhesive films over eyedrops, whereby drug run off and washing away by tears are reduced, with the drug being retained at point of application.

3.8. Delivery of methylene blue to the cornea

The delivery of methylene blue from film and eye drops of equal concentrations to untreated in vitro porcine corneas and cryoprobe-treated porcine corneas (n = 2 or 3; ±SD) is shown in Fig. 11. It can be seen that methylene blue delivered from the thin film (0.072 mMol in untreated cornea and 0.081 mMol in cryoprobe-treated cornea) was significantly greater than the amount delivered from liquid eye drops (0.046 mMol in untreated cornea and 0.066 mMol in cryoprobe-treated cornea) for both untreated ($p < 0.01$) and cryoprobe-treated corneas ($p < 0.05$). The film delivered significantly more methylene blue to the cryoprobe-treated corneas as compared to native corneas ($p < 0.05$) and this was even more apparent for the eye drop-delivered agent ($p < 0.0001$). This is probably a consequence of the cryoprobe-treated corneas being more "leaky" owing to a modulation or disruption the cell and matrix components of the cornea cause by tissue freezing, which allows more drug to penetrate.

3.9. Delivery of ROCK inhibitor Y-27632 to the cornea

The corneal delivery of ROCK inhibitor Y-27632 from films and eye drops of equal concentration into native unfrozen porcine corneas and cryoprobe-treated porcine corneas (n = 2 or 3 ± SD) are shown in Fig. 12. It can be seen that for cryoprobe-treated corneas, the rapid diffusional release (Section 3.2.2) was manifested in the delivery of almost 3 times more Y-27632 (0.52 mMol) when compared to delivery via the equivalent eye drop solution (0.177 mMol) ($p < 0.01$). For native corneas, Y-27632 delivered from the thin film was greater than from liquid eye drops, although this was not statistically significant ($p > 0.05$). The films delivered significantly more Y-27632 to the cryoprobe-treated corneas (0.52 mMol) as compared to native corneas (0.23 mMol) ($p < 0.01$). The eye drops appeared to deliver more Y-27632 across the cryoprobe-treated corneas, although this was not statistically significant ($p > 0.05$). Prior to the development of a 10 mM Y-27632 film, a lower concentration Y-27632 film was produced (containing 10 mg of Y-27632). The delivery onto the cryoprobe-treated corneas of this was much lower (0.083 mMol) due to the lower concentration of the film (n = 3 ± SD).

Y-27632 has been reported to have no direct toxicity or significant effects on cell proliferation (Honjo et al., 2007), and a protective effect was observed for mouse motoneurons in a cytotoxicity assay following Y-27632 treatment (Günther et al., 2014). A clinical study involving another ROCK inhibitor, SNJ-1656 proved that it is a safe topical agent and effective in reducing intraocular pressure in the eyes of human volunteers (Tanihara et al., 2008).

4. Conclusions

This study has compared drug delivery to the cornea achieved via eye drops or release from mucoadhesive thin films. Initially, methylene blue as a model agent, followed by 10 mMol of selective ROCK inhibitor Y-27632, which is a drug used to treat vision loss caused by corneal endothelial dysfunction as would occur in FECD and/or bullous

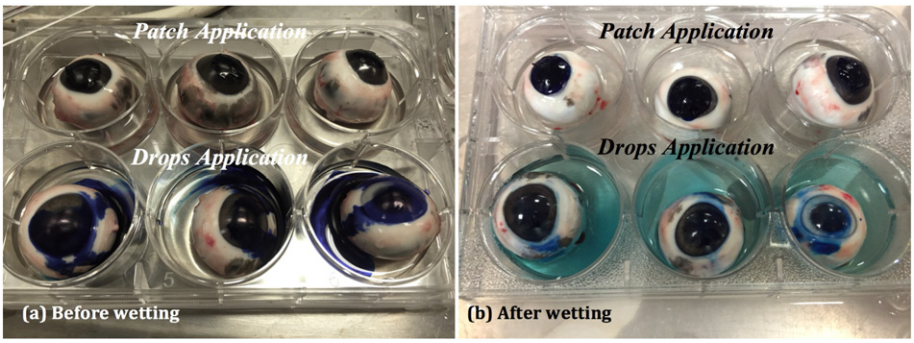

Fig. 10. Porcine eyeballs treated with film patch (upper) or equivalent 'eyedrop' solution (lower) immediately after application (left). Lower left clearly shows run off of significant amounts of MB immediately following application. Lower right shows that upon irrigation further MB is washed away from the eyeball dosed with eyedrops. In contrast, upper right shows no detectable wash out of MB from applied films following irrigation (3 determinations).

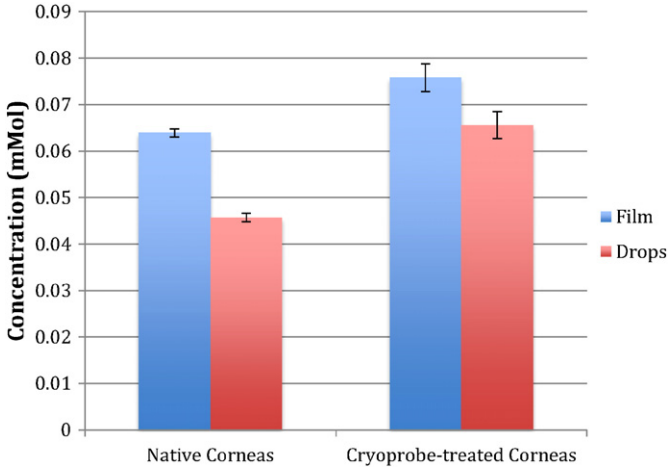

Fig. 11. The corneal delivery of methylene blue from film and 'eye drops' of equal concentration across native porcine corneas and cryoprobe-treated porcine corneas (n = 2 or 3 ± SD).

keratopathy. The major conclusions are: (1) drug delivery to the cornea via thin mucoadhesive films is a superior alternative to eye drop delivery because (at equal concentrations) significantly higher concentrations of Y-27632 were observed in the cornea after delivery from thin films compared to delivery via eye drops. This suggests the delivery of ROCK inhibitor from thin mucoadhesive films may produce better clinical results compared to eye drops. (2) thin mucoadhesive films have advantages over eye drops in that dose is well retained at point of application on thin film application, whereas eye drops are easily washed away by tearing and blinking of eyes. (3) thin films result in a 3-times higher delivery of Y-27632 over and above that achieved by eye drop delivery on cryoprobed-treated corneas. This suggests that targeted delivery of Y-27632 to the cryoprobe-treated, freeze-damaged tissue at the front of the cornea allows the ROCK inhibitor to penetrate more readily into and across cornea. Thus, a combination of transcorneal freezing and topical application of ROCK inhibitor via thin films is a potential non-invasive approach to deliver relatively higher concentrations of drugs into the corneal endothelium. In summary, findings from this study support the further development of mucoadhesive thin films as a modality for the targeted delivery of ROCK inhibitors to the cornea (with or without transcorneal freezing) to treat corneal endothelial dysfunction. The approach also has wider potential in the use of thin mucoadhesive films as an alternative to eye drop application in other scenarios, and could possibly apply to other ocular diseases by incorporating other disease-target drugs.

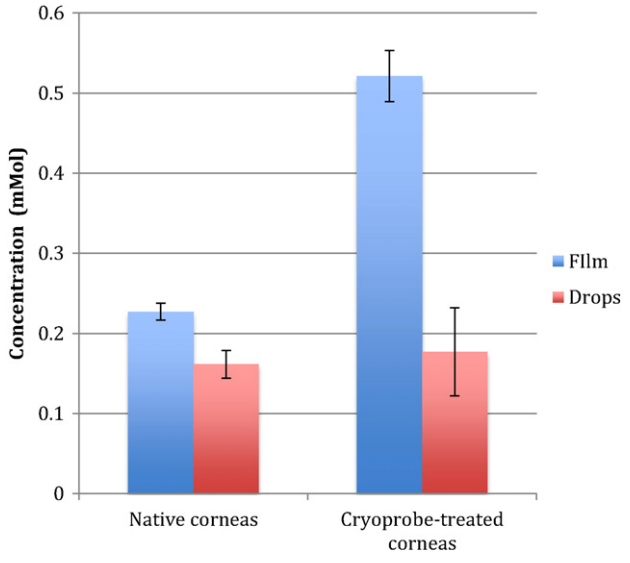

Fig. 12. The ocular delivery of ROCK inhibitor Y-27632 from film and eyedrops of equal concentration across native porcine corneas and cryoprobe-treated porcine corneas (n = 3 ± SD).

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