POLYDIMETHYLSILOXANE RELEASING MATRIX METALLOPROTEASE INHIBITORS AS MODEL INTRAOCULAR LENS MATERIALS FOR MITIGATING POSTERIOR CAPSULE OPACIFICATION

By

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Abstract

Improved materials for implantation as intraocular lens (IOL) devices are needed to minimize the occurrence of posterior capsule opacification (PCO). In this work, novel polydimethylsiloxane (PDMS) loaded with matrix metalloprotease inhibitors (MMPI) were developed as model IOL materials.

PDMS was chosen as silicones are currently used successfully as IOLs. Inhibitor release rates and amount of initial burst of drug-loaded PDMS could be controlled by changing solvent when loading into elastomer base, as well as drug loading method, and release buffer.

Two lens epithelial cell lines were characterized for *in vitro* tests: FHL124 and HLE B3. These cell lines produce different combinations of extracellular matrix proteins when grown on various biomaterial surfaces. Significant differences between the two cell lines were observed both in collagen I/III and α-smooth muscle actin levels, both when cells were unstimulated, and as a result of epithelial to mesenchymal transition induced by treatment with transforming growth factor β2. FHL124 cells were selected in further tests due to their consistent expression of extracellular matrix components when exposed to different materials.

Solutions of synthetic MMPI GM6001 and MMP 2/9 Inhibitor II, known to mitigate anterior subcataract formation, were released from PDMS and found to protect in a modest but significant way against protein profile changes and to delay migration.

Due to the Zn^{2+} dependence of MMPs, chelators, including EDTA, TPEN and 1-10 phenanthroline were examined as alternative inhibitors. Only the latter was found to have a significant effect on cell migration rates *in vitro*. Sulfadiazine, due to its chemical resemblance to synthetic MMPI was determined to be the most efficient at reducing migration rates as well as to have the lowest toxicity.

Overall, sulfadiazine was determined in this work to be a potentially effective solution to mitigating PCO. These results indicate that releasing MMPI molecules from PDMS as a model IOL is a promising way to mitigate aspects of PCO.

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To Izabela and Andy

Just follow your dreams

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List of Abbreviations

ACO	Anterior Capsule Opacification
ASC	Anterior Subcapsular Cataract
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycan
HCEC	Human Corneal Epithelial Cells
HCSF	Human Corneal Stromal Fibroblasts
IOL	Intraocular Lens
IPA	Isopropanol alcohol
LEC	Lens Epithelial Cell
MMP	Matrix Metalloprotease
MMPI	Matrix Metalloprotease Inhibitor
Nd:Yag	Neodymium-doped yttrium aluminum garnet laser
PCO	Posterior Capsule Opacification
PDMS	Polydimethylsiloxane
PMMA	Polymethylmethacrylate
RPE	Retinal Pigment Epithelial cells
α-SMA	Alpha Smooth Muscle Actin
TCPS	Tissue Culture Polystyrene
TGF	Transforming Growth Factor
THF	Tetrahydrofurane
TIMP	Tissue Inhibitor of Matrix Metalloprotease
TPEN	N,N,N',N'-Tetrakis-(2-pyridylmethyl)-ethylenediamine
ZO-1	Zonula Occludens-1

1.0 LITERATURE REVIEW AND SCOPE OF PROJECT

1.1 Background

Cataracts remain the leading cause of treatable blindness in the world [1]. Up to 20% of the cases of blindness and low vision in Canada are also attributable to this condition [2]. Since cataract occurrence increases with age, these numbers are expected to increase [3]. Cataracts are easily treated by replacing the diseased natural lens with an artificial intraocular lens implant (IOL). Cataract surgery is one of the safest and most performed surgical procedures in the world, with more tha one million surgeries performed in Canada alone, annually [1].

However, this common surgical procedure is not without complications. The most common complication is denoted as secondary cataracts, or more specifically posterior capsule opacification (PCO), with a global occurrence of 28% of patients within the first 4 years of surgery [4], with an even higher incidence of 35-78% within the first 3 to 6 years in paediatric patients [5-7]. While secondary cataracts are easily treatable by neodymium-doped yttrium aluminum garnet (Nd: YAG) laser therapy in developed countries, this treatment still poses a major financial burden on the health system [4,8]. In developing countries, treatments for secondary cataracts are simply not accessible. Nd:YAG laser therapy also has complications of its own, with retinal detachment and cystoid macular oedema being the most common, occurring in 2% and 10% of patients,

respectively [9]. Furthermore, some IOL materials can be damaged during the laser procedure [10]. Complications following laser treatment are especially serious in paediatric patients [4,6].

Improvements in surgical procedure and IOL design have reduced the incidence of side-effects remarkably in the last decade [4,8]; however the large numbers of patients that require secondary interventions make it desirable to develop better devices. There is a need for a device capable of reducing the rate of secondary cataracts and minimizing the necessity for secondary interventions such as laser treatments, or ongoing drug therapy. While a drug-releasing IOL material may be a good solution, delivering drugs directly into the lens capsule [11], there are no such devices yet approved for use to prevent secondary cataracts.

1.2 The Natural Lens

The biconvex, transparent crystalline lens shares the role of refracting light and focusing it to the retina, with the cornea. It is comprised of a capsular bag, lined on the anterior surface with lens epithelial cells (LECs), and a nucleus made of fiber cells. The avascular lens receives its nutrients from the aqueous and vitreous humor by diffusion. The capsular bag is one of the thickest basement membranes in the body. It is composed primarily of collagen IV, but is coated on the inside mainly with laminin produced by LECs [12-14]. The capsule is attached to the ciliary muscles by zonular fibers. This allows for accommodation by changing the curvature of the lens.

In the normal lens, a subset of LECs has the potential to divide, transform into fiber cells and migrate, at low rates [14-16]. The single row of cuboidal LECs lining the anterior capsule (A-cells) has a role in maintaining homeostasis in the lens [14,15]. In adult lenses, this anterior layer of LECs has minimal mitotic activity, while the cells in the equatorial region (E-cells) can divide and are responsible for fiber cell production throughout their lifetime [17,18] (see Figure 1-1). Fiber cells, arranged in symmetrical layers at the center of the lens, do not have nuclei or organelles and are mainly filled with soluble crystallin proteins. This creates the nucleus or cortex, responsible for light transmittance and focusing to the retina [14,19].

LECs can undergo a phenotypical change known as epithelial to mesenchymal transition (EMT), [21-23], a process that occurs naturally in the normal aging lens, at very low rates [24]. Low levels of growth factors, particularly transforming growth factors beta (TGFβ) and basic fibroblast growth factors (bFGF), are normally present in the lens and in the aqueous and vitreous humor, which bathe the lens, to perform regular maintenance functions including cell growth and differentiation [25-27]. As the lens ages, these cytokines gradually accumulate due to the low permeability of the lens capsule, thus accelerating the EMT process [12,24].

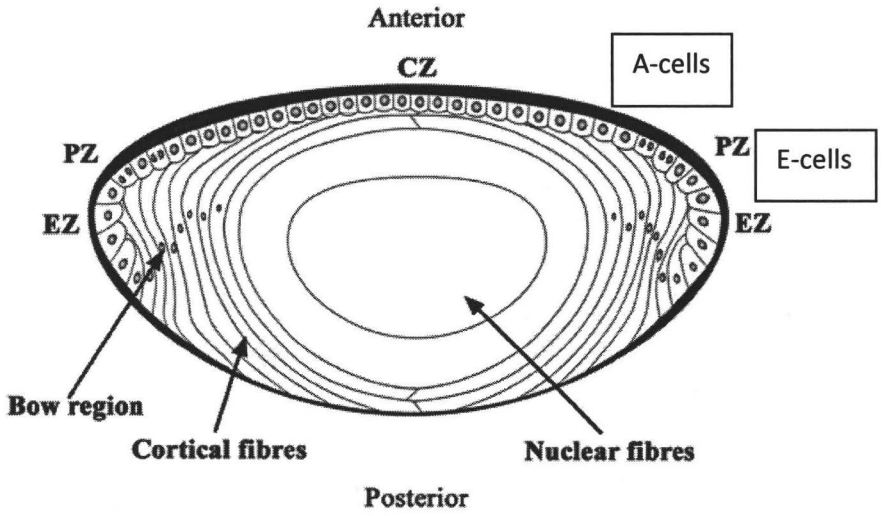

Figure 1-1. Human lens structure [20]. CZ – central optical zone; PZ – pre-equatorial zone; EZ – equatorial zone. Adapted with permission, © 2005 Master Publishing Group

1.3 Cataract Formation

Cataracts are broadly defined as any opacity or loss of transparency of the lens. There are several types of cataracts. Nuclear and cortical cataracts occur when the crystallin proteins in the lens cortex aggregate, creating a decrease in lens transparency. Another type, the subcapsular cataracts, occurs due to changes in epithelial lens cells lining the lens capsule. Cataracts can be caused by a range of factors and medical conditions such as diabetes, smoking, steroids and nitric oxide, as well as environmental factors, particularly UV exposure or genetic predispositions [28-30]. However, the most common cause of cataracts is aging, and the incidence increases with population age [3,30].

1.4 Cataract Surgery

Currently, phacoemulsification is the most common type of cataract surgery, particularly in developed nations [31]. In this procedure, a circular incision commonly reffered to as continuous curvilinear capsulorhexis, is followed by breaking down of the natural lens using ultrasound and aspiration of the lens fragments and a large proportion of lens epithelial cells lining the capsular bag. This is followed by insertion of an IOL into the capsular bag which allows for the retention of most of the lens capsule. This type of incision is done on the anterior side of the capsule, usually by removing a circular piece of the anterior capsule, in a continuous movement, in order to allow shear and stress forces to be distributed evenly on the remaining capsule, to avoid tearing [32,33].

Since the 1970s, cataract surgery has involved not only removal of the clouded natural lens, but also insertion of an artificial IOL. With rigid one piece IOLs, the incision is approximately 12mm in diameter. Availability of foldable IOLs has reduced the incision size to approximately 3.2mm [32].

Updates to surgical technique are being developed, mainly in the interest of increasing the availability of treatments in developing countries, which need to be both cost effective and with minimal side effects, due to lack of post-operative care [32,34,35]. Skill of the surgeon and surgical technique have been linked to several side effects [31,32,36], including secondary cataracts [32,37,38].

1.5 Complications of Cataract Surgery

As with any surgical procedure, complications occur in a small percentage of patients. With cataract surgery, major complications include endophthalmitis [36,39], suprachoroidal haemorrhage [40], corneal oedema and bullous keratopathy [41], vitreous and retinal detachment [42]. However, each of these occurs in less than 1% of patients. Most major complications can be avoided with improved surgical technique and aseptic method [39]. Furthermore, changes to the intraocular lens material have also been used for this purpose [43,44]. Minor complications such as off-centre lens placement, retinal tear, glaucoma, macular edema [36], anterior capsule contraction and secondary cataracts [45] also occur. However, the incidence of secondary cataracts is significantly greater than that of any other complication, occurring in approximately 28% of patients within 4 years and in an even higher percentage of patients younger than 40 years of age [4,46].

Secondary cataracts are defined as opacifications within the axis of view, and were shown to be created by cells migrating and laying extracellular matrix (ECM) on the posterior capsule, together with capsular wrinkling. Histology studies have shown that LECs, fibroblasts, macrophages and iris-derived pigment cells can contribute to the formation of secondary cataracts, although LECs are believed to be the major contributors primarily due to their location, and known ability to transform and become motile in response to stress [22,47,48]. Both anterior capsule opacification (ACO) and posterior capsule opacification (PCO) can occur, as a result of stress-induces changes in A-cells and E-cells, respectively (see Figure 1-1). PCO is the most common and most severe complication.

1.5.1 Anterior Capsule Opacification

ACO usually appears within several months after cataract surgery [45,49,50]. Remnant LECs create fibrotic lesions at the margin of the capsulorhexis. While these lesions cannot directly interfere with vision, they still pose a risk, especially for diagnosis of retinal diseases, as they obstruct inspection of the retina [51,52]. Remnant LECs are mainly A-cells, attached to the anterior capsule. Following the surgery, they divide, transform and migrate, in an effort to re-establish the initial monolayer [22,38] (see Figure 1-2). The number of remaining LECs on the anterior side of the capsule is proportional with the size of the capsulorhexis [53]. Smaller incisions are common with foldable IOLs. It has been established that the preservation of healthy A-cells is necessary to create a strong capsular bend, as these cells are needed for the wound healing response. Thus smaller size incisions are preferable [34,37,38,54]. However, this subset of LECs also participates in the development of secondary cataracts, as polishing the cells off the anterior capsule at the time of surgery has been linked with lower percentages of both ACO and PCO [53].

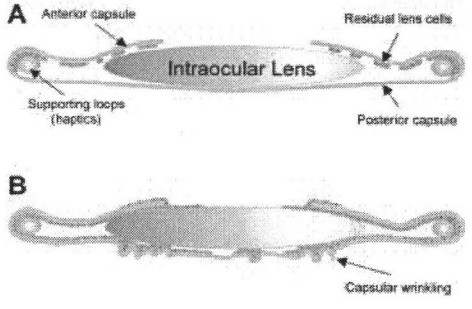

Figure 1-2. Lens cell migration to the posterior after IOL implantation. Reproduced with permission from [23], © 2002 Elsevier Science Ltd.

1.5.2 Posterior Capsule Opacification

Equatorial LECs are considered the main contributor to PCO development. In the literature, PCO is divided in two categories, regenerative, and fibrotic. In regenerative PCO, E-cells continue to actively divide after cataract surgery, creating an organized multi-layered lesion between the capsule and the IOL, denoted as Soemmering's ring [55,56]. This ring is considered to be the response of LECs to the lack of nucleus, in an effort to regenerate the cortex [33,38]. Soemmering's ring continues to grow to fill the space between the capsule and the IOL, for periods of up to two years [38,56]. Soemmering's ring is located around the IOL and outside the axis of view. However, it can cause visual impairment in the form of Elschnig's pearls which occur when small pieces of the Soemmering's ring break off and settle in the line of vision [13,57]. The pearls are unstable globular masses of cells, connected by gap junctions. Since these masses of cells can change shape, size and position in less than 24 hours, it seems that they are not attached to the capsule or the IOL [57,58]. However, Elschnig's pearls can increase in size and number over the years, which can result in the need for secondary interventions [59].

In fibrotic PCO, the most common type of PCO, the wound healing response induced by the stress of the cataract surgery is thought to initiate a series of events that leads to EMT in LECs. In particular, TGFβ2, activated from latent form, and newly released by the lens cells following mechanical stress of cataract removal and IOL implantation [16,17,47,60], has been associated with EMT. Other cytokines, including epidermal growth factor (EGF) and bFGF-2, have also been associated with the EMT

process, as enhancers of the effect that TGFβ2 has on LECs [61,62]. As a result of these cellular changes, LECs become myofibroblasts and display different properties, including increased migration and production of extracellular matrix components. The motile LECs can reach the posterior capsule within 7-11 days after IOL implantation [63-65]. E-cells divide, migrate and deposit excessive aberrant ECM on the posterior capsule (see Figure 1-2). This process, also denoted fibrous metaplasia, can block the light from reaching the retina [17,26,66]. As with Elschnig's pearls, vision is affected only when opacities are located on the axis of view, leading to the need for secondary interventions, typically in the form of Nd:YAG laser treatment [23,46].

The high incidence of PCO and the related high cost of Nd: YAG laser treatment can make the procedure unaffordable, particularly in developing countries. In developed nations, the need for secondary treatment represents an additional burden on the health care system [4]. The cost of equipment together with lack of follow-up facilities in developing countries, especially in rural areas, allow PCO incidence to render cataract surgery unsuccessful. Furthermore, where available, Nd: YAG treatment has its own serious complications, and may be needed more than once, since opacities can return [4]. The most serious side-effects are in paediatric patients, mainly due to the higher rate of LEC proliferation in children [4]; these can include but are not limited to: higher incidence of postoperative inflammation, glaucoma, retinal detachment and most importantly amblyopia [6,67]. Therefore, there is a need to mitigate PCO in an efficient and cost-effective manner.

1.6 Lens Epithelial Cells and PCO

Three main stages have been identified in lens cells resulting in PCO: cell division, migration and transformation. While the order of these events remains unclear, it seems that migration occurs prior to cell division [60,68]. However, the migration and transformation events appear to be tightly interconnected, potentially making it impossible to elucidate a clear temporal difference between these events [69-71].

1.6.1. Cytokines Involved in PCO

The EMT process is triggered by cytokine binding to LEC receptors. Cytokines are provided to the lens cells in a paracrine manner through the vitreous humor and the ciliary body, as well as through autocrine production by the LECs themselves [72,73]. Many of these cytokines exist in the lens in latent form. A balanced combination is necessary for the normal function and development of the lens. However, high concentrations can lead to unwanted growth and differentiation. In normal lenses, A-cells produce low amounts of TGFβ2, while E-cells produce both TGFβ1 and TGFβ2. EMT is triggered by elevated concentrations of growth factors, most notably TGFβ2 [23,60] as wells as TGFβ3 [23,73].

Basic FGF-2 and TGFβ2 work together to enhance cells survival, ECM production and cell multi-layering [74]. Epidermal growth factor (EGF) has been shown to be involved in increasing MMP-2 activity and cell migration, through cellular pathways, similar to those used by TGFβ2 [61], but is not involved in trans-differentiation or cell survival of LECs [13,74]. Other cytokines have been detected in

the normal lens and post-cataract surgery; however, none has been detected to be directly involved in either the EMT or migration events. Because TGFβ2 has been shown to be directly implicated in both LEC trans-differentiation and migration, it has been used to induce EMT like changes in *in vitro* models of secondary cataracts, both in cell culture and in capsular explants [16,26,72,74].

1.6.2 Epithelial Markers

Following EMT, specific proteins that maintain the epithelial phenotype are down-regulated, not longer produced or destroyed. Epithelial cells are characterized by an organized cuboidal appearance, maintained by cell-cell connections. In the lens, cell-cell connections are mostly governed by E-Cadherin, Connexin-43 and Zonula Occludens-1 (ZO-1) [75-77]. E-Cadherin plays a role in cytoskeletal organization, by being connected via catenins to actin fibers in the cytoplasm [16]. As well, epithelial cells express specific ECM proteins. The ECM is intimately interconnected with the cells, creating a three dimensional environment that acts not only as structural support, but just as importantly as a messenger highway and storage system. Cells are anchored in the surrounding ECM by integrins, membrane proteins that bind to specific ECM protein motifs. Each cell type has a specific complex of integrins, and thus flourishes in a specific ECM environment. Research has determined that LECs in the normal lens capsule lay on a bed of collagen IV, covered by a layer of laminin, the most abundant non-collagenous ECM component in the lens capsule [78]. These complex interactions dissolve as result of EMT, via several pathways.

Matrix metalloproteases (MMPs) are endopeptidases capable of degrading major ECM components, but also capable of cleaving other substrates and activating ECM bound cytokines [79]. In the normal lens, MMPs are tightly regulated both by the presence of tissue inhibitors of metalloproteases (TIMPs) and by expression in a precursor form that must be cleaved in order to become activated. Inactivated signaling molecules and MMPs are present within the ECM, becoming activated in an appropriate environment. While the MMPs belong to a large family of zinc-dependent proteins expressed throughout the body, specific MMPs and their natural inhibitors have been shown to be expressed in the lens tissue, at different stages of development and in disease [80,81]. While their specific role in EMT has yet to be elucidated, at the molecular level, in particular, activation of the matrix metalloproteinases MMP-2 and MMP-9 in the lens has been observed as a result of elevated TGFβ levels [26,82] and therefore these molecules have been implicated in EMT and PCO.

Both MMP-9 and MMP-2 can cleave structural proteins of the ECM and components of basement membranes, as well as other substrates. Specifically, MMP-9 has been shown to cleave collagen IV, V and elastin, while MMP-2 cleaves collagen VII, X, XI, laminin-5 as well as fibronectin [77,79]. Besides this very important function of breaking down ECM to allow for cell migration, MMP-9 has also been shown to be able to proteolytically activate TGFβ2 [83]. Recent studies suggest that MMP-9 might have a role in MMP-2 activation and production in TGFβ2 activated rat capsules and human LECs in culture [84]. MMP-2 is also responsible for cleaving pro-MMP-2, and thus feeds back to increase the concentration of active MMP-2 [79]. As well, MMP-2 has been

shown to cleave FGF receptors [85], and to be responsible for the activation of MMP-1 and MMP-13 [79]. Cleavage of the extracellular fragment of E-Cadherin is associated with EMT, presumably as a result of MMP-7 and/or MMP-3 activity (shown for other systems, such as prostate and kidneys) [86]. Other MMPs are active in the lens as well, in what is believed to be an intricate cascade of pathways leading to ECM restructure and loss of cell-cell connections [81]. However it is clear from the body of existing research that MMP-2 and MMP-9 play the most pivotal roles in EMT and therefore in PCO [26,60,80-82,84].

1.6.3 Mesenchymal Markers

Mesenchymal markers have been shown to increase following EMT. Of particular interest are fibronectin [16], tenascin [16], collagen I, the primary component of the aberrant PCO ECM [66,87], α-SMA (internal cytoskeleton protein) [16,88] and RhoA (intracellular GTP-binding protein, activated prior to migration) [89]. Fibronectin is an ECM protein that is the natural ligand of the integrin β1 [90]. It has been demonstrated *in vitro* that adhesion of LECs to fibronectin is increased following stimulation with TGFβ2 [70]. Furthermore, increased expression of the integrin β1is necessary for migration in lens cells that have undergone TGFβ2 induced EMT [70,90]. As well, deposition of both fibronectin and collagen I in PCO are associated with the white opacities that block light passage to the retina [91]. The expression of α-SMA is a common hallmark of the EMT process and the myofibroblast phenotype, in all cell types where EMT has been studied [75,84,92,93] although its role in PCO is not clearly

defined. α-SMA has a role in collagen fibril contraction [94] but has been suggested as another factor which may be responsible for capsular wrinkling that occurs in PCO [88]. Thus its role in PCO is not clearly defined.

Cell migration is another characteristic of a trans-differentiated lens cell. The mechanism of cell migration is not yet completely established but it has been demonstrated *in vitro* in human capsule experiments that LECs migrate as a sheet [68]. The RhoA member of the Rho family of GTPases (family of enzymes that can bind and hydrolyze GTP) has been shown to be involved in migration of several cell types, including in lens, in TGFβ induced EMT [89]. RhoA is also a known marker of cell sheet migration, being expressed in high amounts especially in the outer front of the migrating sheet, following fibronectin adhesion [68,89,95]. This sequence of molecular events might indicate that migration abilities follow cellular trans-differentiation, as fibronectin is an ECM component, and its cellular binding partner integrinβ1, are only expressed as a result of the EMT event. In LEC cell culture studies, measuring RhoA amounts will provide relevant information regarding the cell's readiness to migrate, as levels of this protein will be expected to rise.

1.7 Recent Improvements to Reduce Complications

Secondary cataracts are complex and, as a result, there are numerous potential strategies that have been and can be used to reduce the incidence of PCO [4]. The most important and widely reported methods include improvements to surgical technique, IOL

design and IOL material biocompatibility which have resulted in significant improvements in outcome.

1.7.1 IOL Design

The most important advance in IOL design is without doubt the squared edge design [8,45,46,55,96]. This design, first used in AcrySof® lenses, produced by Alcon Laboratories in the early 1990's, was a manufacturing quirk that resulted in significant reduction in the incidence of PCO [97,98]. The first clinical results to observe the effect of the AcrySof® lenses on PCO rates came out in 1998 [99,100]. In response, laboratory analyses of the lenses and their effect on lens migration were conducted, and Nishi and colleagues determined that sharp-edged acrylic IOLs reduce the incidence of PCO, independent of IOL material, mainly by blocking LEC migration to the posterior capsule via contact-inhibition that occurs at the capsular bend [55,64,101] (see Figure 1-3).

Further studies revealed that *in vitro* or in rabbit eye models, the sharpness of the IOL posterior edge [102] and the haptic design [50] were critical elements for reducing PCO. It has been suggested that these allow for more complete barrier formation, important in impeding LEC access to the posterior capsule. Recent studies and meta-analyses of controlled trials are highlighting the added benefits of sharp-edged IOL design in clinical patients. Since 2000, clinical trials have validated laboratory data in clinical settings, confirming that patients with sharp-edged IOLs had significantly lower PCO rates two years after IOL implantations than those with round-edged IOLs [103]. In 2007, a study of 23 randomized controlled trials further validated earlier clinical results,

by concluding that sharp-edged IOLs have a dramatic effect in reducing PCO rates [46]. Additionally, in a long-term study of twenty-six randomized trials, it was concluded that visual acuity was better in sharp-edged IOLs than in round-edged IOL at more than one year after surgery [98].

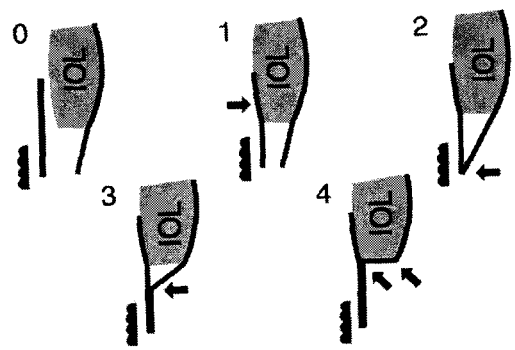

Figure 1-3. Development of capsular bend and physical barrier with square-edged IOLs. Reprinted from [64] with permission, © 2002 ASCRS and ESCRS.

Due to previous laboratory results correlating LEC migration with the formation of the capsular bend, it is believed that migration of LECs to the posterior prior to capsule seal formation is a major factor in PCO [55]. The variation in the time of PCO diagnosis is thought to be so broad because it is directly proportional to the number of LECs that reach the posterior [33] and their ability to divide, both of which are known to be dependent on the age of patient [55,65]. Therefore, a novel method of blocking LEC migration past the capsular bend in the first two weeks after surgery may lead to the mitigation of PCO.

1.7.2 IOL Material

The first IOL material, initially used in the 1950s, was poly (methyl methacrylate) (PMMA). This material was inadvertently discovered for its inert properties, during World War II, when Dr. Harold Ridley noticed that shards of airplane canopies did not produce an apparent reaction in the body [104]. The material was proven sufficiently inexpensive and malleable to be used as an IOL for decades. Today, PMMA IOLs are still used in some developing countries due to their low cost and ease of manufacture [18].

Notwithstanding the above, and the fact that hard PMMA IOLs pioneered the field of cataract surgery, the current market is overwhelmed by soft IOLs, made of acrylics and silicones. Soft IOL materials have dominated the market since they were approved for use, approximately 20 years ago [7,105]. Some obvious advantages include the change in surgical technique, as soft, foldable IOLs allow for initial incisions as small as 3.2 mm, in comparison to the 13mm incision needed for rigid lenses [32]. This has resulted in a lower incidence of inflammatory complications and thus better overall outcomes. To date, only hydrogel acrylic IOLs have the disadvantage of showing significantly higher rates of PCO when compared to the other materials [46,106].

LEC responses to silicone and acrylic materials, as well as responses to hydrogels, are different both *in vivo* and *in vitro*. While clinical studies show no significant differences in PCO rates between acrylic and silicone IOLs [46,106,107], hydrogels lead to more PCO than either acrylics or silicone IOLs [46]. LECs exhibit better adhesion, less migration and reduced apoptosis on acrylics *in vitro* [108]. A minor disadvantage to

silicone IOLs in a clinical setting, is that they are more prone to Nd:YAG laser damage [109].

1.7.3 Surgical Advances

Numerous variations for performing extracapsular cataract surgery have been or are currently being explored in an effort to minimize cost and maximize efficiency. Approaches include variations in shape and size of the capsulotomy. Others explore the possibility to extract the cataractous lens without the use of an expensive ultrasound, and practice manual techniques. While these advances are particularly important for surgical availability in developing countries, they have not resulted in significant changes in PCO outcomes [32].

As the occurrence of PCO has been established to be correlated to the number of residual LECs in the capsule after IOL insertion, many strategies have focused on attempting to eliminate all remnant LECs. There have been successes with respect to minimizing the number of remnant cells after cataract surgery, although none has succeeded in completely eliminating the cells [4]. Larger surgical openings in the anterior lens capsule, or buttonholing the IOL, have been tried, as these approaches were thought to remove a larger percentage of remnant LECs [34]. Capsular wash with drug solutions, particularly a 15 mM solution of ethylenediaminetetraacetic acid (EDTA) [110] prior to IOL insertion, was effective at reducing PCO in rabbits. However, while promising, none of these approaches has yet been approved for patient use. Recently, a sealed capsule

irrigation mechanism was demonstrated to be clinically safe in children; however, using distilled water to irrigate the capsule did not significantly change the PCO outcome [111].

1.7.4 Pharmacological Therapeutic Approaches

Pharmacological approaches have been suggested as a potential solution to PCO [110,112-115]. Initial studies focused on finding pharmacological agents that have desirable effects on LECs *in vitro* or *ex vivo*. Several groups focused on this approach, concluding that solutions of lidocaine [116], calcimycin [117] and salmosin [118] gave promising results. However, when human trials started, lidocaine raised some serious safety issues [119]. One drug solution is currently in human clinical trials. Immunotoxin 4197X-ricin A, is lens specific toxin that binds specific glycoproteins of the surface of the cells, is internalized and then blocks protein synthesis [120]. Current clinical trials, involving injecting a 1ml volume of toxin in the capsular bag at the time of IOL insertion showed a reduced PCO rate after 2 years [121]. This treatment however, is not yet approved, and long-term side effects are not yet clearly established.

However, the nature of the drug as well as the method of drug delivery and required delivery duration must be considered. It is generally agreed that a treatment delivered at the time of the surgery is preferred to an eye-drop regime, since the latter depends on patient compliance [115]. Ideally, a drug-release mechanism localized to the lens capsule would deliver drug, for prolonged periods of time and would minimize potential side-effects of the therapeutic agent on the other ocular cell types.

Using the Perfect Seal (Milvella Ltd. Sydney, Australia) capsule irrigation system devised by Maloof *et al* in 2003 [122], several research groups have demonstrated that cytotoxic agents such as Triton X-100 [123] and thapsigargin [124] can successfully delay PCO significantly in rabbit eyes, without compromising the neighbouring cells. While the irrigation system has been proven safe to use in human eyes, it has not yet been tested in combination with any pharmacological agent [111,123].

There have been very few attempts to modify the chemical structure of the lens materials in order to further reduce PCO [45,125]. Most studies on drug-releasing IOLs or other ocular drug-releasing devices have focused on anti-inflammatory and antibiotic drugs with a goal of reducing the incidence of major complications, such as endophthalmitis [39,43,44].

There have been attempts to develop drug-releasing IOLs specifically for PCO prevention. Controlled delivery of drugs including daunorubicin [126], indomethacin [126,127], diclofenac sodium, tranilast, mitomycin C, colchicines, ethylene diamine tetraacetic acid [11], 5-fluorouracil [11,128] and fibroblast growth factor (FGF) 2-saporin [62] has been proposed as a method of reducing PCO in animal studies, with limited success. Few studies were done on IOL materials modified with drug formulations that reduce human LEC proliferation *in vitro* [125]. While the drugs selected did not lead to significant decreases in PCO, it may be that combining this approach with other pharmacologic agents is needed as in none of the studies was it clear that the delivery of the agent was the issue.

However other factors are also critical to the success of a drug delivering IOL. For example, in most cases, the therapeutic dose is not known. As well, the effect of the cells on neighbouring ocular cells must also be considered since it is likely that the released drug may diffuse out of the capsule. As well, parameters such as the ability of the material to release the drug at an appropriate concentration and over an appropriate period of time must also be considered.

Of particularly interest in the current work is a series of target molecules aimed at inhibiting the MMPs released during the surgical procedure. Initial studies done on anterior subcapsular cataracts (ASC) have shown that blocking MMP-2 and MMP-9 selectively in the lens by using specific inhibitors and general inhibitors is sufficient to mitigate ASC [60]. Previous research has shown that ASC and PCO are both fibrotic disorders, with similar LEC behavior. Therefore, it is hypothesized that inhibition of MMPs through the use of appropriate MMP inhibitors may be efficient against PCO.

1.7.5 Other Surgical Devices

While there have been attempts to design devices which, implanted at the time of cataract surgery, minimize the access of the lens epithelial cells to the posterior capsule, most attempts have not resulted in a reduction in the incidence of PCO. One exception is the capsular bending ring device [4,129], which acts as an additional mechanical barrier to impede LEC migration to the posterior capsule. Regardless, while manufacturing advances show promise, it is clear that PCO still occurs, and that other approaches must be used in combination to completely inhibit PCO.

1.8 Scope of Work

The goal of this project is to develop novel drug-releasing materials which have the potential to alter LEC function and therefore which may ultimately minimize the incidence of PCO. Poly (dimethyl siloxane) PDMS was selected as a model material for these studies as, in addition to its use as an IOL material, it has been previously used successfully in rate controlled drug delivery applications, in multiple formats, for both protein and low molecular weight drugs e.g. [130-132].

It was thus hypothesized in this work that using an IOL material such as the model silicones used in the current work, with the potential to deliver MMP inhibitor molecules directly to the remaining LECs, at a controllable dose and for a known period of time, may mitigate the incidence of PCO. Solutions of MMP inhibitor drugs have been shown to inhibit LEC migration and transformation *in vitro* [82,133]. It is believed that providing a drug dose via drug-releasing silicone materials will be sufficient to delay LEC migration enough to allow for capsular bend formation, and thus full development of a mechanical barrier.

In order to facilitate the delivery of a broad range of potential MMP inhibiting drugs, a silicone based platform capable of delivering molecules with a range of molecular weights was developed. The system must potentially maintain desirable optical properties (light transmittance and refractive index), and allow for controlled release of the drugs with various rates, as it is currently not clear what drug concentrations and duration of release are appropriate. The former property is not critical

as it may be possible to deliver the molecule from the lens haptic with no effect on the visual pathway. Results from this study are presented in Paper 1 (Chapter 3).

Ultimately, based on this work, two different approaches were used to develop silicones with the potential to release MMP inhibitor drugs. The first method involved incorporating a solution of the drug in the PDMS base prior to curing. The other method involved soaking previously cured PDMS in drug solutions. This latter method would allow previously fabricated IOLs to be loaded with MMPIs after fabrication. Various methods were used to assess EMT and potential for PCO, using an *in vitro* model and two human lens cell lines.

As a tool for cellular toxicity screening, cell migration and EMT, an appropriate cell line had to be used for this study. However, issues with cell lines demonstrate that an appropriate cell line must be selected for *in vitro* screening of the systems prepared in this work. Therefore a comparison was made between FHL124 and HLE B3 lens epithelial cell lines in order to assess their response to TGFβ2 and to the various MMP inhibitors. These differences may prove important in further studies that compare adult and paediatric lens epithelial cellular responses. Results from this study are presented in Paper 2 (Chapter 4).

In the current work, three different classes of MMPIs and their delivery from PDMS as a model IOL material were investigated. Studies of the effect of the inhibitors on neighbouring ocular cells were performed.

1.8.1 Synthetic MMP Inhibitors

The first category of drugs selected for testing were three synthetic MMP inhibitors, a general inhibitor, GM6001 [134,135], and two specific inhibitors, MMP 2/9 Inhibitor I and MMP 2/9 Inhibitor II [136]. Research has demonstrated the importance of blocking active MMPs in EMT and LEC migration using GM6001. This has been shown to delay LEC migration and MMP-2 production [77,82,84,133]. These inhibitors were incorporated into silicones as model lens materials. The results of this work are presented in Paper 3 (Chapter 5).

1.8.2 Chelators as MMP Inhibitors

The second drug category studied included a series of chelators. Since MMPs are zinc-dependent proteases, it was hypothesized that release of chelators in a controlled manner has the potential to inhibit MMP activity. Other studies have shown that EDTA rinses of the capsule just prior to IOL insertion significantly delays PCO incidence [110], while IOL soaking in EDTA prior to incubation with LECs *in vitro*, significantly reduces LEC adhesion [11,45]. Chelators act on a number of other enzymes, since many proteins require a divalent cation for function. Other possible targets are cell-cell and cell-ECM connections, which may become loose after chelator treatment, and therefore may impede cell sheet migration [137-139]. EDTA, 1-10 phenanthroline and a zinc-specific chelator, TPEN, were examined in this work. The results from this study are outlined in Paper 4 (Chapter 6).

1.8.3 Sulfadiazine

The third type of drug examined is somewhat related to the synthetic MMP inhibitors previously described. Sulfadiazine is a sulphonamide antibiotic routinely used to treat infections such as toxoplasmosis [140]. While this compound has not been used to inhibit MMPs, the work described herein demonstrates that this drug has proven LEC migration and MMP inhibitor activities that may surpass those of synthetically developed MMP inhibitors. Similar classes of drugs, such as the synthetic MMP inhibitors previously described are N-sulfonyl amino acid derivatives [136] and they have been shown to inhibit LEC migration *in vitro* [82]. Sulfonamide drugs with similar chemical structure, such as sulphasalazine have been also demonstrated to have potential role in PCO prevention, by blocking activation of nuclear factor kappa B [141]. However the drug sulfadiazine has never before been studied for its ability to inhibit MMPs and LEC migration. The results of this study are presented in Paper 5 (Chapter 7).

1.9 References

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2.0 CONTRIBUTIONS TO ARTICLES

I was responsible for all literature searches, experimental design and data analysis. I prepared the first draft of each manuscript and I worked with my supervisor on subsequent manuscript drafts. Most of the experimental work was conducted by me, with some minor exceptions where the work was performed by either technical staff or summer students under my supervision. For Paper 1, TEM and confocal microscope sample preparation was done by Marcia Reid in the McMaster Electron Microscopy Laboratory. For Paper 2, several ECM composition analysis experiments were performed by Rebecca Kalfleish, a summer student in Dr. Heather Sheardown's laboratory at McMaster University, using protocols developed by me. For Paper 4, released chelator MMP-9 activity assays were performed by Larissa Schudlo, a summer student in Dr. Heather Sheardown's laboratory at McMaster University, using protocols developed by me.

In addition to the papers included in this thesis, I have also participated in writing the following book chapter:

David Morrison, Bettina J. Klenkler, Diana Morarescu, Heather Sheardown Advances in intraocular lens development leading to better vision and fewer complications. For publication in Ophthalmic Biomaterials and Regenerative Medicine Traian Chirila Editor 2009.

3.0 PAPER ONE: SOLVENT EFFECT ON THE RELEASE OF SMALL MOLECULES FROM PDMS

Authors: **Diana Morarescu**, Heather Sheardown

Objectives:

This work focuses on developing a method of controlling drug release from PDMS such that the resulting material will be suitable for use in ophthalmic applications. Control over release was achieved by changing the properties of the polymer matrix by loading the drug using solvents. The release rates and initial burst amounts of several model molecules of different molecular weight were investigated. Five different solvents were examined. Model drugs were loaded by mixing into the PDMS elastomer base, prior to addition of curing agent.

Main Scientific Contributions:

1. While there are studies using water as a hydrophilic solvent in PDMS, there are not systematic studies of the effects of different solvent drug combinations on drug release from silicone matrices.

2. The results demonstrate that mixing low molecular weight model drugs with hydrophilic solvents and elastomer base prior to adding curing agent can be used to control release, resulting in a small initial burst and low gradual rate of release. In contrast, hydrophobic solvents lead to release profiles which are generally characterized by a large initial burst followed by a high release rate.

3. While molecular weight of the drugs is important when drugs are loaded in powder form, with larger molecules releasing more slowly than smaller molecules, when loaded in dissolved form, solvent was found to be the main influence on the release profile.

Abstract

Silicone materials have been widely used as drug delivery matrices and rate controlling membranes. In the current work, we examined the effect of curing the silicone in the presence of various solvents on both the burst and release rate of model compounds including hydrophobic and hydrophilic dyes with molecular weights ranging from 141-1018 Da. Regardless of the compound examined, similar trends were observed. It was found that, under these loading conditions, the release kinetics were more dependent on the nature of the solvent than on the nature of the drug. Specifically, a low burst and relatively steady long-term release was observed when the compounds were mixed with water or phosphate buffered saline at pH 7.4 (PBS) and this mixture was incorporated into the PDMS during the curing process. Mixing the same dyes with solvents which were more compatible with the silicone prepolymers and which can potentially swell the final PDMS network, led to higher initial bursts, higher release rates and shorter release times overall. Therefore, this study suggests that release from silicone matrices can, to some extent, be controlled by incorporating a drug in solution into the matrix during synthesis.

Keywords: silicone elastomer, small molecule drug delivery, controlled release

1. Introduction

Silicones were among the first materials used for controlled drug release and have been used in this capacity since the late 1960s, primarily as rate-controlling membranes surrounding small molecule drug reservoirs [1]. From catheters and shunts coated with silicone containing antibiotics [2-5], to patches [6,7]and vaginal rings [8,9], to drug-releasing contact lens materials [10-12]; silicone based materials continue to be widely studied in drug delivery applications. Control of release kinetics, particularly from newer silicone based materials such as silicone hydrogels remains an area of significant research.

Designing better silicone based drug-delivery devices, with customized release rates and physical properties, involves an in depth knowledge of the properties of the drug of interest and its interactions with the silicone matrix. Characterization of release kinetics and diffusion properties of several small molecular drugs through poly(dimethyl) siloxane (PDMS) membranes have been studied [5,13-19]. The most common methods of loading the drugs include mixing powdered drug with the elastomer base [3,13,17,20] or swelling the silicone membrane with a nonpolar solvent containing the drug [2,10-12,21,22]. Alternatively, silicones have been used as rate controlling membranes surrounding a liquid [7-9,14,16,18,23] or a solid drug reservoir core [19]. While silicones are usually prepared neat, the use of solvents has been exploited in casting PDMS films [13,24], for the generation of silicone based interpenetrating networks [25] and for the release of specific compounds [5,13,24]. However the effect of adding drug containing solvents to curing PDMS on drug release kinetics has not been systematically studied.

Herein, we investigated the use of solvents on the the release rate of model drugs of various molecular weights from PDMS. Specifically, we examined small drugs (MW between 144 and 1018 Da) with various properties. Since our primary interest is ultimately the delivery of drugs to the eye, solvents examined included both non-polar and polar solvents at levels that did not alter the transparency of the system.

2. Materials and Methods

2.1 Drug Loading and Release

All reagents, unless otherwise specified, were purchased from Sigma Aldrich (Oakville ON). PDMS was prepared using a Sylgard 184 kit from Dow Corning (Midland, MI), according to the manufacturer's instructions, using 10:1 ratio of elastomer base to curing agent, with and without the solvent containing the drugs. Fluorescent model drugs of various molecular weights were dissolved in dimethyl formamide (DMF), ethanol, isopropanol (IPA), water or phosphate buffered saline (PBS). These solvents were selected based on solubility of the PDMS base and the potential interactions in the system. Solvent volumes of 0.8-1.0% v/v or 4.5-5.0% v/v containing an appropriate drug were mixed with the PDMS elastomer base prior to the addition of the curing agent. Following the addition of the curing agent, the films were first kept in the fume hood for 1 hour at room temperature, then kept in a Isotemp Model 280A vacuum (Fisher Scientific, Ottawa, ON) at 2.46psi and room temperature for 1-2 hours, after which they were cured for 7 days at 37°C in the dark. Compositions of the various materials prepared are summarized in Table 1. To examine the preparation of the dye containing

films neat, solid powdered dye was added to the elastomer base with no solvent prior to curing.

Table 1: Composition of drug-loaded PDMS films.

Drug name	**Solvent**	**Amount of drug per ¼" disk (µg)**
6-Aminoquinoline MW 144.17 Da	Water (5% v/v)	1.1
	PBS (5% v/v)	0.4
	DMF (1% v/v)	2.9
	Ethanol (5% v/v)	12.6
	IPA (3.5% v/v)	9.4
Fluorescein MW 332.32 Da	Water (5% v/v)	0.1
	PBS (5% v/v)	0.5
	DMF (1% v/v)	0.1
	Ethanol (5% v/v)	0.7
	IPA (3.5% v/v)	0.3
Rhodamine B MW 479.02 Da	DMF (1% v/v)	3.7
	Ethanol (5% v/v)	14.1
Calcein MW 622.53 Da	DMF (1% v/v)	0.1
	PBS (5% v/v)	0.6
Rose Bengal MW 1017.65 Da	Water (5% v/v)	12.8
	PBS (6% v/v)	2.7
	DMF (1% v/v)	1.1

Disks with a diameter of approximately ¼" and a thickness of approximately 0.5mm were punched from the final PDMS films. Release studies were performed using n=6 disks. Each drug-loaded silicone disk was incubated in 600µl PBS, pH 7.4 at 37°C with mild agitation, in the dark. At regular time intervals, for periods of 2 to 3 months, the disks were transferred to fresh buffer solution, and the releasates were analyzed. To

maintain sink conditions, the transfer was done every 30 minutes for the first 6 hours, and every 8 hours over the following 72 hours. A 24 hour interval was used to change release buffer for the following 5 days, and then longer intervals as appropriate (typically 120 hours) until the end of the release. The concentration of the drug was measured by fluorescence, based on a standard curve, corrected at all time points for quenching and solvent effects [26]. Sink conditions were maintained by changing the solution before reaching 10% of maximum solubility.

2.2 PDMS Analysis

The optical properties of the material were determined by measuring transmittance at 405nm, 540nm, 595nm and 630nm with a Bio-Rad Plate Reader. Statistical analysis of the light transmittance data was done using the Kruskal-Wallis test followed by Dunn's post hoc test, using SigmaStat software.

3. Results

3.1 Drug Loading and Release

Model drugs were loaded in various solvents at concentrations of 1% and 5% (v/v) of the elastomer base. These percentages were chosen based on the maximum solvent volume that allowed the PDMS to cure under the described conditions. Larger volumes of solvent rendered the elastomer mix translucent and were found to inhibit curing. The addition of ethanol and IPA resulted in the fastest rates of curing, with solid membranes produced in 4-5 days; however, water, PBS and DMF containing disks

needed the entire 7 days for complete curing. For consistency, all films were kept at 37°C in the dark for the entire 7 day period.

While testing the release rates, sink conditions were maintained throughout the experiments, with concentrations of the model drug in PBS lower at all times than 10% of the drug solubility in PBS. Release of various model drugs is presented in Figures 1-5. The cumulative percent release profiles outline the visible differences in release when the different solvents were used to synthesize the drug containing membranes.

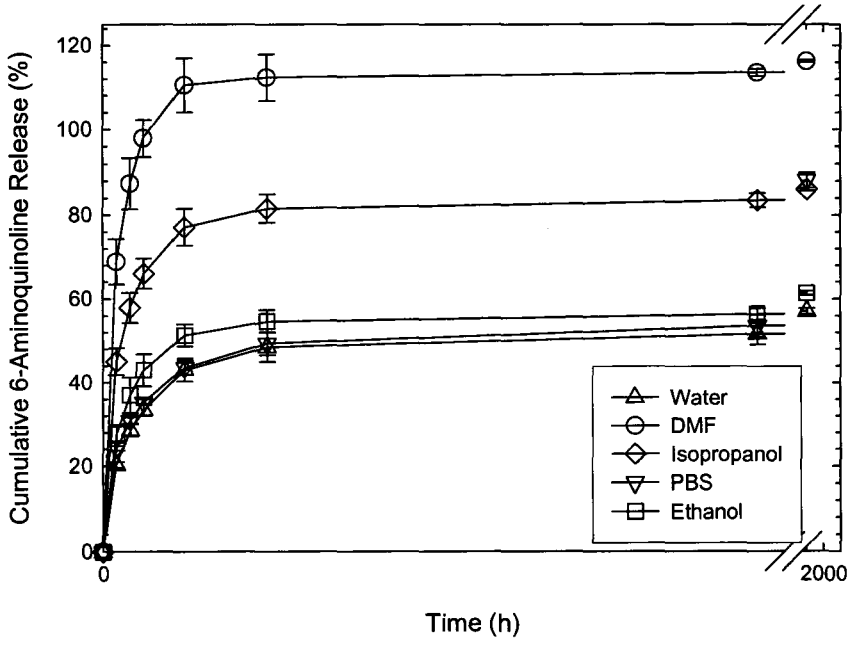
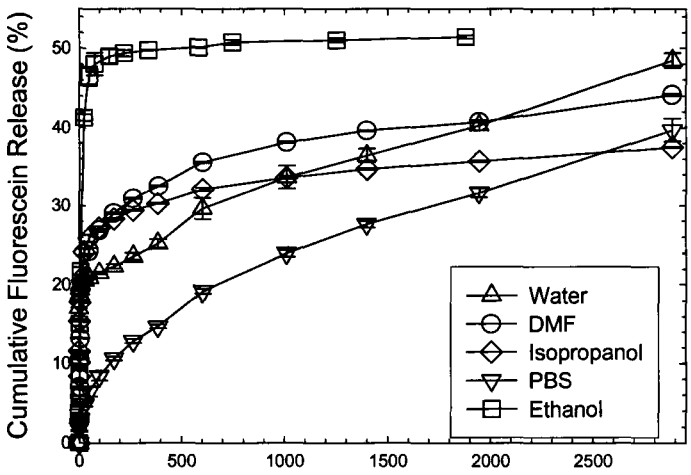


Figure 1: Cumulative release of 6-Aminoquinoline (MW 144.17 Da) when loaded as a solution. The highest burst is achieved in DMF. A smaller burst with a longer period of release is observed when water or PBS is used as the solvent. $n \geq 4 \pm SD$.

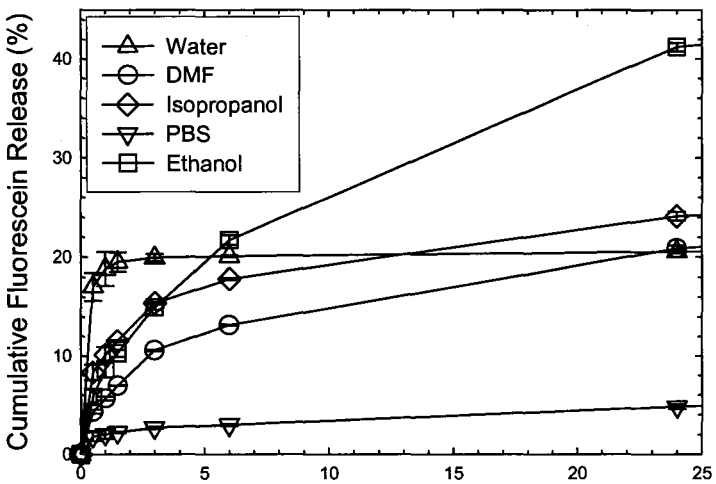
In Figure 1, the cumulative release of 6-Aminoquinoline when using the DMF solvent for loading appears to be above 100%. This is due to large error bars in the early release time points. This model drug is the only amine-containing substance.

In general, regardless of the model drug or its solubility in the solvent used, higher bursts are observed with IPA, ethanol and DMF, with lower bursts when water and PBS were used as solvents. While there are some differences in the loading of the model compounds depending on the solvent, the results suggest that solvent effects play a greater role than drug type to the changes observed. Specifically, it can be seen that the release of fluorescein, loaded at similar levels, showed very different release kinetics depending on the solvent used to generate the membrane as shown in Figure 2. in particular, it can be observed form Figure 2, panel B, that water has the highest initial burst, while PBS has the smallest burst, while the other solvents have a similar burst, in between these two values. However, in the long term, water and PBS dissolved fluorescein maintain a release rate for the 2500h period, while there is little to no release observed for the other solvents past the initial burst.

In general, the release can be approximated by square root time kinetics (Figure 6). Only 6-aminoquinoline shows two distinct diffusion regimes, with a steeper slope in the first 24 hours. Greater rates of release are typically observed with ethanol and isopropanol relative to water and PBS for the same drug.



A.



B.

Figure 2: Cumulative release of Fluorescein (MW 332.32 Da) when loaded as a solution. The highest burst is achieved in one of the swelling solvents, ethanol. Panel A shows the overall aspect of the release, for the entire 2500h period. Panel B shows in detail the release profile in the first 24 hours. A smaller burst with a longer period release is observed especially when PBS is used as the solvent. $n \geq 4 \pm SD$.

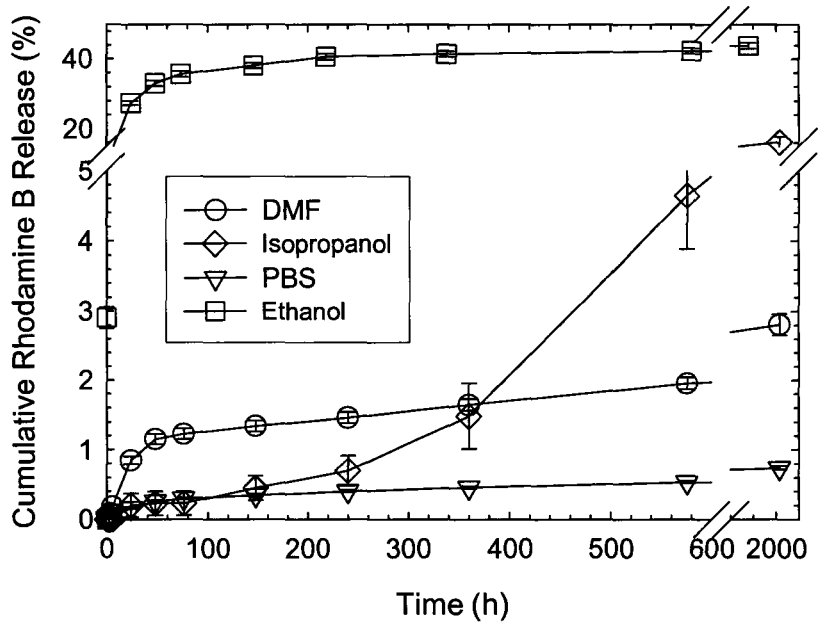


Figure 3: Cumulative release of Rhodamine B (MW 479.02 Da) when loaded as a solution. The highest burst is achieved in one of the swelling solvents, ethanol. $n \geq 4 \pm SD$.

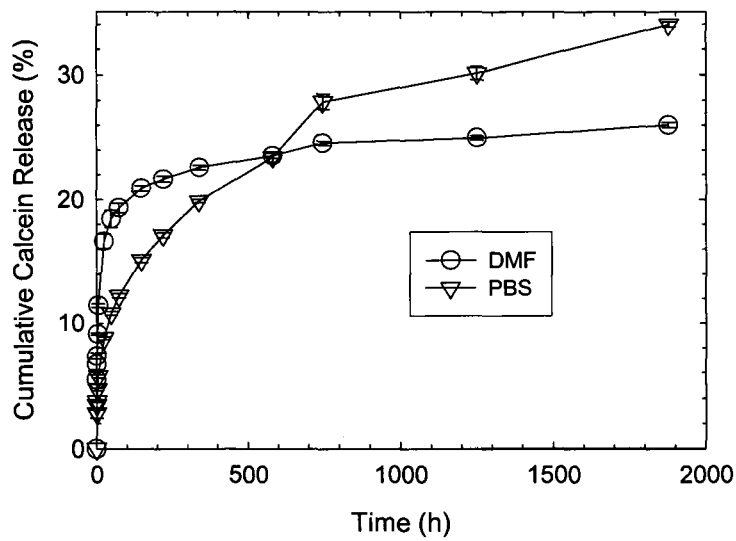


Figure 4: Cumulative release of Calcein (MW 622.53 Da) when loaded as a solution. The highest burst is achieved in DMF. A smaller burst with a longer period of release is observed when PBS is used as the solvent. $n \geq 4 \pm SD$.

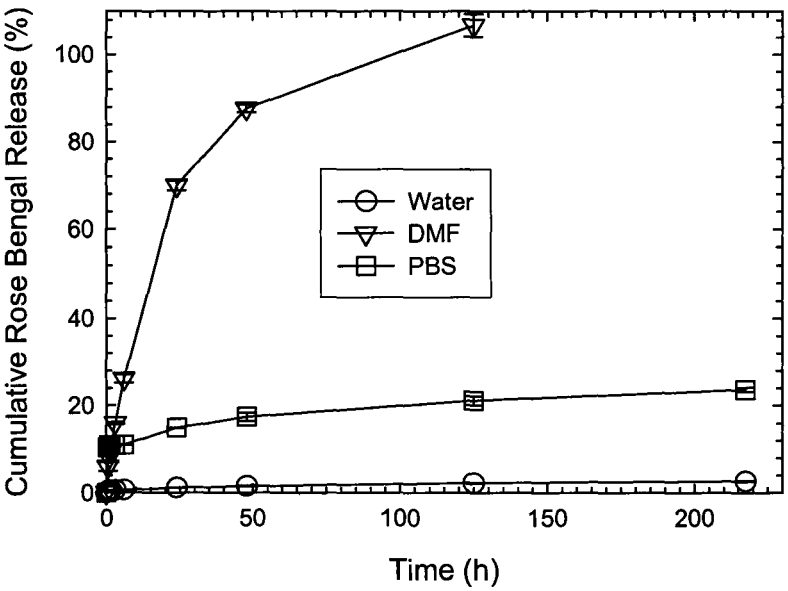


Figure 5: Cumulative percent release of Rose Bengal (MW 1017.65 Da) when loaded as a solution. The highest burst is achieved in DMF. A smaller burst with a longer release is observed when either water or PBS is used as the solvent. $n \geq 4 \pm SD$.

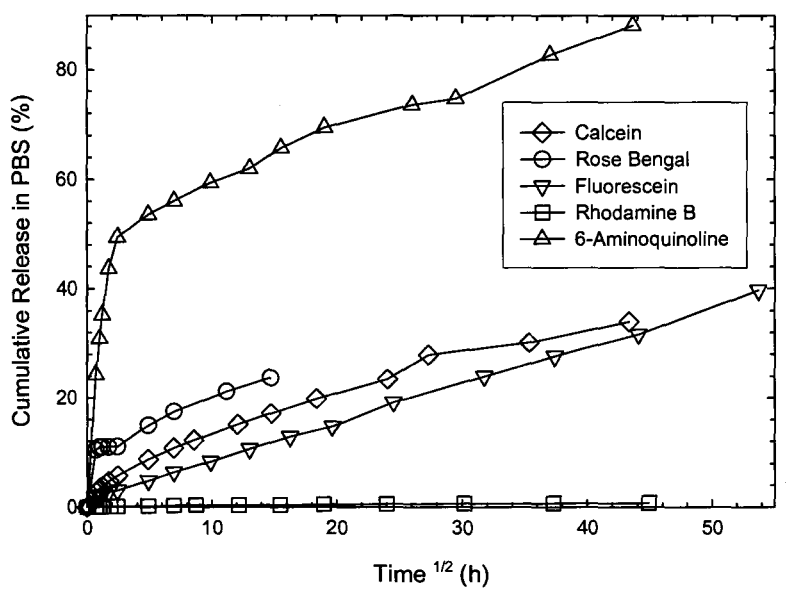


Figure 6: Cumulative percent release of all model drugs when loaded as a PBS solution in PDMS. All drugs present linear square root time kinetics. 6-Aminoquinoline is the only molecule that exhibits two distinct rates of release. $n \geq 4 \pm SD$.

Interestingly, as shown in Figure 7, the role of the drug properties is more important when the drugs are loaded into the matrices in powder form. Fluorescein, the smallest molecule, continued to show significant release at 100 hours. Rhodamine B, the next in size, also exhibits measurable release at 100 hours. However, the largest drug tested, Rose Bengal, has a near-flat slope after the initial burst with limited release after 100 hours.

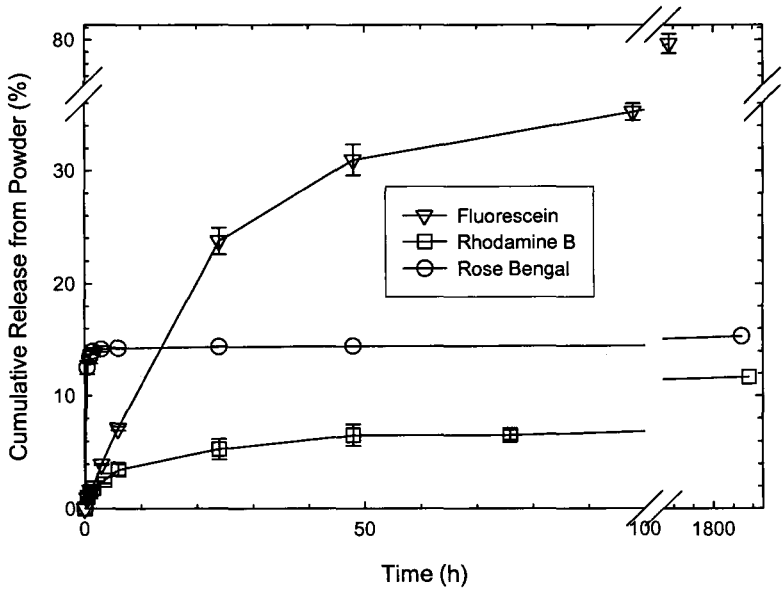


Figure 7: Cumulative release of model drugs when loaded in the PDMS in powder form. Long term, relatively constant release is best achieved using the more hydrophobic Fluorescein, and Rhodamine B. Initial burst can also be correlated to drug size and water solubility. Notably, Rose Bengal, one of the largest molecules examined, shows the highest burst.

Dramatic differences in the initial burst of the drugs are also observed, with the most hydrophilic molecule, Rose Bengal, with a solubility of 36g/100ml, water showing the highest burst, with nearly 14% released within the first three hours. Rhodamine B

and fluorescein, which had much lower water solubility, at 0.8g/100ml and 0.08g/100ml respectively, show much lower bursts with less than 4% released in the first three hours. However, given that only three drugs were examined, it is not possible to draw detailed conclusions about the effect of the nature of the drugs including size and hydrophilicity on release.

3.2 Film Properties

Qualitatively, the PDMS films prepared showed different textures depending on the solvents used. All of the films prepared were visually transparent, solid and easily manipulated. PDMS modified with solvents alone did not show a significant deviation in light transmittance ($p<0.05$) in the range of 405nm to 630nm (see Figure 8), with the exception of PDMS with 1% PBS, which showed significantly lower values ($p<0.05$) than PDMS prepared in the absence of solvent.

Transparency of the disks is not affected, indicating that the drug is likely not precipitating and the size of these drug pockets is smaller than the wavelength which leads to the scattering of light. Under these conditions, release is thought to be facilitated by partitioning and dissolution in channels produced by the solvent rather than diffusion through the PDMS membrane.

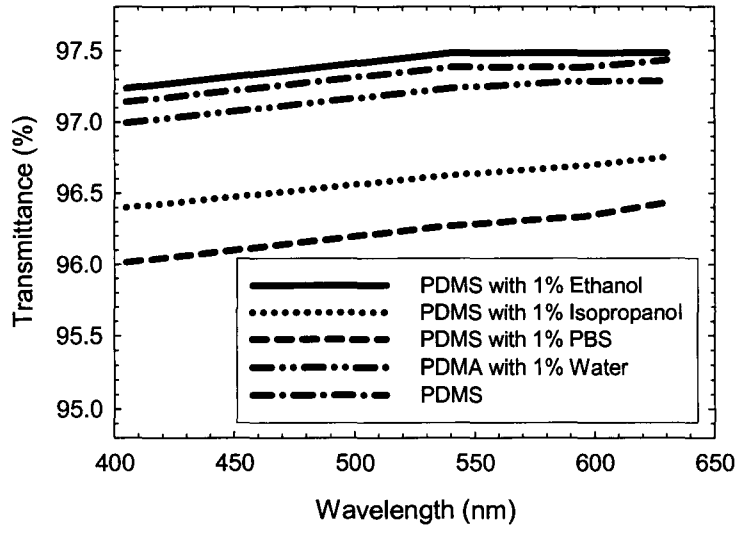


Figure 8: Light transmittance through PDMS matrices at wavelengths within visual range. The values for transmittance are very high similar to those of the crystalline lens of a 4-5 year old child [29]. This indicates material is suitable for ophthalmic applications.

4. Discussion

The design of effective drug delivery systems involves an understanding of the interactions between the drug and matrix and ultimately the ability to be able to control these interactions. While many properties of drug-loaded silicone materials have been described [5,13-19,23], delivery of hydrophilic drugs from a silicone matrix has been a challenge not easily overcome. There are relatively few reports of the release of hydrophilic molecules from silicone matrices. Similarly, there are few studies which examine the effect of using solvents to generate the drug loaded PDMS and how this alters the kinetics of release.

In this work we have described a method which may be used to control drug release from PDMS while maintaining transparency. Based on previous work [25], it

was hypothesized that casting the PDMS in solvents would alter the structure of the matrix produced, thereby altering the kinetics of release without significantly altering mechanical or optical properties. Depending on the solvent used, it is thought that its presence may lead to the creation of interconnecting holes and/or drug pockets although this remains to be demonstrated. In particular, solvents that are capable of swelling PDMS to some extent, specifically isopropanol, and to a lower extent ethanol and DMF, allow for the drug to be more evenly distributed throughout the polymer. Water based solvents cannot swell PDMS [22], but can lead to the formation of pores in the matrix [27].

Interestingly, however, the use of water and especially PBS also seems to alter the structure of the hydrophobic silicone elastomer relative to that obtained when the films are prepared neat, resulting in the possible formation of large pores in the network where the drug may be deposited. Since water is commonly used as a porogen for PDMS [22,27], it is not surprising that preparation of drug loaded PDMS membranes with vastly different properties is possible using this method and that this method can be used to alter drug release kinetics of these membranes. However, it is surprising that the materials retain their transparency when prepared in aqueous solutions and that the release is relatively controlled with only a small burst. Even though the transparency of the PDMS film with 1% PBS is significantly lower than that of PDMS prepared neat, it remains sufficiently high to be used as an ophthalmic material [29].

While the use of aqueous solvents to incorporate particularly hydrophilic drugs into PDMS matrices has been previously reported [13], this is, to our knowledge, the first

systematic study of the effect of different solvents on the release of model compounds of varying molecular weight and hydrophilicity. Furthermore, in the previous study, rapid release of a small and highly hydrophilic molecule was observed when membranes were prepared in the presence of water, presumably the result of the high loading used. However, in the current work it can be seen that altering parameters such as volume ratio or drug concentration, can be used to tailor the release profile to some extent, leading to longer release times with lower bursts and more constant release. Better solvents for the PDMS such as ethanol and isopropanol led to more even drug distribution in the matrix and generally to a slower rate of release regardless of the properties of the model drug tested. It is therefore clear that network structure can be easily manipulated using solvents to alter the kinetics of drug release. Others have altered the morphological features of PDMS in order to facilitate release or to alter release kinetics so it is not surprising that membrane morphology can affect release [30].

Analysis of the drug release profiles of the 5 model drugs (Figure 1-5) shows a clear trend. The highest burst and lowest rate of release are achieved with the solvents that have the best PDMS swelling ability – IPA, ethanol and DMF. Contrarily, the lowest burst but longest steady state release rates are observed with the solvents that are the least compatible with the PDMS – water and PBS. These effects seem to be relatively independent of the size and charge of the molecule released. The release of molecules when initially dissolved in PBS are different than those of water, since additional salts remain incorporated in the PDMS matrix, which lead to an osmotically driven release, which can go on for longer time periods than most other solvents (Figures 1-5).

Combined with its small size, 6-aminoquinoline acts as an aminated solvent and is released rapidly form the matrix, as most drug is quickly eliminated in the first 48 hours (Figure 1). This molecule is also the only one showing two distinct slopes in the release relationship with the square root of time (Figure 6). This behavior could be explained if the substance crystallizes on the disk surface, possibility sustained by the fact that this highly hydrophobic molecule was exposed to an aqueous solution for release – PBS [22].

Factors such as size, solubility in the solvent and solubility in the matrix were varied in this study, but results indicated that the major factor controlling the release in these membranes is membrane morphology which is controlled by the solvent used to dissolve and load the drug. Significantly different results were obtained when the model drugs were loaded in powder form with the properties of the molecules and their interactions with the PDMS presumably having a greater effect on the release kinetics. The data suggest that by altering the structure of the PDMS network, control over the long-term release of both hydrophobic and hydrophilic drugs can be achieved.

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4.0 PAPER TWO: DIFFERENCES IN PHENOTYPE AND RESPONSE TO MMP INHIBITORS OF TWO LENS EPITHELIAL CELL LINES

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Submission Information: To be submitted to the Experimental Eye Research.

Objectives:

In order to be able to test the effect of proposed molecules on human LECs, two cell lines have been investigated. The two cell lines, HLE B3 and FHL124, have been most widely used *in vitro* to study lens development and disease. However, these cells have not been well characterized with respect to response to stimulants that may precipitate changes consistent with posterior capsule opacification. The differences in donor age and mode of cell line preparation were tested for their influence on the extra cellular matrix laid by the cells and their response to MMP inhibitors and different seeding surfaces.

Main Scientific Contributions:

1. The two cell lines were found to produce different ECMs in unstimulated conditions, demonstrating the basal levels of some of the common EMT markers is significantly different.

2. While the cell lines react differently to MMP inhibitor treatments, they can still provide useful information in drug testing studies, particularly if the *in vitro* results can be correlated with *in vivo* results from age matched donors.

3. Due to the response differences between the two cell lines, more conclusive analysis needs to be done either using animal models *in vivo* or by using human eye capsules.

Abstract

FHL124 and HLE B3 are two commonly used human lens epithelial cell lines for *in vitro* studies, particularly for studies associated with posterior capsule opacification (PCO). The two lines have distinct properties, arising from the fact that the former is transformed while the latter is a primary line. They have different origins; FHL 124 cells are derived from adult cells, while HLE B3 cells are derived from infants. It is therefore of interest to determine how the behavior of the two lines may impact study conclusions. The two cell lines deposited different extracellular matrix and matrix deposition appeared to be material dependent. Furthermore, cell numbers were found to be both cell type and substrate dependent. Relevant to studies of PCO, differences in basal epithelial and myofibroblastic marker protein levels, including laminin, E-Cadherin and α-SMA for these cell lines were significant. Response to MMP inhibitors and TGFβ2 treatments were also shown to be affected by the cell line tested. The markers which behave most similarly in the two cell lines were fibronectin and collagen I/III levels. As differences between the two cell lines could possibly account for some of the normal phenotypical differences in human populations, novel treatments which can be validated using both cell lines in some way may indicate increased potential for success in a greater range of patients. Testing with both cell lines prior to animal studies may also avoid the need for extensive use of animal models.

1. Introduction

Cell lines are valuable research tools, allowing researchers to test hypotheses as well as to learn about various diseases or normal cell function and metabolic pathways. For example, in the fields of drug development, biomaterials, and drug delivery, they are especially useful for exploring novel treatment methods and testing possible therapeutics. However, the use of an appropriate cell line is critical to obtaining relevant results. Currently there are three widely used lens epithelial cell (LEC) lines described in the literature [1-3]. In addition to their use in studies aimed at the understanding of normal lens development, these cells have been applied to better understand and test potential therapeutic options for such lens disorders as anterior subcapsular cataracts (ASC), and posterior capsule opacification (PCO). The latter is of particular interest as cataract surgery is the most common surgery performed worldwide [4,5] and it is been estimated that as many as 40% of patients who undergo cataract surgery will develop PCO requiring secondary treatment within the first five years of lens implantation [4,6].

Researchers have long tried to identify a relevant *in vitro* PCO model both to better understand the conditions which lead to its formation and to develop viable preventative options. Studies have been performed using organ explants, mostly using rabbit, porcine and bovine lens capsules [7-11]. While animal lens explants may be a good choice due to their availability, the significant differences in PCO etiology between animal and human eyes may lead to results which are less relevant in a human population [12]. Studies using human lens capsules are considered ideal; however their availability and the preparation process make this model unfeasible, particularly for toxicity studies,

where high-throughput conditions are necessary. For this type of study, a cell line has the advantage of ease of propagation, thereby minimizing supply limitations, as well as ease of manipulation. The challenge in the case of LECs is that one of the hallmarks of PCO is a change in phenotype; however, they also undergo epithelial to mesenchymal transition (EMT) in response to stimuli that are often present under tissue culture conditions.

EMT is a process that is encountered in embryonic development [13,14], as well as in several types of mature epithelial cells including liver, kidney and lung in addition to in the lens [13-19]. EMT is caused by TGFβ2 stimulation of LECs, which change protein profiles of the cells. EMT is also the process thought to be responsible for cell migration to the posterior capsule, which subsequently leads to the deposition of aberrant extracellular matrix (ECM) and capsular wrinkling that are hallmarks of PCO [6,19]. However, in the case of PCO, EMT is presumably initiated by the mechanical stress imposed on the capsule by the cataract surgery [24,25] which subsequently leads to increased production of TGFβ2, the primary cytokine involved in EMT [14,20].

Studies of the EMT process in LECs and other epithelial cell types have established several markers which are characteristic of either the epithelial or the mesenchymal phenotype. Specifically, changes are induced in the expression of ECM and cytoskeletal proteins as well as integrins and in the migration ability of the cells [13,14,19,26]. The most notable mesenchymal marker however, is the acquired ability of the cells to produce alpha smooth muscle actin (α-SMA) [25,27,28], a protein associated with increased contractile properties of the cell, essential to the process of fibrosis and tissue remodeling in wound healing [28]. While one study has demonstrated that in the

lens, that α-SMA is not necessarily associated with the capsular wrinkling that happens in PCO [29], most other research still associates this protein to the capsular wrinkling process [4,10,14,19,25].

The three human lens epithelial cell lines widely used in the literature have different origins and properties. In the current work, two of the three cell lines, HLE B3 and FHL 124, were characterized based on their differences relevant to PCO; the SRA 01/04 cell line was not characterized, due to similar origins with the HLEB3 line. HLE B3, developed from infant lenses and immortalized with SV40, was the first human lens epithelial cell line to be established [3] and is currently the only commercially available human lens epithelial cell line. SRA 01/04 cells are similar in that they are also SV40 immortalized infant lens derived cells [1]. A major disadvantage to using either of these two cell lines in PCO studies is that they are poorly characterized in literature with respect to gene expression, their ability to be stimulated by TGFβ2, and their response to cytokine stimulation [1,3]. However, their crystallin protein expression has been investigated [1,3].

In contrast, FHL124 cells are untransformed primary cells derived from adult lens epithelial cells [2]. This cell line has been studied in depth, and its gene expression profile has been characterized and compared to normal human LECs [2,30]. While proven responsive to TGFβ2 treatment, there is a limit to the number of passages that can be used [31], as, unstimulated, they express abnormally high levels of α-SMA at high passage [2,32]. This may indicate that their potential to undergo EMT due to cytokines in growth medium is somewhat heightened.

Based on the nature of the cells, two major important differences between the cell lines can be assumed. Transformed and primary cells can differ in DNA methylation, karyotype, protein profiles and responses to stimuli, especially as a result of SV40 transformation [33,34]. Furthermore, primary cell lines, while untransformed, and thus not genetically altered, are less able to resist prolonged manipulation due to culturing, and have a limited life span, as they undergo senescence, due to the inability of normal cells to maintain telomere length and chromosome stability [35]. The third major difference is the age of donor tissue. Infant epithelial cells have a greater potential for mitosis and are more receptive to stimuli such as FGF [22]. Many cellular responses and activities are changed in adult tissue, due to normal senescence [35]. Thus the age of the donor, in primary cell lines especially, is important in establishing cellular response to treatment. Therefore, it is clear that additional studies are necessary to establish the properties of the cells which make them conducive for studies of PCO. In this study, the HLE B3 and FHL124 cell lines were compared for relevance to studies of PCO, both by looking at EMT markers, and at ECM composition on two relevant biomaterial surfaces: tissue culture poly(styrene) (TCPS) and poly(dimethyl siloxane) (PDMS) as model IOL materials.

2. Materials and Methods

2.1. Surface preparation

All reagents, unless otherwise specified, were purchased from Sigma Aldrich (Burlington, ON). Sheets of poly(dimethylsiloxane) (PDMS) were prepared using the

Sylgard 184 kit (Dow Corning, Midland, MI), according to manufacturer's instructions, using a 10:1 ratio of elastomer base to curing agent. Disks with a diameter of approximately ¼" and a thickness of approximately 0.5 mm were punched from the PDMS films. Tissue culture poly(styrene) (TCPS) dishes were purchased from Becton Dickinson (BD, Mississauga, ON, cat# 353047).

The MMPIs, GM6001, MMP 2/9 Inhibitor I and MMP 2/9 Inhibitor II (Calbiochem, San Diego, CA) were dissolved in either dimethyl formamide (DMF) or ethanol and prepared as described (see Chapter 4, Section 2.1).

2.2. Cell culture

Unless otherwise mentioned, reagents were purchased from Invitrogen (Burlington, ON). HLE B3 lens epithelial cells were grown in MEM-F15 media (Sigma Aldrich, Oakville ON), with 20% FBS, 0.01mM L-glutamine, 0.06% (w/v) sodium bicarbonate, 1mM sodium pyruvate for complete media conditions. FHL 124 lens epithelial cells were grown in MEM media with 10% FBS, 0.01mM L-glutamine for complete media conditions. In addition, the response of the lens cell lines to ECM laid down by a variety of other cells was examined. L929 and 3T3 cells were grown in DMEM medium; CHO and RPE cells were grown in DMEM-F12 medium; human corneal epithelials (HCEC) were grown in KSFM medium; human corneal stromal fibroblasts (HCSF) were grown in M199 medium; all cell lines were grown in media with 10% FBS and a 1% antibiotic-antimycotic mix. Cells were washed in PBS prior to medium change, which was done every 2-3 days.

To obtain ECM coated wells, cells were grown to confluence (approximately 2-4 days) which was followed by starvation via incubation with PBS for 14 days. Cells detached as they died, leaving ECM attached to the wells. Dying and detaching cells were aspirated and washed every other day. The composition of the ECM remaining was also tested, as described in section 2.3.

To test the response of the lens epithelial cells lines to the ECM laid down by the various cells, 30,000 cells, either B3 or FHL 124, were seeded onto ECM coated wells, obtained as described above and the cells were grown for 5 days under serum free conditions. At the end of the interval, cells were detached using Tryple Express and counted with a Beckmann Coulter cell counter. For growth on surfaces, 11,000 cells were seeded per ¼ inch disk.

2.3. Testing for ECM components

All reagents for immunostaining were purchased from Sigma Aldrich (Oakville, ON), unless otherwise specified. The ECM-coated wells and disks were blocked with 5% goat serum in PBS for 30 minutes. This was followed by incubation with the appropriate primary antibody for 4 hours. All incubations were performed at room temperature. Specifically, 1:300 dilutions of rabbit anti-fibronectin primary antibody, rabbit anti-laminin primary antibody or sheep anti-vitronectin antibody (Abcam, Cambridge, MA) were used. An appropriate fluorescent secondary antibody (1:100 dilution for the anti-rabbit secondary antibody and 1:1000 dilution for the anti-sheep antibody) was subsequently added, and incubated for 2 hours at room temperature with mild shaking.

Concentrations of the various components on the surfaces were determined fluorimetrically. For both secondary antibodies, fluorescence readings were set for λ_{ex} 485 nm; λ_{em} 520 nm. The relative amount of bound antibody was calculated based on the change in fluorescence units (ΔRFU).

For glycosaminoglycan (GAG) staining, two alizarin blue solutions were prepared. Sulfated GAGs were determined using a 1% solution in sulfuric acid (pH 0.2) [36]; total GAG was quantified using a 1% solution in acetic acid (pH 2.5) [37]. Alizarin blue concentrations were assessed by reading at 540nm using a Bio-Rad microplate reader.

2.4. Response of cells to TGFβ2 and MMP Inhibitors

Due to the established link between the presence of TGF-β2 and PCO [19,21] and the known effect of MMP inhibitors on the processes which lead to cell transformations consistent with PCO, it was of interest to determine the response of the cell lines to these molecules. Cells were grown to ~ 80% confluence in complete media. TGFβ2 (Peprotech, Rocky Hill, NJ) was added at a final concentration of 2ng/ml media, after preparing a stock solution as recommended by the manufacturer. MMP inhibitors were added to the medium simultaneously with the TGFβ2, at a concentration of 90 nM, in DMSO. Protein composition was tested after 48hr of treatment, as described above. In addition to fibronectin and laminin, combined collagen I and III amounts were determined using Sirius Red, as previously described [38,39], and alpha smooth muscle actin (α-SMA) was detected fluorometrically using an FITC-labeled primary antibody. E-

cadherin staining was performed on formalin fixed cells, using a primary antibody (Sigma, Burlington, ON) and the appropriate FITC-labelled secondary antibody. Quantification was preformed using a Victor 3 fluorometer and its software.

3. Results

3.1. ECM composition: effect of substrate material

Basal ECM compositions for unstimulated cells are shown in Figure 1. When comparing ECM composition on tissue culture polystyrene (TCPS) with ECM composition on PDMS, it is interesting to note that the adult derived cell line, FHL124, does not modify its structural protein composition significantly, while the infant derived cell line, HLE B3, produces a very different ECM for each material. Both cell lines show significant variation in GAG composition ($p<0.05$) in response to the different growth substrates. The relative fluorescence is proportional to the amount of protein detected, thus overall, HLE B3 cells appear to deposit more structural proteins on the same surface area, than FHL124 (Figure 1).

3.2. Cell survival on different ECM

In order to determine general responsiveness of the two cell lines to similar culture conditions, cells were seeded on the ECM produced by various cell lines. Both cell numbers and differences in preference for different ECM substrates were examined and the results are shown in Figure 2.

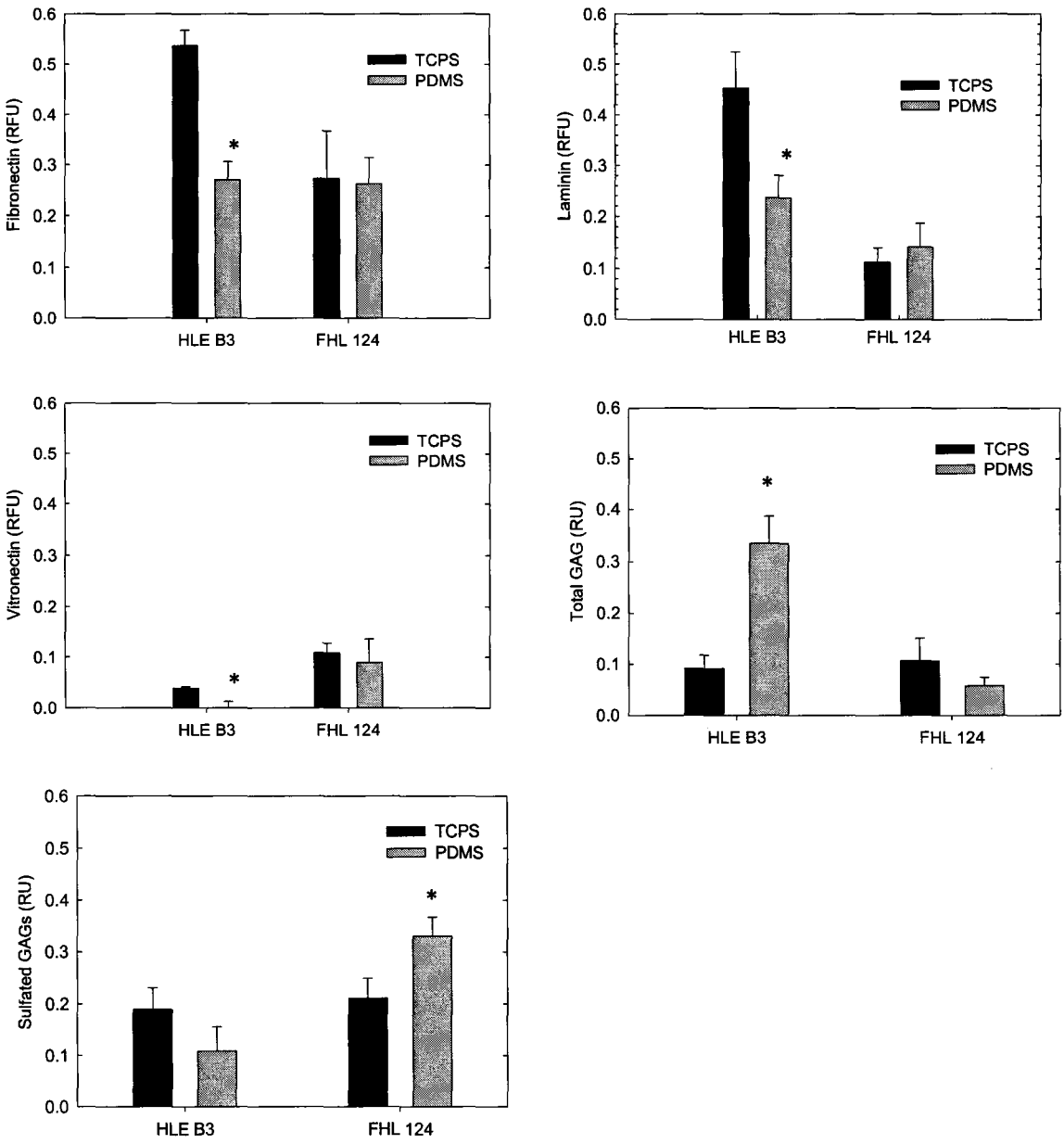

Figure 1: Comparison between HLE B3 and FHL124 extracellular matrix composition. HLE B3 cells produced higher levels of structural proteins on TCPS than on PDMS (panels a, b, c), while FHL124 protein production was not affected by the differences between these materials. GAG amounts in the ECM fluctuated for both cell lines in response to changes in surface (panels d, e). * $p<0.005$

Cell numbers were measured after 5 days of culture in serum free conditions.

Significant differences were observed between the two cell lines under the same growth

conditions ($p<0.0005$). It should be noted that, under serum free conditions, HLE B3 cell numbers are approximately 10 times higher than those of FHL124.

Somewhat surprising, both of the lens cell lines tested showed a preference for ECM produced by L929 cells over their own ECM, and to a lesser extent ECM produced by CHO cells. The trend is more pronounced for FHL124 cells, which show a 6 fold increase in cell number on this matrix than on matrix previously deposited by FHL 124 cells; HLE B3 cells show only a two fold increase in cell numbers on the L929 matrix than on matrix produced by previously seeded HLE B3 cells. The L929 ECM was the only ECM to contain detectable levels of residual mRNA, of up to XXng/ml.

3.3. Response of cells to TGFβ2 and MMP Inhibitors

As shown in Figure 3, cell viability, as measured by MTT assay, is decreased in the presence of TGFβ2, which is expected [40]. However, somewhat surprisingly, the effects of the growth factors were not decreased by the presence of the MMP inhibitors. A combination of MMP 2/9 Inhibitor II and TGFβ2 resulted in the largest decrease in cell viability, by approximately 28%.

Also of interest is the effect of growth factor and MMP inhibitor treatment on the production of relevant marker proteins by the cells. Specifically, changes in fibronectin, laminin, collagen and α-SMA in LECs are associated with EMT. As shown in Figures 4 and 5, the only two markers that behave similarly in both cell lines are fibronectin and collagen. Differences in collagen I/III levels were also noted (Figure 4), with FHL124 cells showing a 38% increase in collagen production upon TGFβ2 stimulation ($p<0.05$);

HLE B3 cells show a lower but still significant increase of 21% ($p<0.05$). Both cell lines show significantly reduced collagen production after TGFβ2 and MMP inhibitor treatment ($p<0.02$); however, HLE B3 cells respond more effectively, showing complete prevention of collagen I/III production. FHL 124 cells, under the same conditions and treatment, still show a 12%-14% more collagen I/III than untreated controls when co-treated with TGFβ2 and either inhibitor.

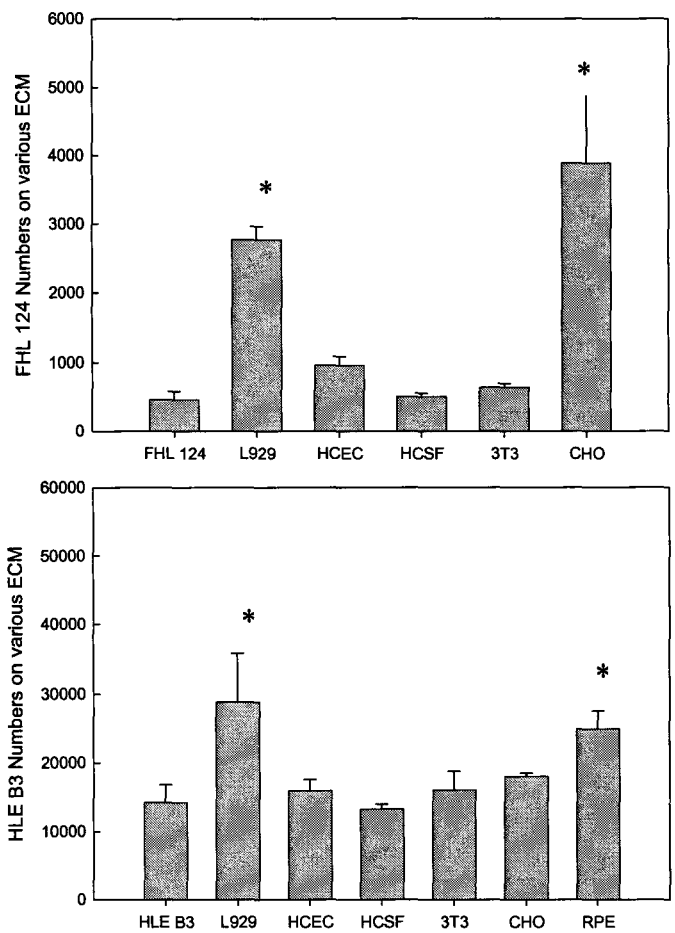

Figure 2: Cell numbers after 6 days of incubation on ECM produced by a variety of cell lines. Both FHL124 and HLE B3 cells grew better on ECM produced by L929 cells, compared to their own ECM. FHL124 also grew significantly better on HCEC and CHO produced ECM, while HLE B3 cells showed preference for RPE ECM. (* $p<0.0005$).

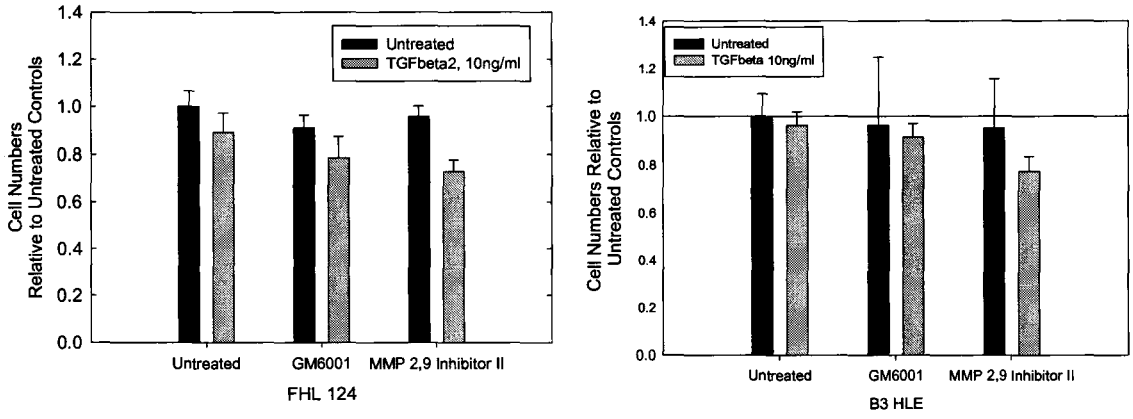

Figure 3: Cell numbers decreased significantly when both cell lines were co-treated with TGFβ2 and MMP 2/9 Inhibitor II solution for 6 days (* p<0.005). However, th ' oad inhibitor GM6001 did not cause a * nificant decrease in cell numbers (p=0.68,.

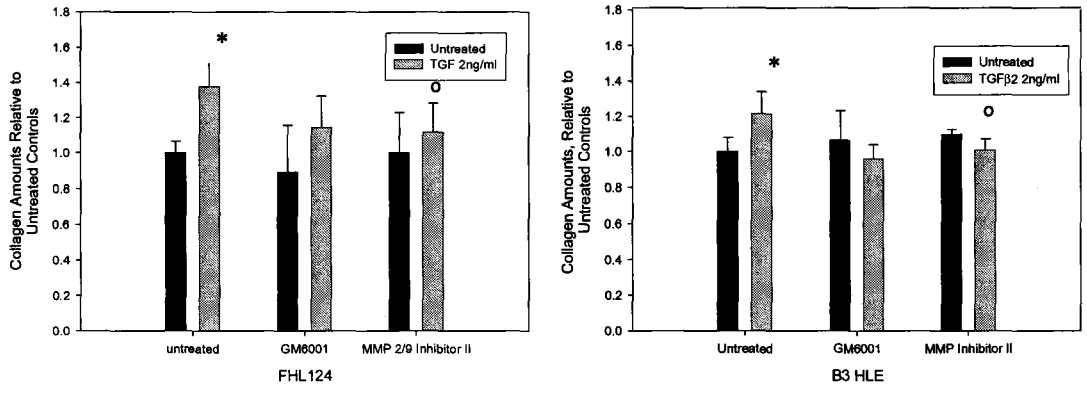

Figure 4: Collagen levels increased with TGFβ2 treatment in both cell lines, after 6 days. However, while both drugs were capable of minimizing this increase, the effect was more pronounced in HLE B3 cells. Significance was calculated relative to untreated controls (* p<0.05), and relative to TGFβ2 treated controls (o p<0.02).

Differences in the basal levels of laminin are depicted in Figure 6. While none of the treatment conditions led to changes in HLE B3 expression of laminin, the FHL124 cells show diminished laminin levels after TGFβ2 treatment, a response that is prevented with the addition of MMP 2/9 Inhibitor II. Fibronectin levels were significantly (p<0.005) elevated (approximately by 65%) in both cell lines by the presence of TGF-β2; none of the inhibitor treatments was able to suppress this increase (Figure 5).

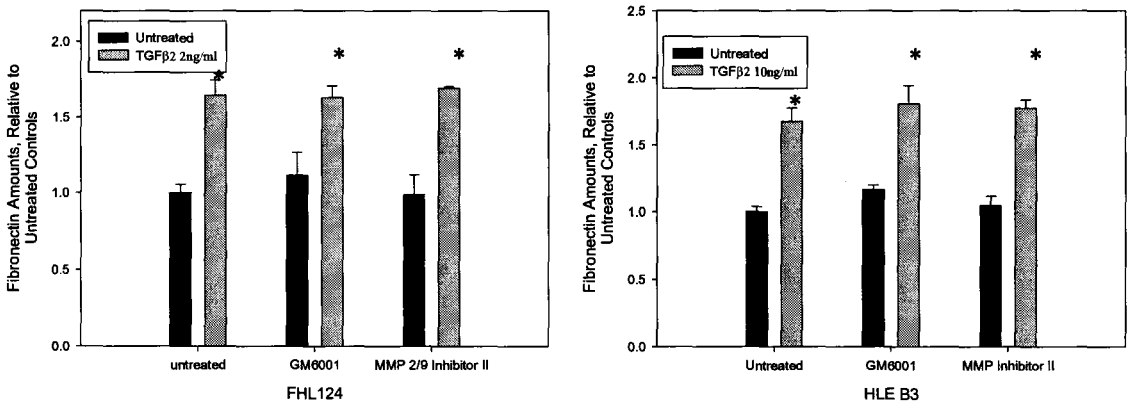

Figure 5: Fibronectin levels significantly increased in both cell lines by TGFβ2 treatment, after 6 days, and neither MMPI treatment was able to reverse this effect in either cell line. (* $p<0.005$)

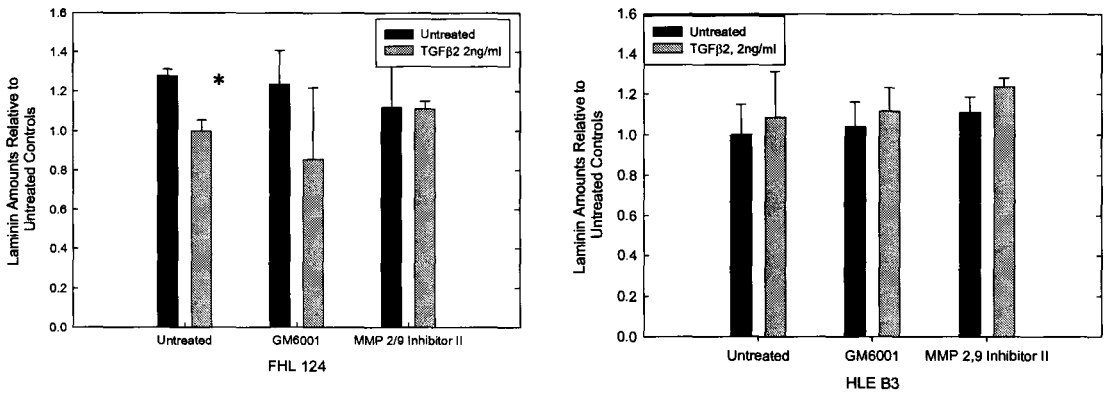

Figure 6: Laminin levels fluctuated significantly only for FHL124 cells when treated with TGFβ2 for 6 days ($p<0.005$). Only MMP 2/9 Inhibitor II was able to protect the cells from this change. HLE B3 cells did not show any significant fluctuation in their laminin levels in the same conditions.

Differences in E-Cadherin, shown to be an important mediator of cell migration in lens epithelial cells, are presented in Figure 7. While FHL124 cells show no differences with the various treatments, HLE B3 show reduced levels of membrane-bound E-

Cadherin in the presence of TGFβ2, which is prevented by treatment with the MMP inhibitors.

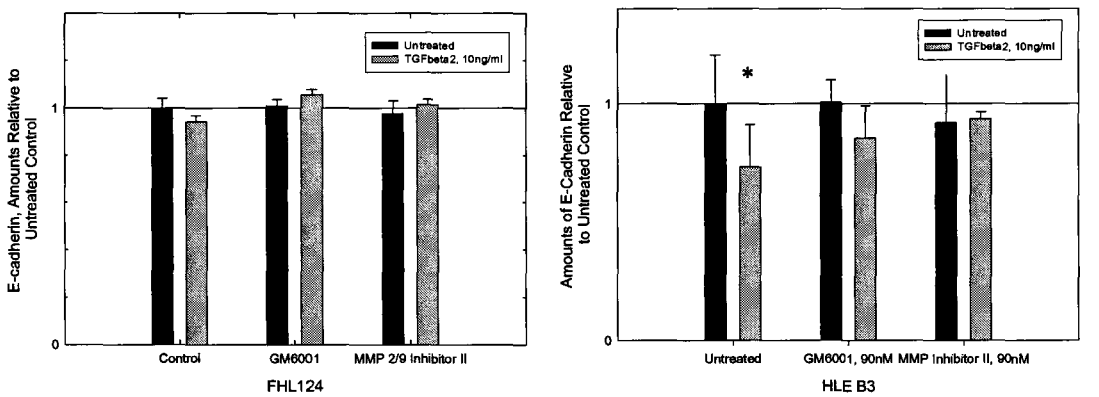

Figure 7: E-Cadherin levels remained unchanged regardless of treatment in FHL 124 cells. In HLE B3, TGFβ2 caused a significant reduction in membrane bound levels of E-Cadherin protein ($p<0.05$), avoided by MMP 2/9 Inhibitor co-treatment. Protein levels were measured after 6 days of treatment.

Figure 8 emphasizes the known ability of TGFβ2 to stimulate α-SMA production in both cell lines. Results are normalized to the amounts of α-SMA present in cells 48 hr prior to the treatment, as both cell lines have been shown to produce basal amounts of α-SMA under normal culture conditions. Both cell lines show a minor significant elevation in α-SMA levels (20%-25%) as a result of TGFβ2 treatment ($p<0.05$). However, this increase can only be blocked by MMP 2/9 Inhibitor II in both cell lines; the other MMP inhibitors tested were not effective at reducing these levels. Overall, it should be noted that MMP 2/9 Inhibitor II had a greater impact than GM6001 in preventing EMT associated changes in both cell lines. The effect of TGFβ2 was more pronounced on FHL124, as it induced significant ($p<0.05$) changes in four out of the five EMT markers tested.

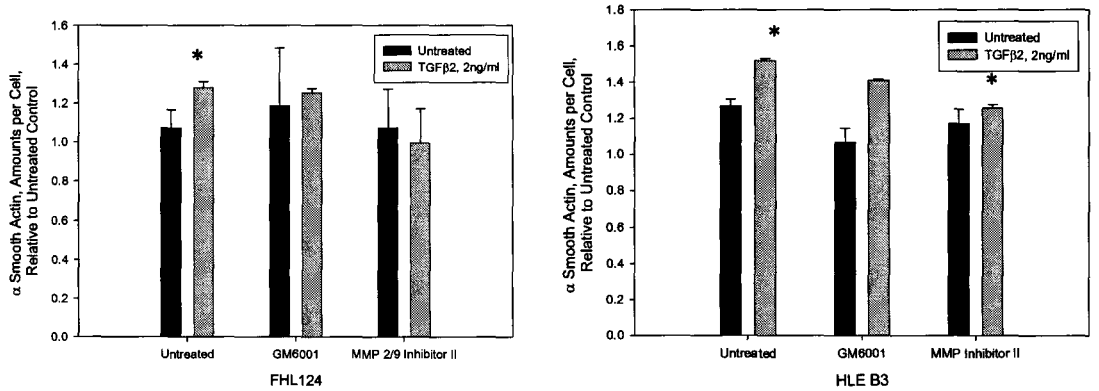

Figure 8: The levels of α-SMA increased by TGFβ2 treatment for 6 days in both cell lines. Neither treatment was able to produce a significant reduction in FHL124 cells. Only the specific inhibitor minimized significantly the increase in α-SMA levels in HLE B3 cells, relative to TGF β2 treated controls. (* $p<0.05$)

4. Discussion

Both HLE B3 and FHL124 cell lines have potential to be used for the development of *in vitro* models for PCO research, as both cell lines present cellular changes consistent with PCO when exposed to TGFβ2. However, these two cell lines have several major differences as a result of their origins and prior treatments. Differences are expected between a primary line and a transformed line, in terms of viability and long-term passaging ability. Another important source of expected differences in behavior stems from the fact that one line is derived from adult cells (FHL 124), while the other is derived from infant cells (HLE B3). Infant epithelial cells have a greater potential for mitosis and are receptive to more stimuli [22,41,42]. This major difference between these established cell lines has not been clearly recognized in literature, and the impact of this difference on research results that utilize only one of the two cell lines has not been acknowledged.

To highlight differences between the unstimulated cell lines, the ECM produced when cells were exposed to two common materials: TCPS and PDMS were examined. PDMS in particular was selected based on the fact that silicone is commonly used in the manufacture of intraocular lenses. ECM is remodeled during growth, and ECM composition is significantly different in the stationary phase than in growth state [54]. Not surprisingly, the ECM composition for the two cell lines varied significantly ($p<0.005$), both in structural protein and GAG composition, in unstimulated conditions. Specifically, the infant-derived cell line HLEB3 showed higher structural protein production on TCPS compared to PDMS, while the adult-derived cell line showed no significant differences (Figure 1, panels a,b,c).

To outline the fact that the two cell lines respond differently to the same growth conditions and stimuli, we exposed the cells to ECMs produced by different cell lines and compared cell numbers on the various surfaces. These systems were examined as it has been shown that ECMs obtained by similar methods are able to still store some cytokines [10,43,44]. While the cytokine composition has not been tested for the various ECM created in this work, it is evident that the two cell lines respond very differently in serum free conditions when grown on the ECM produced similarly. Together with the results from exposure to either TCPS or PDMS, this suggests that the two cell lines studied behave differently when exposed to different surfaces in the same conditions, potentially implying that pediatric and adult patients may react differently to different IOL materials. This is particularly important given that, to date, there have been no reports of IOL

materials or PCO treatments prepared specifically for pediatric patients, despite the fact that extremely high rates of PCO are observed in pediatric patients [55].

When considering these cell lines in studies of factors associated with PCO, it is important to understand their behavior in the presence of TGFβ2, as this cytokine has been widely demonstrated to play a significant role in EMT in lens epithelial cells [6,19]. While the response to TGF β2 has been explored in each cell line individually, the results have not been compared directly. This study shows that while some common EMT markers change in a similar way in both cell lines, there are some important differences. Collagen IV and laminins are the main ECM components of epithelial cells, while fibronectin, collagen I and vimentin are hallmarks of mesenchymal cells [14,27]. While lens epithelial cells are tightly bound to each other via E-Cadherin and ZO-1 molecules, both of these anchors have been found to be degraded in the mesenchymal cell phenotype [14,19,45-47] where anchorage via the integrinβ1-fibronectin is dominant [48]. Mesenchymal cells also become mobile in the lens via RhoA mediated rearrangement of the cytoskeleton as well as integrinβ1-fibronectin connections [49,50]. It is clear for example that when using HLE B3 cells, laminin should not be used as an epithelial marker in a test for PCO, while E-Cadherin protein levels should not be used in FHL124 cells, when testing for PCO. On the same note, this research indicates that fibronectin levels are not a good measure of MMP inhibitor ability to prevent PCO related changes in either cell line. Furthermore, this work suggests that the specific MMP 2/9 Inhibitor II is more efficient than the general MMP inhibitor GM6001, in both cell lines. GM6001 is potent against several MMP enzymes [51,52]. Since MMPs other than MMP-2 and

MMP-9 are active in the lens cells [30,32,53], it is expected that similar concentrations of GM6001 will be less effective than MMP 2/9 inhibitor II specifically against MMP-2 and MMP-9 as there is a high potential for the GM6001 inhibitor to bind to other active MMPs that it can bind. Because of its broader spectrum of activity, it is also expected that GM6001, at higher concentration would be capable to affect more aspects of LEC phenotype and migration, than the specific inhibitor.

In conclusion, while this research clearly shows that HLE B3 and FHL 124 cell lines show some major differences in the unstimulated state, as well as showing different behaviors and different responses to TGFβ2 stimuli, data obtained using lens epithelial cell lines may provide an important screening tool in assessing material and drug specific effects. However care should be taken in selecting an appropriate marker for each cell line. Ultimately, to better understand the response to potential treatments, capsule explants should be examined and the results compared to those obtained in the current work.

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5.0 PAPER THREE: EFFECT OF DELIVERY OF MMP INHIBITORS FROM PDMS AS A MODEL IOL MATERIAL ON PCO MARKERS

Authors: **Diana Morarescu**, Judith A. West-Mays, Heather Sheardown

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Objectives:

The objective of this work was to develop silicone based systems capable of releasing synthetic MMP inhibitors in active form over a prolonged time period.

Main Scientific Contributions:

1. MMP inhibitors GM6001 and MMP 2/9 inhibitor can be released in active form from a PDMS matrix for up to 5 months in some cases; however, activity of released inhibitors decreased significantly after 1 month.

2. Released MMP inhibitors have the potential to inhibit TGFβ2 stimulated increases in collagen I/III production in HLE B3 cells *in vitro*. Therefore, these molecules have the potential to counter TGFβ2 induced EMT related changes in lens epithelial cells.

3. Several preparations were also found to have the ability to significantly reduce FHl124 *in vitro* cell migration using scratch assays. The specific MMP-2/9 inhibitor was found to be more potent than the generic MMP inhibitor, at similar concentrations.

4. While a cell migration reduction of up to 80% has been observed with solutions of the specific inhibitor, the released formulations were found to significantly reduce migration by 20-30%. However, this may not be sufficient to block the transformation and migration of all LECs to the posterior prior to formation of the physical capsular bend barrier.

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Effect of delivery of MMP inhibitors from PDMS as a model IOL material on PCO markers

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ABSTRACT

Posterior capsule opacification (PCO) or secondary cataract formation, following intraocular lens implantation, is a significant complication affecting an estimated 28% of cataract patients. Matrix metalloproteinases (MMPs) have been demonstrated to play a role in the formation of anterior subcapsular cataracts and it has been shown that the presence of MMP inhibitors (MMPI) decreases subcapsular cataract formation *ex vivo*. Since the mechanisms responsible for anterior subcapsular cataract formation and posterior capsule opacification are similar, it is reasonable to suggest that MMP inhibitors may also mitigate PCO. One of the most effective ways of delivering the inhibitors may be from the implanted intraocular lens (IOL) material itself. In the current work, delivery of three different MMP inhibitors from silicone rubber as a model IOL material was examined. Loading methods were developed which allowed continuous release of active MMPI for periods of over 5 months in some cases. Reduced migration rates were observed in human lens epithelial cells *in vitro*, suggesting that an effect on PCO may be possible. While further studies are necessary to tune the systems to achieve the desired rates of release, this work demonstrates that delivery of MMPI from silicone IOL materials has the potential to decrease the incidence of PCO.

1. Introduction

Intraocular lenses (IOLs) have been used successfully for over 50 years to replace the cataractous human lens. However, the frequent occurrence of secondary cataracts (posterior capsule opacification, PCO) requiring subsequent treatment, recently estimated at 28% within 5 years after the surgery, with rates as high as 70% and 78% reported in young (<40 years old) and pediatric patients respectively [1–3], suggests the need for improvements. Recent advances, including redesign of IOLs to incorporate square edges and special haptics [3–6], combined with improvements in surgical technique [7–10], have reduced the occurrence of PCO, but unacceptably high incidences remain. Secondary cataracts are relatively easily treated by Nd:YAG laser but this procedure, aside from the obvious financial burden to the health care system and inconvenience for the patient, can lead to such complications as recurrence of the opacity, cystoid macular edema, retinal detachment, and glaucoma [11–14]. Furthermore, PCO is of particular concern in pediatric patients, due to the high incidence and that fact that delay in diagnosis can cause irreparable amblyopia [2,15].

Secondary cataracts are the result of a variety of cellular changes that lead to excessive deposition of extracellular matrix components, particularly type I, III and IV collagen, as well as wrinkling of the capsular bag, linked to the presence of α-smooth actin (α-SMA) [8,16–18]. Lens epithelial cells (LECs) remaining in the equatorial region of the lens capsule following cataract surgery, migrate postoperatively due to a stress-induced phenotypical change known as epithelial to mesenchymal transition (EMT) [17,19]. The EMT process is thought to be initiated by the presence of transforming growth factor β2 (TGFβ2) [19,20] or epidermal growth factor [21] or which are released by these remaining lens cells as a wound healing response, following the mechanical stress of cataract removal and IOL implantation [8,16,17]. While the complete cascade of events that leads to EMT has yet to be elucidated, activation of the matrix metalloproteinases MMP-2 and MMP-9 in the lens has been observed as a result of the increased growth factor levels [16,20]. Treatment of anterior subcapsular cataracts, which have similar pathology to PCO with MMP inhibitors (MMPI) has been shown to minimize secondary cataract formation [20]. Therefore it is hypothesized that delivery of

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MMPI to any remaining LECs after cataract surgery might have therapeutic potential improving the long-term success of IOL devices.

Silicones with indices of refraction varying between 1.382 and 1.600 are commonly used in the manufacture of IOL materials. This wide range of refraction is advantageous for drug release, as the thickness of the IOL can be adjusted to incorporate variable amounts of drug [22]. Furthermore, silicone IOLs are preferred by some surgeons for their rapid unfolding times, relative to acrylic materials [1,23]. While some studies have demonstrated significantly lower PCO rates with silicone IOLs than with their acrylic counterparts [1,6,24] there is no consensus in the literature about which of the two materials has superior performance. Release of drugs from IOL materials, particularly acrylic IOL materials, has been previously examined as a means of reducing post-operative infection and inflammation [25–29]. Controlled delivery of agents such as daunorubicin [30], indomethacin [30–32], diclofenac sodium, tranilast, mitomycin C, colchicines, ethylene diamine tetraacetic acid [33], 5-fluorouracil [33,34], flurbiprofen [28], gatifloxacin and levofloxacin [29] and fibroblast growth factor 2-saporin [35,36] has been proposed as a method of reducing PCO in canine, rabbit or bovine eyes, although success to date has been limited. In addition to their widespread use as IOL materials, silicones were selected in the current work due to their previous extensive use in controlled drug delivery applications, in patches or coatings, with successful outcomes for both protein and low molecular weight drugs [29,37–39].

In this study, release of three MMPIs, a general inhibitor, GM6001 [40], and two specific inhibitors, MMP 2/9 Inhibitor I and MMP 2/9 Inhibitor II [41] from silicone rubber (poly(dimethyl)siloxane (PDMS)), as a model lens material, was investigated. Previous studies have demonstrated that treatment of lens epithelial cells with GM6001 may reduce both MMP-2 and MMP-9 levels, and the ability of lens epithelial cells to migrate [42–44]. The latter is important as some researchers suggest that inhibiting cell migration in the first month post-surgery may be sufficient to prevent PCO, since after this time, the formation of a mechanical barrier known as the capsular bend is thought to minimize access of the LECs to the posterior capsule [45,46]. Therefore, the current work focuses on investigating whether the MMPIs can be delivered in active form over periods of one month or more and whether these released inhibitors have the potential to impact lens epithelial migration. Since the released inhibitors may diffuse to other ocular tissues, the effect of MMPIs on neighboring ocular cells was also examined.

2. Materials and methods

2.1. Sample preparation

All reagents, unless otherwise specified, were purchased from Sigma Aldrich (Oakville, ON). PDMS was prepared from Sylgard 184 from Dow Corning (Midland, MI), according to the manufacturer's instructions, using 10:1 ratio of elastomer base to curing agent. Disks with a diameter of approximately 0.6 cm and a thickness of approximately 0.5 mm were punched from the PDMS films. The MMPIs, GM6001, MMP 2/9 Inhibitor I and MMP 2/9 Inhibitor II (Calbiochem, San Diego, CA) were dissolved in either dimethyl formamide (DMF) or ethanol. The inhibitors are of similar molecular weight, but differ in functional groups (see Table 1). The appropriate inhibitor solution was mixed with the PDMS elastomer base prior to the addition of curing agent. Following addition of the curing agent, drug-loaded films were cured for approximately 48 h at 37 °C.

On average, the disks with high concentration of drugs, (HC) contained approximately 55 nmols of inhibitor per disk (GM6001 and MMP 2/9 Inhibitor II) and approximately 86 nmols per disk for MMP 2/9 Inhibitor I. Approximately 6.4 nmols of inhibitor GM6001 and MMP 2/9 Inhibitor II were loaded per disk for the lower concentration samples (LC). These loadings were determined based on the solubility of the drug in solvent.

An alternative loading method involved soaking PDMS disks in solutions of inhibitors in ethanol (250 μM for GM6001 and 150 μM for MMP 2/9 Inhibitor II) for 4 days with mild shaking, followed by evaporation of the solvent for 48 h in the fume hood. Disks prepared in this way are denoted as SOAK.

2.2. Refractive index and light transmittance measurements

A digital hand-held pocket refractometer, (Atago, Bellevue, WA) was used to measure refractive index of the cured disks. The optical properties of the material were also determined by measuring transmittance at 405 nm, 540 nm, 595 nm and 630 nm with a BioRad plate reader. All measurements were done in triplicate.

2.3. Inhibitor release

Release studies were performed in triplicate, using 6 disks, produced from the same sheet of PDMS, or soaked in the same inhibitor solution. Each inhibitor-loaded silicone disk was incubated in a known volume (400–600 μl depending on release time) of phosphate buffered saline (PBS, pH 7.4) at 37 °C with mild agitation. This relatively small release volume was selected to mimic the relatively low volume of fluid that would be expected in the lens capsule. At regular time intervals, the disks were transferred to a known volume of fresh buffer solution, and the releasates were analyzed. The concentration of the drug was measured spectrophotometrically based on a standard curve.

2.4. MMPI activity assay

Activity of the released MMPIs from disks prepared with high concentration (HC) solutions of inhibitors was measured using a modified version of the assay for MMP-2 activity [41]. Briefly, human active recombinant MMP-9 enzyme (Calbiochem, San Diego, CA) was diluted to 200 ng/ml in PBS; 2 ng was used per reaction. MMP Substrate III Fluorogenic (Calbiochem, San Diego, CA) was diluted to 12.5 mM in PBS. The reactions were set in 50 mM TrisHCl, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM ZnCl, 0.01% Brij35. A 250 μl volume of sample was placed into a 96-well plate and the substrate and enzyme added. The change in the relative fluorescence units (ΔRFU) was measured using an excitation wavelength of 340 nm and an emission wavelength of 485 nm. The initial reading was made immediately following addition of the substrate, and the final reading was performed after 18 h. Calibration and quantification of the results was based on fresh inhibitors of known concentration.

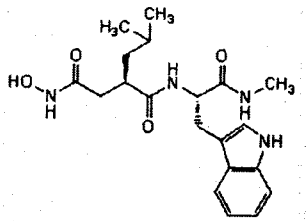
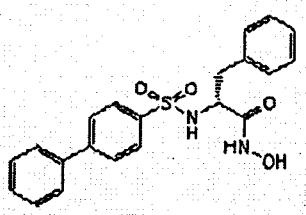
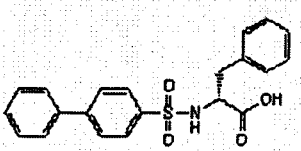
Due to the lower concentrations of inhibitor present in the solution, activity of the released MMPIs from LC and SOAK samples was measured using a modified version of the Invitrogen EnzCheck gelatinase activity kit (Invitrogen, Burlington, ON), using human active recombinant MMP-9 enzyme (Calbiochem, San Diego, CA), and DQ-gelatin substrate (Invitrogen, Burlington, ON). The enzyme was diluted to 75 ng/ml in reaction buffer, and 10 μl was used per reaction. The DQ-gelatin substrate was diluted to 250 μg/ml in reaction buffer, and 20 μl were used per reaction. The reactions were performed in 50 mM TrisHCl, 150 mM NaCl, 5 mM $CaCl_2$, and 1 μM ZnCl. An 80 μl volume of sample was placed into a 96-well plate and the enzyme and substrate added. The change in the relative fluorescence units (ΔRFU) was measured using an excitation wavelength of 495 nm and an emission wavelength of 518 nm. As above, the initial reading was made immediately after adding the substrate, while in this case, the final reading was performed after 2 h. Calibration of the results was based on fresh inhibitors of known concentration.

2.5. Cell toxicity assay

To examine potential effects of released MMPI on ocular cells, cell toxicity studies were performed. All cell media and supplements were purchased from Invitrogen (Burlington ON) unless otherwise stated. Human corneal epithelial cells were grown in keratinocyte serum-free media with complete supplement. Human corneal stromal fibroblasts were grown in M199 media with 1% ITS supplements (Becton Dickinson, Mississauga, ON). Human retinal pigment epithelial (RPE) cells were grown in DMEM:F12 media, with 5% FBS, 1% L-glutamine and 0.8% sodium bicarbonate. The human lens epithelial cell line, FHL 124, was grown in MEM media, with 1% L-glutamine, 10% FBS and 50 μg/ml gentamycin. The human lens epithelial cell line B3 was grown in MEM:F15 media (Sigma, Oakville, ON), with 1% L-glutamine, 0.8% sodium bicarbonate, 20% FBS, 1% sodium pyruvate. All media preparations also contained 1% penicillin–streptomycin solution. Cells were seeded in 24-well plates at a density of 30,000 cells per well. They were incubated in the appropriate growth medium for 3 days at which time the medium was replaced with fresh medium containing 90 nM of either GM6001 or MMP 2/9 Inhibitor II. The concentrations were chosen based on the average concentration of released drug in the first 3 days and based on results obtained previously in the West-Mays lab. The cells were incubated for 1 day and 5 days. On days 1 and 5 following addition of the inhibitor, the cells were detached using Tryple-Express (Invitrogen, Burlington, ON) and were counted using a Beckman Coulter cell counter. Untreated cells were considered 100%, and the treated cells were represented as percentages compared to untreated controls. Significance between treatment groups and untreated controls was assessed by one way ANOVA tests.

Cell viability was also tested using the MTT assay. Briefly, cells were grown in appropriate medium in 96-well plates for 1 or 5 days, in presence or absence of inhibitors (90 nM for GM6001, 0.1 mM for MMP 2/9 Inhibitor I and 900 nM for MMP 2/9 Inhibitor II). MTT reagent was added and cells were incubated for a further 16 h period. At the end, the medium was removed and the purple formazan precipitate

Table 1
Physical properties of the MMP inhibitors used in the study and amounts loaded in each batch.

Inhibitor	Chemical structure	Molecular weight (Da)	Batch	Amount per disk (nmols)	Solvent	Release buffer
GM6001		388.47	HC	55.0	DMF	PBS
			LC	6.4	DMF	PBS
			LC	6.4	DMF	TBS
			SOAK	Unknown	Ethanol	PBS
MMP 2/9 Inhibitor II		396.5	HC	54.0	DMF	PBS
			LC	6.3	Ethanol	PBS
			LC	6.3	Ethanol	TBS
			SOAK	Unknown	Ethanol	PBS
MMP 2/9 Inhibitor I		396.5	HC	86.0	DMF	PBS

was dissolved in 100 µl DMSO. The concentration was measured using a BioRad 96-well plate reader at 570 nm, with a reference filter at 630 nm. Viability was assessed by comparison to the untreated controls. Significance between treatment groups and untreated controls was assessed by one way ANOVA tests.

2.6. Cell migration

While a variety of markers can be used for PCO, migration was selected as the primary method of assessing the effect of the released inhibitors on lens cells since migration is critical to PCO. Following a previously published protocol [48], FHL 124 were grown to confluence, under the conditions described above. The cell monolayer was scratched with a pipet tip and the gap was monitored microscopically using a 20× magnification on a Zeiss Axiovert 200 microscope. The cells were treated with an 800 µM aphidicolin solution, to inhibit mitosis [49]. TGFβ2 levels in the media were measured using an ELSA kit (R&D Systems, Minneapolis, MN). The distance migrated by the cells was measured after 48 h and analyzed using the AxioVision 3.1 software. Significance between treatment groups and untreated controls was assessed by one way ANOVA tests.

2.7. Protein quantification

Collagen presence was detected by staining with a 1% Direct Red 80 solution in water, based on a protocol adapted from Taskiran [47]. Briefly, B3 human lens epithelial cells were exposed to 90 nM solution of inhibitor and 10 ng/ml TGFβ2 (Calbiochem, San Diego, CA) for 48 h. Cells were then either fixed with 10% formalin solution (Sigma, Burlington, ON), or detached and counted on a Beckmann Coulter cell counter. Fixed cells were incubated with a 1% Direct Red 80 solution for 1 h, at 22 °C, with mild agitation. The dye was then quantified by absorbance at 630 nm on a BioRad plate reader. Untreated controls were set as reference. Results were normalized to reflect collagen amounts/cell number.

Immunostaining experiments were done to assess the expression of fibronectin. Cells were fixed (using 4% formalin) and blocked with 5% goat serum in PBS for 30 min. This was followed by incubation with a 1:300 dilution of rabbit anti-fibronectin primary antibody (Abcam, Cambridge, MA) for 4 h at room temperature. Anti-rabbit secondary antibody (1:100 dilution) was subsequently added, and incubated for 2 h at room temperature with mild shaking. The relative concentration of fibronectin on the was determined fluorimetrically using the Perkin–Elmer Victor 3 fluorometer and the stained surfaces were observed using fluorescence microscopy at 20× and 40× magnification. Fluorescence readings were set for λ_{ex} 485 nm; λ_{em} 520 nm. Alpha-smooth muscle actin (α-SMA) was detected fluorometrically using an FITC-labeled primary antibody (Sigma, Oakvile, ON). The relative amount of bound antibody was calculated based on the change in fluorescence units (ΔRFU). Significance between treatment groups and untreated controls was assessed by one way ANOVA tests.

3. Results

3.1. Refractive index and light transmittance measurements

The drug-loaded disks have refractive properties similar to those of PDMS, with values ranging between 1.4107 and 1.3640. All disks were within the refractive index limit for IOLs [22], with the exception of MMP 2/9 Inhibitor II LC samples. There was no significant difference between the GM6001 samples in terms of refractive index, regardless of solvent or amount of drug loaded ($p < 0.05$). However, MMP 2/9 Inhibitor II disks revealed a significant difference in refractive index ($p < 0.05$) when different solvents were used in the preparation of the samples, even when lower amounts of drugs were present (Table 2). Transparency of the materials was not visibly altered in most cases. However, the GM6001 HC disks had significantly lower light transmittance than plain PDMS, however, the disks were still transmitting more light than the normal adult lens [50] (Fig. 1).

Table 2
Refractive index measurements of drug-loaded IOL materials.

Drug	Solvent		Average ± sd
None	DMF	Control	1.4001 ± 0.007
GM6001	DMF	HC	1.4102 ± 0.0001
GM6001	DMF	LC	1.4107 ± 0.001
GM6001	Ethanol	LC	1.4107 ± 0.002
MMP 2/9 Inhibitor II	Ethanol	LC	1.3640 ± 0.007
MMP 2/9 Inhibitor II	DMF	HC	1.3722 ± 0.0016

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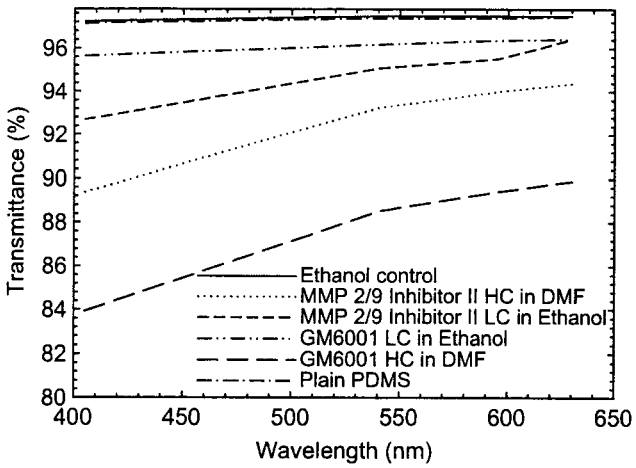

Fig. 1. Light transmittance through PDMS materials at wavelengths within visual range. The values for transmittance are very high similar to those of the crystalline lens of a 4–5 year old child [50]. This indicates material is suitable as an IOL device.

3.2. Inhibitor release

In order to determine the potential of lens materials for the delivery of MMP inhibitors, release from PDMS, as a model material, was monitored over periods of approximately 5 months for HC samples, 6 weeks for LC samples, and 2 weeks for SOAK samples. The release profile for GM6001, a general MMP inhibitor, from HC samples, (Fig. 2a) shows an initial burst of 12–15% of the total amount loaded in the first 24 h. After 6 days, the drug release occurred at a nearly constant rate of approximately 61 pmols/cm^2/h, and continued for another 144 days (see Table 3). For LC, the profile remained similar but the steady state release rate dropped to 8–11 pmols/cm^2/h, a value which is somewhat dependant on the release buffer as shown in Table 3. The steady state release rate could be altered to some extent by changing the concentration of MMPI in the solvent for PDMS loading, as it can be observed in the difference between the curves of HC and LC disks (Fig. 2a). Not unexpectedly, the disks loaded by soaking released the inhibitor at a much faster rate over a shorter time, reaching a relatively constant release rate of approximately 71 pmols/cm^2/h after 72 h of release, and continuing for only another 32 days (Fig. 3).

Similarly, the release of the specific MMP 2/9 Inhibitor II from the HC samples shows a slightly lower initial burst of approximately 8–10% of the total amount in the first 24 h (Fig. 2b). After 6 days, the specific MMP 2/9 Inhibitor II was released at a nearly constant rate of 56 pmols/cm^2/h, a rate relatively similar to that observed with the general inhibitor. The drugs continued to release at this steady rate for another 140 days (Fig. 2b). Likewise, as expected, the steady state release rate could be altered to some extent by changing the concentration of MMPI in the solvent for PDMS loading. For LC disks the constant rate was somewhat more variable but decreased to between 3 and 11 pmols/cm^2/h, in TBS and PBS respectively (Table 3), and continues to release for 45 days. The disks loaded by soaking released the inhibitor at a constant release rate of approximately 3 pmols/cm^2/h after 48 h of release, similar to the LC samples; however these samples only continued release for an additional 14 days (Fig. 3).

Of note, with the LC samples, there is a marked difference in the release of MMP 2/9 Inhibitor II in the two different buffers, a difference that is not observed for the GM6001 inhibitor. Similarly for SOAK, MMP 2/9 Inhibitor II is released much slower, with less of a burst in comparison with GM6001 in the PBS relative to the TBS. The reason for this is as of yet unknown. These buffers were selected to mimic biological fluids and are widely used in drug release studies.

The other specific inhibitor tested, MMP 2/9 Inhibitor I, behaved in a slightly different way, with an initial burst of 25% in the first 24 h. Release of this inhibitor reached a steady rate of approximately 88 pmols/cm^2/h after two weeks and continues to release at approximately this rate for another 48 days (Fig. 3). Other formulations of this drug were not tested, as the release rate obtained with the HC loading conditions was not believed to be sufficient to efficiently inhibit the MMP-2 and MMP-9 naturally present in the lens, since this particular inhibitor has an IC_{50} between 240 and 310 nM [41] and these concentrations may not be easily maintained in active form (data not shown).

3.3. MMP inhibitor activity

To demonstrate that the inhibitors retain functional activity following release from PDMS, an MMP activity assay was developed based on a commercially available assay and performed on random samples of the released GM6001 and MMP 2/9 Inhibitor II. Activity was assessed based on cleavage of a fluorescent substrate. Table 4 summarizes relative activity results based on MMP-9 in the

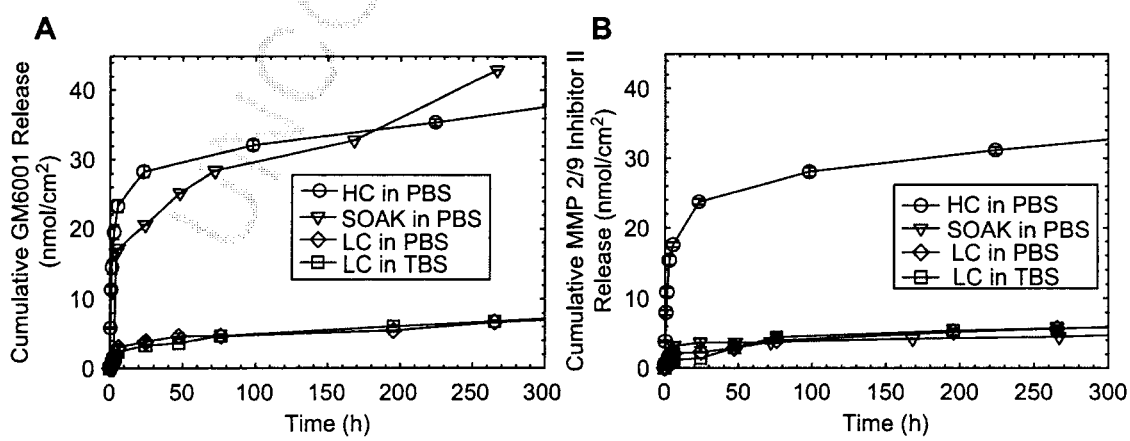

Fig. 2. Release profiles of MMP inhibitors from PDMS. GM6001 release profiles reveal that HC and SOAK formulations have a similar high burst and constant release, while no visible difference is noted for the LC batch, when released in either TBS or PBS (A). In contrast, panel B shows that MMP 2/9 Inhibitor II shows a high burst only in the HC formulation, while the SOAK batch is releasing similarly to the LC formulation.

Table 3
Release details for all inhibitors. Notable differences are seen between loading methods and release buffers for the first two inhibitors.

Batch	GM6001				MMP 2/9 Inhibitor II				MMP 2/9 Inhibitor I
	HC	LC PBS	LC TBS	SOAK	HC	LC PBS	LC TBS	SOAK	HC
Constant rate of release (pmol/cm^2/h)	61.0	8.4	10.8	71.4	56.0	10.7	3.0	3.5	88.2
Initial burst (nmols/cm^2 released in first 5–6 h)	23.3	3.8	2.4	17.1	17.6	2.1	1.1	6.3	15.4
Percentage released after 24 h	24.9	45.6	32.5	n/a	21.1	23.2	14.4	n/a	4.7
Percentage released after 7 days	31.3	63.5	59.8	n/a	27.8	52.1	58.1	n/a	9.4
Percentage released after 4 weeks	40.9	97.2	98.7	n/a	34.8	63.2	69.2	n/a	13.4

presence of fresh and released inhibitor from different samples. The assay was performed on the release samples after various time periods. Controls were inhibitors in PBS samples for appropriate times. Blank samples incubated in the presence of PDMS disks containing no drug were also tested. No effect on enzyme activity was observed (results not shown).

The potency of the freshly prepared solutions was greater than that of the released drug. Regardless, the released MMP inhibitors retained significant activity. The general inhibitor, GM6001, was found to retain approximately 85–95% activity for sampling times below 100 h where the increment of time between sample points was small; activity decreased to approximately 20% at times over 1000 h, where sampling increments were larger. Similarly, the MMP 2/9 Inhibitor II retained 91.6% activity at the initial sample points but the activity was found to decrease to between 20% and 30% with sample times greater than 400 h, and even lower to 4% when sampling times were over 1000 h. However, MMP 2/9 Inhibitor II activity, when released into PBS, was dramatically reduced, with an initial remaining activity of only 40.4% after 1 h, that drops to 30% after only 23 h. Unlike GM6001, the specific inhibitor maintained most of its activity in the first hours of release from SOAK samples, and still retained over 25% activity after a week of release, while GM6001 lost all activity after 24 h of release (Table 4).

3.4. Toxicity to neighboring ocular cell types

Since the released inhibitors could potentially diffuse out of the capsular bag, which is compromised during the surgical procedure, the potential effect of the inhibitors on neighboring ocular cell types was tested. Various human ocular cell lines were examined including corneal epithelial cells, corneal stromal fibroblasts, two lines of lens epithelial cells as well as retinal pigment epithelial cells. Response clearly differed depending on the cell type and inhibitor in question as shown in Fig. 4. Specifically, no response was observed for corneal epithelial and RPE cells with any of the three inhibitors at early times, B3 cells showed decreased viability in the presence of all three inhibitors ($p < 0.05$) while FHL 124 cells showed only a small response to GM6001 (data not shown). By 5 days a dramatic and significant ($p < 0.001$) decrease in cell viability of approximately 40% was observed with corneal epithelial and FHL 124 cells, in response to all three drugs while the RPE cells showed decreased viability in the presence of MMP 2/9 Inhibitor II and GM6001 ($p < 0.0006$) but not MMP 2/9 Inhibitor I. However, the B3 cells were found to be very sensitive to all of the molecules tested, showing a significant ($p < 0.05$) viability decrease in all cases. The corneal stromal cells remained unaffected in all cases.

The concentrations of the inhibitors used in this study were approximately 100 times greater than those reported in the literature to affect cell expression of posterior capsule opacification specific markers [17,20]. However, even at these high concentrations, the inhibitors failed to decrease either cell numbers or cell viability of the various ocular cells examined by 50% or more. Therefore, despite the significant decreases noted in this work in some cases, it is unlikely that the surrounding ocular cells would be significantly affected by the released MMPI.

3.5. Effect of MMPI on protein production

There was no significant change in fibronectin expression in either of the lens cells when treated with TGFβ2 with or without

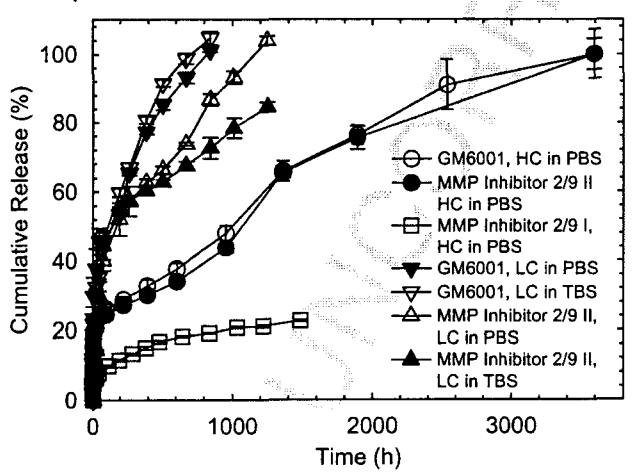

Fig. 3. Percentage release profiles of MMP 2/9 Inhibitor I, MMP 2/9 Inhibitor II and GM6001 outline the possible longevity of the drug-releasing IOL device. The HC formulations show the longest constant release, lasting over 145 days, while the LC formulations release all drug in 35–52 days. Notably, only the release profile of MMP 2/9 Inhibitor II LC is visibly affected by the release buffer, showing a potential longer release in TBS, compared to PBS. Also of interest, the MMP 2/9 Inhibitor I release profile has the longest expected lifetime, however, the current loading capacity of the device might not be sufficient, as this drug is needed in higher concentrations to have the same effect as its counterparts on the target enzymes.

Table 4
Activity profiles of the released inhibitors.

MMP 2/9 Inhibitor II			GM6001		
% Activity	Time (h)[a]	Δt (h)[b]	% Activity	Time (h)[a]	Δt (h)[b]
HC in PBS					
22.0% ± 0.8%	1359.8	408.5			
29.4% ± 3.3%	1890.1	530.3	19.9% ± 3.7%	2535.8	1176.0
3.9% ± 0.5%	3592.8	1702.8	22.4% ± 0.7%	3592.8	1057.0
LC in PBS					
40.4% ± 1.0%	1.0	1.0			
29.8% ± 1.6%	47.3	22.8	85.4% ± 9.2%	265.3	69.9
15.4% ± 0.2%	195.3	119.3	58.2% ± 19.1%	385.0	120.0
LC in TBS					
91.6% ± 3.4%	4.2	1.7			
57.9% ± 2.3%	24.4	18.4	94.2% ± 13.5%	76.0	28.8
45.6% ± 0.8%	385.3	120.0	72.5% ± 11.0%	195.3	119.3
SOAK in PBS					
97.8% ± 7.1%	4.5	1.8	19.3% ± 1.8%	2.8	1.8
34.7% ± 16.1%	72.0	24.0	3.2% ± 0.4%	72.0	24.0
25.6% ± 4.5%	384.0	117.3			

[a] Time elapsed since the start of the release.
[b] Time elapsed between samplings of releasate.

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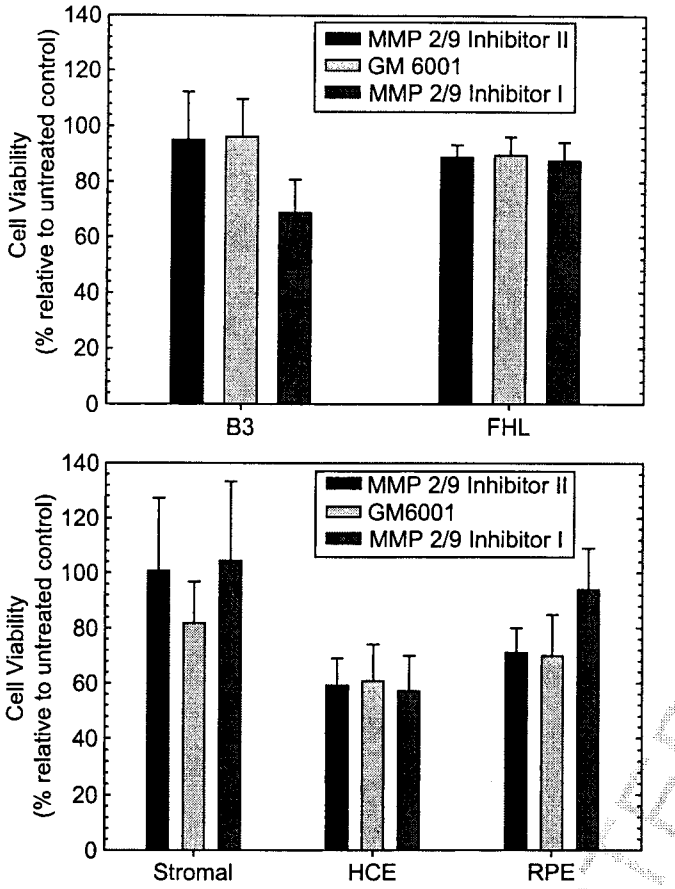

Fig. 4. MMP inhibitors show little or no toxic effect on neighboring ocular cell types. There were slight decreases in the viability of FHL 124 cells remains unchanged with inhibitor treatment while only MMP 2/9 Inhibitor had a significant effect on the viability of B3 cells. While slight but significant decreases in viability were observed in HCE and RPE cells with inhibitor treatment, the corneal stromal fibroblasts were unaffected by even the relatively high concentrations of inhibitor used. Error bars represent standard deviation.

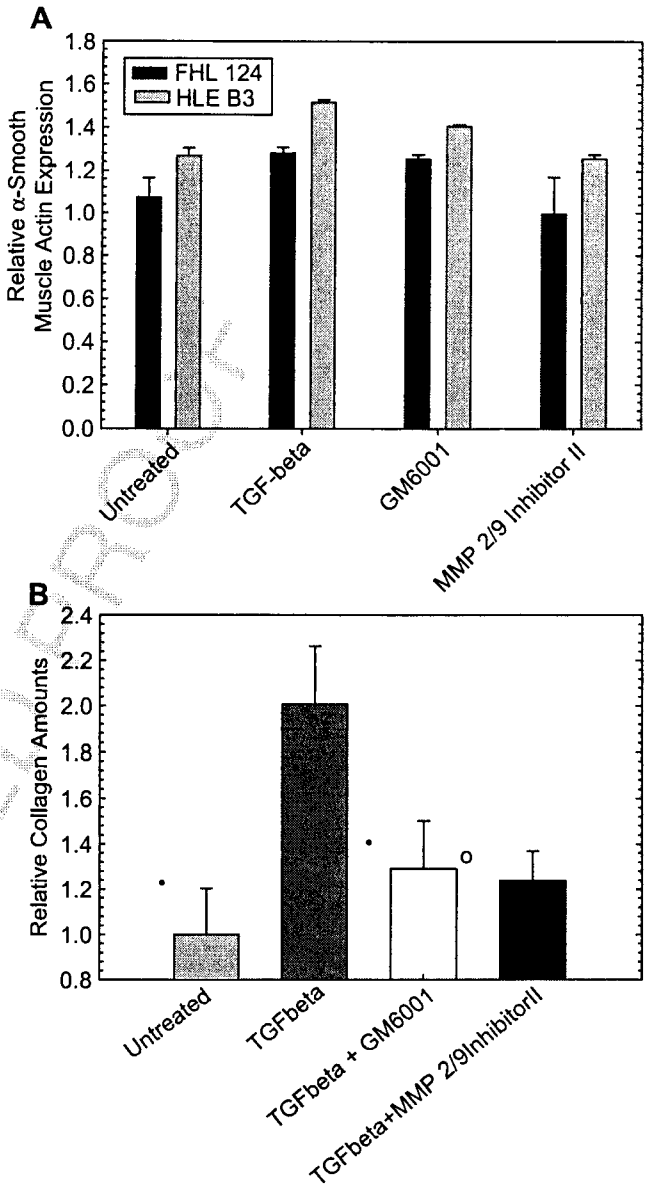

Fig. 5. The MMP inhibitors have the potential to significantly alter the expression of fibrotic markers. A) Treatment of the cells with TGFβ2 results in a significant increase in the expression of α-smooth muscle actin. However, presumably due to the relatively high basal production of this marker, only MMP 2/9 Inhibitor II was found to decrease expression in FHL 124 cells. Both inhibitors tested resulted in a significant reduction in expression in the B3 cells. B) The inhibitors were also able to reduce the amount of collagen I/III produced by B3 HLE *in vitro* after 48 h exposure to both drugs and TGFβ2. While TGFβ2 is capable of increasing the amount of collagen production, as expected, co-treatment with MMPIs reduce the amount of collagen even in comparison to untreated controls. Error bars represent standard deviation. * Indicates $p < 0.0008$; o indicates $p < 0.009$; • indicates $p < 0.05$.

the various MMP inhibitors. With both lens cell lines, a significant increase in the expression of α-smooth muscle actin was observed with TGFβ2 treatment. There was no change in α-SMA expression with inhibitor treatment with the FHL cells but MMP 2/9 Inhibitor II decreased TGFβ2 induced α-smooth muscle actin production by a small but significant ($p < 0.05$) amount (Fig. 5a). Similarly, cells treated with TGFβ2 show an increase in collagen production (Fig. 5b). However, treatment of the TGFβ2 stimulated cells with a 90 nM solution of the MMPI molecules GM6001 and MMP 2/9 Inhibitor II for periods of 48 h leads to a significant ($p < 0.05$) 10–15% decrease in collagen production in HLE B3 cells compared to TGFβ2 treatment alone. These results suggest that these molecules may potentially alter PCO markers in a desired fashion although the relatively high basal production of the markers masks the effects.

3.6. Cell migration

In addition to aberrant matrix deposition, cell migration is a key component of the process which leads to PCO; therefore, a migration assay was used to determine the effect of the inhibitors on lens epithelial cells *in vitro*. After scratching and in the absence of the inhibitor, the FHL 124 cells produce increased amounts of TGFβ2 (Table 5) and migrate into the opening. Of note, these cells reach a 2 ng/ml level of cytokine (the treatment level used in assays other than a scratch assay) by day 3.

Treatment with a 90 nM solution of the GM6001 general inhibitor did not significantly reduce migration as shown in Fig. 6. However, treatment with a 90 nM solution of MMP 2/9 Inhibitor II, resulted in a significant 82% reduction in migration. When the cells were exposed to GM6001 containing disks, migration was reduced by 14% for LC disks, 29% for HC and 31% for SOAK disks, a significant reduction relative to controls ($p < 0.0005$). When exposed to MMP

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Table 5
Amounts of TGFβ2 (pg/ml) in the media following cell sheet scratch demonstrate that cells are capable of producing high levels of TGFβ2 after inducing mechanical stress.

Day	Untreated	MMP 2/9 Inhibitor II, SOAK	GM6001, SOAK	MMP 2/9 Inhibitor II, HC
0	49.4 ± 2.5	49.4 ± 2.5	49.4 ± 2.5	49.4 ± 2.5
2	877.3 ± 43.9	917.5 ± 45.9	383.3 ± 19.2	1203.7 ± 60.2
3	1807.5 ± 90.4	n/a	n/a	n/a
7	4414.3 ± 220.7	n/a	n/a	n/a
9	4312.1 ± 215.6	3672.5 ± 183.6	4859.9 ± 243.0	3461.7 ± 173.1

2/9 Inhibitor II disks, migration was significantly reduced by 42% for HC disks and 33% for SOAK disks ($p < 0.005$). Therefore, delivery of the inhibitors from the disks in all cases led to a significant reduction in migration in the first 48 h following creation of the scratch.

4. Discussion

In this work, MMP inhibitors were demonstrated to reduce LEC migration rate and increase in collagen I/III after TGFβ2 stimulation, both characteristic features of PCO suggesting that delivery of these molecules may have the potential to minimize PCO. While the potential of IOLs for delivering drugs to the eye has been previously demonstrated, the use of drug-releasing IOL materials for mitigating posterior capsule opacification, the main complication of intraocular lens surgery [8,16], has not been widely investigated. A potentially efficient method of delivering MMP inhibitors directly to the migrating lens cells was hypothesized to be using the IOL itself. To assess the potential of this as a treatment methodology, the release of three MMP inhibitors, GM6001, a general inhibitor, and two MMP 2/9 specific inhibitors, from silicone rubber (PDMS) as a model IOL material was examined.

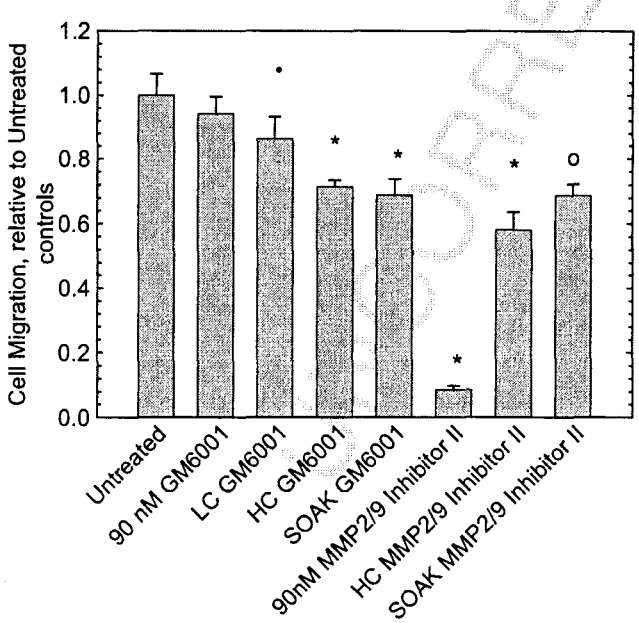

Fig. 6. FHL 124 cell migration is significantly reduced with all prepared disk treatments. A 90 nM solution of GM6001 inhibitor does not significantly reduce migration, while a 90 nM solution of MMP 2/9 Inhibitor II has a very pronounced effect. Error bars represent standard deviation. * Indicates $p < 0.0008$; ○ indicates $p < 0.009$; • indicates $p < 0.05$.

Some research has suggested that MMP-9 activation and production precedes MMP-2 activation and production in TGFβ2 stimulated cells, in ASC studies [51]. As it remains unclear whether MMP-2 or MMP-9 becomes activated first in the process that leads to PCO, the effect of both inhibitors is of interest as they elicit differential preferences for MMPs, with MMP 2/9 Inhibitor II having a stronger influence on MMP-2 and GM6001 having a stronger influence on MMP-9. Of note, the current study included only release profiles and toxicity data for MMP 2/9 Inhibitor I, as the data demonstrate that this compound is much less efficient in inhibiting the target enzymes and would therefore need to be delivered at relatively high rates for efficacy.

For the most part, the loaded materials maintain sufficient transparency to be useful in ophthalmic applications as shown in Fig. 3 and Table 3. From previous studies done in our lab (unpublished data) and from the data presented in Fig. 1 and Table 2, it is clear that loading drugs in combination with solvents can influence the structure, refractive index and transparency of the PDMS network. Therefore, it was felt that the addition of sufficient MMP 2/9 Inhibitor I to affect cellular response would negatively affect the materials properties and make the system unusable in the intended application.

The release profiles presented in Figs. 2 and 3, demonstrate that, following an initial burst, the various inhibitors can be delivered at a relatively constant rate over a period of up to 5 months depending on such factors as loading amounts, conditions of loading and inhibitor properties. For example, the LC sample releasing GM6001 showed virtually complete release after a period of one month, while under similar conditions, the MMP 2/9 Inhibitor II LC samples were found to release over a period of 2 months. Desired duration of release is not known at this time. There is evidence that the wound healing response after IOL implantation in rabbit eyes is approximately 8 weeks in length [52] although there are reports of MMP enzyme and inhibitor activity in human lens explants for periods of up to 18 months [53]. It has also been shown that the production of MMP enzymes is initially decreased in the presence of GM6001 inhibitor, with slow restoration after 2 weeks [43]. Regardless, the variation in the release profiles suggests that efficacious delivery of the inhibitor may be possible by tuning the system and that delivery over a sufficiently long period of time might be feasible.

As expected, release profiles (Fig. 2) show a large initial burst of inhibitors in the first 24 h; the amounts released during this burst period may actually be desirable as they would presumably be sufficient to inhibit the higher amounts of MMP present following cataract surgery. Ultimately, however, more detailed studies will be necessary to determine for example if a relatively short period of release immediately following implantation is sufficient to inhibit EMT. As presented in Fig. 5, both inhibitors have the capacity to reduce collagen I/III production and to a lesser extent α-smooth muscle actin in presence of TGFβ2, after 48 h.

A hallmark of TGFβ2 stimulated lens cells is their capability to migrate following EMT [54]. MMP levels, especially MMP-2 and MMP-9 protein and mRNA levels, also increase following EMT [55,56]. It was thus hypothesized that MMP inhibitors would play a major role in inhibiting the cell migration process. Research has shown that *in vivo*, a period of 7–11 days of migration is necessary for the remaining lens epithelial cells in the anterior capsule to reach the posterior [57–59]. With a square-edged IOL, the best PCO prevention method to date [3,60], the capsular bend, formed in 2–4 weeks [57,58,61], creates a physical barrier preventing the LECs from reaching the posterior [61,62]. It is thus speculated that it is crucial to aggressively reduce migration and LEC viability during the first 2–4 weeks post-surgery, in order to prevent the cells from reaching the posterior prior to capsular bend formation.

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The released inhibitors were able to significantly ($p < 0.005$) decrease LEC (FHL 124) migration in the first 48 h, in all formulations (Fig. 6). The results represent only cell migration, as the cells have been treated with aphidicolin, a mitosis inhibitor that allows for mRNA production and protein synthesis, but blocks DNA gyrase [49]. Preliminary studies demonstrated that a 800 μM concentration of aphidicolin was sufficient to block incorporation of labeled nucleotides into LEC DNA, as tested with the Click-IT Edu kit (Invitrogen, Burlington, ON). The most efficient formulation proved to be the MMP 2/9 Inhibitor II HC disks, which reduced migration by 42% ($p < 0.005$). However, of note, SOAK formulations of both inhibitors were also able to reduce migration by approximately 30%. This result is of particular interest, since manufacturing of such IOL materials does not have to interfere with current fabrication processes, and it may therefore be possible to incorporate the inhibitors as a final step in IOL preparation. While these results are promising, additional studies are needed to determine the impact on migration over longer periods of time. As well, studies on human capsule explants and *in vivo* testing will also be necessary to further validate these preliminary results.

A significant advantage to using MMP inhibitors for mitigating PCO is that the effects of these compounds are mainly on cellular transformation and therefore cellular toxicity is not expected to be significant. To test this hypothesis, the effect of the active MMP inhibitors on various ocular cells was examined (Fig. 4). The general inhibitor, GM6001 had the greatest effect on the cell populations tested, as expected, since this molecule can affect several pathways by inhibiting a large number of enzymes. However, even at high concentrations, this potent inhibitor did not reduce cell numbers by more than 30%, with the most affected being the corneal stromal fibroblast line. The MTT viability assay demonstrated both slower growth and reduced mitochondrial function in some cases. Slower growth is a more desirable side effect as cells in the eye are mostly in a fully differentiated state, and are not actively growing. Immediate effects of drugs, after one day exposure were observed and exposure for 5 days was found to cause significant decreases in viability in most cell lines, as expected. In all cases, the concentrations of drugs tested in the viability assay were high based on the total amounts loaded and released; accumulation in ocular compartments other than the lens capsular bag is not anticipated. Therefore, the relatively low levels of toxicity that were observed with the very high concentrations of MMP inhibitors examined suggest that delivery of the inhibitors from the IOL has potential to affect cellular function of the remaining lens epithelial cells without significantly adversely affecting other cell types in the eye.

It is clear that both release duration and amount of inhibitor released can be altered by changing relatively simple key loading parameters. Furthermore, as shown in Table 4, it is clear that the inhibitors can be released in active form although in most cases, some activity was lost, particularly when the inhibitors were released over much longer durations. However, this loss of activity was thought to be at least in part due to hydrolysis which occurred during the long incremental time periods between samplings [41,63]. Together with the released inhibitor capacity to reduce collagen I/III production and LEC migration rates, this research demonstrates that the delivery of MMP inhibitors from IOL materials has great potential to mitigate PCO.

5. Conclusions

In the current work release of MMP inhibitors from silicones as model lens materials was demonstrated. Release durations of more than 5 months were possible. Inhibitors were active and resulted in cellular changes consistent with decreased EMT. While further investigations are needed to demonstrate the potential of these released inhibitors in ablating PCO *in vivo*, these results suggest that MMP inhibitors can be released from IOL materials at concentrations appropriate for inhibition of MMP-2 and MMP-9 activity in the human lens capsule, which may mitigate anterior subcapsular cataract formation *in vitro*. Furthermore, these molecules at high concentrations were found to have only a relatively small effect on other ocular cell types, presumably slowing growth. The disks produced in this experiment were able to significantly reduce both collagen levels, and lens epithelial cell migration after 48 h of exposure *in vitro*. Further work will focus on examining the effect of the released inhibitors on lens cells, specifically related to the inhibition of EMT and long-term LEC migration. Therefore, delivery of MMPI drugs directly to the LECs from the IOL may represent a very promising solution to reduce the incidence of secondary cataract formation.

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6.0 PAPER FOUR: CHELATORS FOR MITIGATING SECONDARY CATARACTS: DELIVERY FROM PDMS AS A MODEL INTRAOCULAR LENS MATERIAL

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Objectives:

The objective of this work was to determine whether, chelators can be used as MMP inhibitors block migration of LECs in the first 4-6 weeks after surgery based on the fact that MMP binding to MMP inhibitors is a zinc dependent process. Furthermore, release of biologically active chelators from a PDMS matrix, for sufficient periods of time was also tested.

Main Scientific Contributions:

1. Chelators were found to be potent MMP inhibitors which maintain high activity even after prolonged periods of release.

2. The released chelators induced cell detachment and lowered the viability of several ocular cell lines, indicating the potential for toxicity in case of accumulation

3. 1-10 phenanthroline was the only chelator tested that was able to significantly reduce LEC migration rates. The effect was greater than that observed with the synthetic MMP inhibitors. However, these molecules were found to be more toxic to other ocular cell types that the MMPIs.

Abstract

Posterior capsule opacification (PCO) remains the most common complication of cataract surgery, with 20-40% of patients needing additional intervention in the first 5 years post-surgery. Matrix metalloproteinases (MMPs) are thought to play an essential role in migration of any lens epithelial cells (LECs) to the posterior capsule, which is ultimately thought to lead to the occurrence of PCO. In the current work, we explore the hypothesis that the release of chelator molecules from model intraocular lens materials may be effective at inhibiting LEC migration based on the mechanism of action of MMPs. Three different chelators, ethylenediaminetetraacetic acid (EDTA), phenanthroline and N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), loaded and released from silicone rubber as a model intraocular lens (IOL) material, were examined. Using two different methods of loading, continuous release of active chelator molecules for periods of over 3 months in some cases was observed. The activity of released chelators was not influenced by loading conditions, but was found to be altered by the composition of the release medium. Reduced migration rates were observed in the human lens epithelial cell line FHL124 *in vitro* upon exposure to the released inhibitors. While accumulation of these molecules in the ocular tissues is not expected due to the effective drainage processes in the eye, toxicity tests demonstrated that high concentrations of the released chelators may have damaging effects on most ocular cell populations. Therefore control over the amounts of the chelators released would be necessary. Further studies are therefore necessary to optimize the systems in order to achieve efficacious rates of release. However, since delivery of chelators from model

silicone IOL materials has the potential to decrease migration of lens epithelial cells, this treatment method may ultimately be useful for decreasing the incidence of PCO.

Keywords: EDTA, TPEN, 1-10 phenanthroline, polydimethylsiloxane, intraocular lens, controlled drug release, lens epithelial cell migration

1. Introduction

While cataract surgery is currently the most performed surgery in the world, the high incidence of secondary cataracts, and specifically posterior capsule opacification (PCO), still creates a major burden to both health care systems and patients. In past years, advances in IOL design have dramatically reduced the rate of PCO requiring follow up treatment to 20-40% of patients within 5 years of surgery [1]; however, rates as high as 78% are still reported in pediatric patients [2]. While PCO is relatively easily treated with Nd:Yag lasers in the developed world, this is not a feasible option in developing nations [3,4]. Furthermore, this treatment is not without risk, with associated side effects including retinal detachment and endophthalmitis [5,6]. Therefore, there is clearly a need for better intraocular lenses with fewer side effects.

Secondary cataracts are the result of the migration and trans-differentiation of lens epithelial cells remaining in the lens capsule following surgery [7]. The latter process, known as epithelial to mesenchymal transition (EMT), is followed by aberrant extracellular matrix (ECM) deposition [8]. The combination of this matrix and wrinkling

of the capsule leads to the visual distortions. Research has shown that square edged IOLs lead to the formation of a mechanical barrier to LEC migration known as the capsular bend within one month post-surgery [9,10]. Therefore proposed treatments which prevent these remaining cells from breaching the posterior capsule within the first 4 weeks following surgery may successfully minimize the complication [10]. Two possible approaches are envisioned – reducing cell migration or minimizing cell viability. To date, many studies have focused on the latter approach. In the current work, we propose to examine the former as a means of improving outcomes of cataract surgery.

While lens epithelial cells typically have limited migration ability, following cataract surgery, initiation of migration is thought to stem from the LECs undergoing EMT [11-13]. In the lens, this process can occur due to the normal aging process or can be the result of the stress of the surgical manipulation. Regardless, these have in common an accumulation of activated growth factors, particularly transforming growth factor beta 2 (TGFβ2) [8,12,14]. As a result of TGFβ2 stimulation, expression of matrix metalloproteases (MMPs), particularly MMP-2 and MMP-9, becomes elevated [15,16]. MMP enzymes are a large class of proteases capable of cleaving specific extracellular matrix components whose activity has been associated with the PCO process [16,17]. *In vivo*, it has been shown that lens epithelial cells (LECs) produce elevated levels of MMP-2 and MMP-9 up to 18 months following cataract surgery [17]. Blocking MMP-2 and MMP-9 activity *in vitro* with the synthetic inhibitors GM6001 and MMP 2/9 Inhibitor II has been shown effective in reducing migration rates of LECs [17,18]. However, while effective, this may not be sufficient, as the inhibitors used are somewhat unstable in

aqueous conditions [19,20]. Thus, while blocking MMP enzyme activity seems to have potential, alternative methods of inhibiting these molecules should be considered.

Silicone based IOLs have rapid unfolding times [10,21] and may exhibit lower rates of PCO compared to other IOL materials [22]. Furthermore, silicone materials, especially poly (dimethyl siloxane) (PDMS) have been successfully used in controlled drug release applications [23,24]. Therefore, these may be suitable model materials for the release of MMP inhibiting molecules from IOLs. Incorporating drugs which have the potential to reduce PCO directly into the IOL material may be an effective way to deliver the agent directly to the lens, avoiding additional trauma to the patient. There are a limited number of published studies on drug-releasing IOL materials for PCO prevention; none have used silicone IOL materials [25-31]. While the initial, mainly *in vitro*, results of these studies showed promise, in most cases the focus was drugs that affected cell viability. In the current work, chelators were examined as potential MMP inhibitors. We have previously demonstrated that release from a PDMS matrix is affected by the addition of small amounts of drug containing solvent added prior to curing [32] which will be exploited in the current work to allow for flexibility of manufacturing and for controlling the initial burst and the rate of release.

As MMPs are dependent on divalent cations for their activity, particularly Zn^{2+}, chelators with high zinc activity were selected in the current work for MMP inhibition. N,N,N',N'-Tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) is the most cited Zn^{2+} specific chelator used in literature while ethylenediaminetetraacetic acid (EDTA) was

chosen due to its previous use in studies involving lens cell migration and adhesion properties [29,33]. Commercially available kits for MMP-2 and MMP-9 activity use 1-10 phenanthroline as a positive inhibitor control. The chelators were loaded in PDMS either as part of the manufacturing process, or by soaking after the PDMS was prepared. Activity against MMP-2 and effect on LEC migration *in vitro* were measured.

2. Materials and Methods

2.1. Drug Loading and Release

All reagents, unless otherwise specified, were purchased from Sigma Aldrich (Burlington, ON). PDMS was prepared from Sylgard 184 from Dow Corning (Midland, MI), according to manufacturer's instructions, using 10:1 ratio of elastomer base to curing agent. Disks with a diameter of approximately 0.6 cm and a thickness of approximately 0.5 mm were punched from the PDMS films. To incorporate the chelators during PDMS manufacture, each inhibitor was dissolved in either dimethyl sulfoxide (DMSO) or ethanol at concentrations of 0.3 M. The chelator solution was subsequently added to the PDMS elastomer base (1% v/w) followed by addition of curing agent. For example, the resulting 1-10 phenanthroline disks loaded in this manner (denoted MIX) contained approximately 4.6nmols.

An alternative loading method involved soaking plain PDMS disks in 0.7 M ethanol solutions of each inhibitor. Disks were soaked for 4 days with mild shaking, at

room temperature, followed by evaporation of the solvent for 48 hours in the fume hood (denoted SOAK).

Release studies were performed using 2 separately produced sheets of PDMS, with n=8. For release, each inhibitor-loaded silicone disk was incubated in 600µl phosphate buffered saline (PBS, pH 7.4) at 37°C with mild agitation. This relatively small release volume was selected to mimic the relatively low volume of fluid that would be expected in the lens capsule. At regular time intervals, the disks were transferred to fresh buffer solution, and the releasates were analyzed, either for concentration, for activity, or both. The releasate concentration of 1-10 phenanthroline and TPEN was determined by mixing 100µL of releasate with 100µL of $FeCl_2$ solution and the mixture incubated at room temperature for 30 minutes. The solution was then read at 450nm, with a reference at 630nm. A standard curve was prepared with varied amounts of 1-10 phenanthroline or TPEN and used to determine concentration in releasate solutions.

2.2. MMP-9 Activity Assay

Activity of the releasates was measured using a modified version of the Invitrogen EnzCheck gelatinase activity kit (Invitrogen, Burlington, ON), using human active recombinant MMP-9 enzyme (Calbiochem, San Diego, CA), and DQ-gelatin substrate (Invitrogen, Burlington, ON). The enzyme was diluted to 75 ng/ml in reaction buffer, and 10 µl was used per reaction. The DQ-gelatin substrate was diluted to 250 µg/ml in reaction buffer, and 20 µl was used per reaction. The reactions were set in 50 mM

TrisHCl, 150 mM NaCl, 5 mM $CaCl_2$, and 1 μM ZnCl. An 80 μL volume of sample was placed into a 96 well plate and the enzyme and substrate added. The change in the relative fluorescence units (ΔRFU) was measured using an excitation wavelength of 495 nm and an emission wavelength of 518 nm. The initial reading was made immediately after adding the substrate, while the final reading was performed after 2 hours. Calibration was based on fresh inhibitors of known concentration.

2.3. Cell Toxicity Assay

All cell media and supplements were purchased from Invitrogen, Burlington ON, unless otherwise stated. Human corneal epithelial cells were grown in KSFM media with complete supplement [34]. Human corneal stromal fibroblasts were grown in M199 media with 1% ITS supplements (Becton Dickinson, Mississauga, ON) [35]. Human retinal pigment epithelial (RPE) cells were grown in DMEM:F12 media, with 5% FBS, 1% L-glutamine and 0.8% sodium bicarbonate [36]. The human lens epithelial cell line, FHL 124, was grown in MEM media, with 1% L-glutamine, 10% FBS and 50μg/ml gentamycin [37]. The human lens epithelial cell line B3 was grown in MEM:F15 media (Sigma, Burlington, ON), with 1% L-glutamine, 0.8% sodium bicarbonate, 20% FBS, 1% sodium pyruvate [38]. All media preparations also contained 1% penicillin-streptomycin solution.

Cell viability was tested using the MTT assay. Briefly, cells were grown in appropriate medium in 96 well plates for either 1 day or 5 days, in presence or absence of

the inhibitor solutions (3.5mM of TPEN and 1-10 phenanthroline and 2.5mM EDTA). MTT reagent (Sigma, Burlington, ON) was added and cells were incubated for a further 16 hour period. At the end of the incubation period, the medium was removed and the purple formazan precipitate was dissolved in 100 µl DMSO. The concentration was measured by a BioRad 96-well plate reader at 570 nm, with a reference filter at 630 nm. Viability was assessed by comparison of each test result to the untreated controls, and significance was computed by a t-test.

2.4. Cell Migration

FHL124 were grown to confluence, under the conditions described above. The cell monolayer was scratched with a pipette tip and the gap was monitored microscopically (Zeiss Axiovert 200) at 20x magnification. The cells were treated with a 800µM aphidicolin solution, to inhibit mitosis [39], so that the entire analysis is based on migration of already existing cells. Distance migrated by cells was measured after 48 hr, using AxioVision 3.1 software. Significance was computed by a t-test.

3. Results

3.1. Inhibitor Release and MMP Inhibitor Activity

The release profiles of 1-10 phenanthroline and TPEN are presented in Figures 1 and 2. MIX and SOAK batches of 1-10 phenanthroline show different release profiles, with the SOAK batch having a lower burst and lower release rate.

There is a difference in the burst amount of the MIX batches, between the ethanol dissolved chelator and the DMSO dissolved inhibitor, in concordance with results from previous studies [32]. As ethanol has a higher ability to swell PDMS than DMSO [40], it allows for higher bursts and faster rates of release [32]. MIX batches of EDTA and TPEN were not analyzed, as the PDMS did not cure in the presence of these chelators at any of the concentrations tested. The release profile of 1-10 phenanthroline SOAK and TPEN SOAK were analyzed for concentration and release profiles are presented in Figure 2.

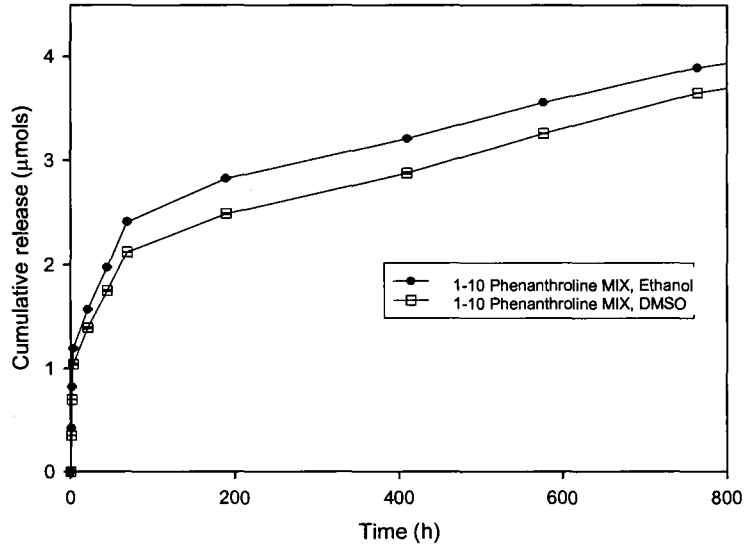

Figure 1: Release of 1-10 Phenanthroline MIX batch. The chelator displays a slight variation in burst rate when loaded dissolved ion ethanol compared to DMSO. Constant release after 200 hours is achieved at a similar rate for both formulations.

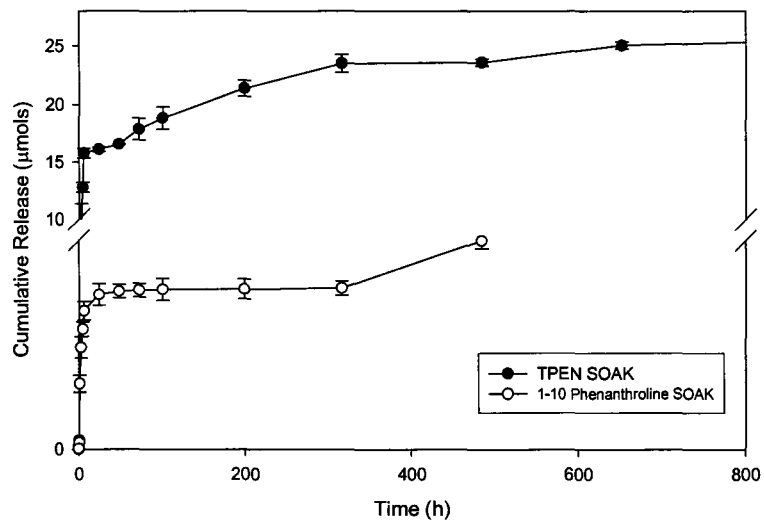

Figure 2: Release of SOAK batches. Even with soaking in equal concentration of inhibitor dissolved in ethanol, TPEN shows both a higher initial burst and a higher constant rate of release in the first 400 hours. This may be an indication of inhibitor/silicone interaction with 1-10 phenanthroline, resulting in a slower prolonged release rate.

3.2 MMP-9 Inhibitory Activity

Released 1-10 phenanthroline and TPEN maintain full activity as expected, since these molecules are not susceptible to hydrolysis [41,42] (Figure 3). The activity assay was performed on release samples after various time periods. Controls consisting of PBS solutions obtained after a mock release consisting of incubation of PDMS disk without the loaded chelators were also tested as controls, and showed no effect on enzyme activity.

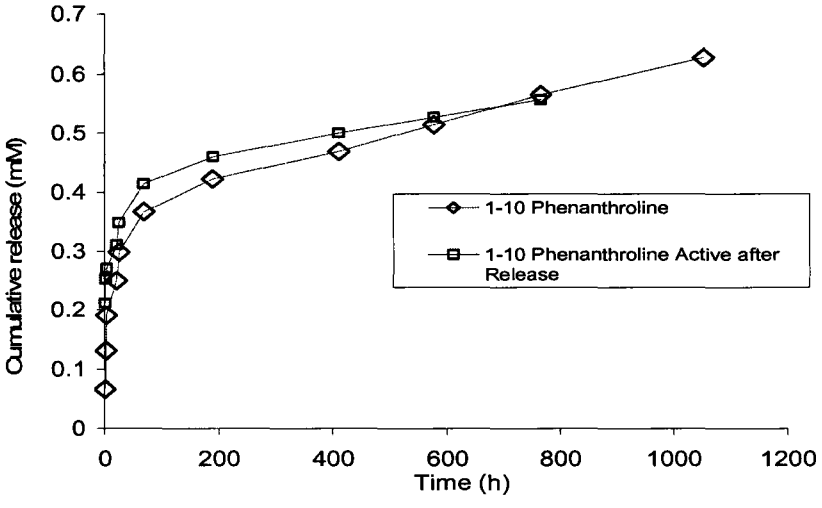

Figure 3: 1-10 Phenanthroline maintains full activity after release. The slight variation in the plots is most likely due to the variation in sensitivity of the two ways used to measure concentration and activity.

3.4. Toxicity to Neighboring Ocular Cell Types

The inhibitors, once released from the lens material, can diffuse out of the capsular bag, which is compromised during the surgical procedure. Therefore the potential toxicity of high concentrations of the chelators was tested with various ocular cells using an MTT assay. As noted from Figure 4, both at Day 1 and at Day 5, treatment with EDTA shows the highest remnant cell viability, while treatment with both TPEN and 1-10 phenanthroline resulted in a significant decrease in cell viability for all ocular cell types tested ($p<0.005$). The tested chelator concentration for these tests was approximately 100 times higher than the initial 3 day average daily release from the disks

prepared. Cell detachment due to inhibition of cell-cell interactions may also have skewed the results.

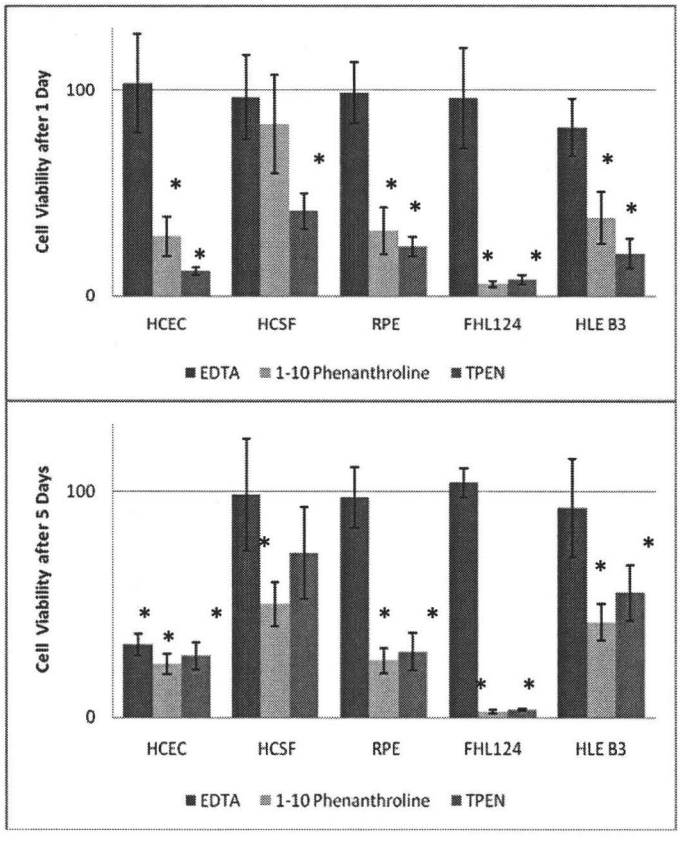

Figure 4: Toxicity of chelators on lens epithelial cells and other ocular cell types after 1 day and 5 days of treatment with 3.5mM of TPEN and 1-10 phenanthroline and 2.5mM EDTA. Prolonged exposure to high concentrations of TPEN and 1-10 phenanthroline result in very low results, which may indicate a risk for toxicity. (* $p<0.005$)

3.5 Cell Migration

After scratching, the FHL124 cells migrate into the opening. When the scratched cells were exposed to the prepared disks, only the 1-10 phenanthroline MIX preparations are capable of significantly reducing migration ($p<0.002$). Both EDTA and TPEN SOAK preparations were found to have no significant effect on cell migration after 24 hours.

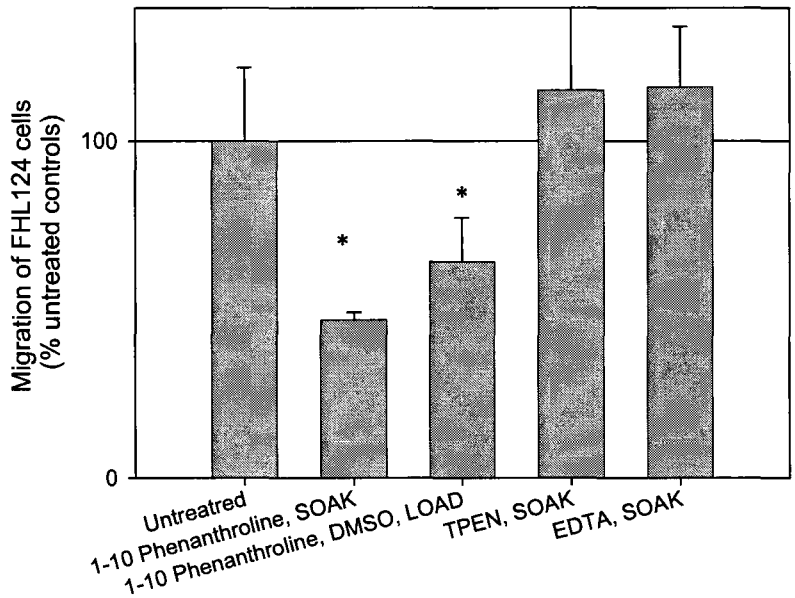

Figure 5: Migration of FHL124 cells is influenced significantly by 1-10 phenanthroline inhibitor-loaded silicone disks. Neither EDTA nor TPEN were able to affect migration rates. Cells were grown to confluence, then scratched and their migration in the gap was monitored by microscopy, in the presence of an anti-mitotic agent, aphidicolin. Both 1-10 phenanthroline preparations were able to significantly reduce migration rates (*$p<0.002$).

4. Discussion

While the potential of IOL materials for drug delivery to the eye has been previously demonstrated [26,27,29], the use of drug-releasing IOL materials for mitigating posterior capsule opacification, the main complication of intraocular lens surgery, has not been widely investigated. Releasing drugs constantly over long period of time, in a constant manner or at least in a controlled manner may be a desirable way to deliver preventive treatment to the lens capsule.

Recent studies have shown that MMP inhibitors influence LEC migration and the EMT process [17,43]. Previous research from our laboratory demonstrated that synthetic

MMP Inhibitors GM6001 and MMP 2/9 Inhibitor II released from PDMS as a model lens material are able to significantly reduce both the migration of LECs and the production of collagen I/III production following TGFβ2 treatment *in vitro*. However, while significant changes in migration were possible, the synthetic MMP inhibitor formulations were able to reduce migration rates only by 20-30%. Therefore, it is of interest to examine alternatives which have the potential to induce desired cellular changes in lens epithelial cells and which might have a more pronounced effect on LEC migration.

Migration, proliferation and transformation are the three main changes in LECs which occur after cataract surgery, and prior to PCO. While a combination of all of these events is necessary for PCO, it has been suggested that delaying or blocking LEC migration, particularly during the first four weeks post-cataract surgery might be an effective approach [1,7,21]. This four week timeframe is based on the fact that with square-edged IOL designs, a period of 2-4 weeks is thought to pass prior to the formation of the capsular bend which produces a permanent physical barrier for LECs, presumably preventing migration to the posterior of the capsular bag [9,10,22].

MMP enzymes play a crucial role in cell migration, not only in the lens, but also in other tissues [44]. In previous work, two common and efficient synthetic MMP inhibitors were examined; *in vitro* results suggested that while this approach seems to be promising, the effects of these inhibitors may not be sufficient to counteract the migration that is known to lead to PCO. As with any enzyme that uses a divalent cation as a co-factor, MMPs are inhibited by chelators. The chelators selected for this work were TPEN, a Zn^{2+} ion specific chelator, 1-10 phenanthroline, used in commercial kits as an MMP-9

inhibitor (Invitrogen, Burlington, ON) and EDTA, a common chelator, selected based on the fact that its impact on LECs has briefly been investigated [29,33,45,46]. It has shown success in preventing LEC adhesion to an EDTA-soaked acrylic IOL [29]. Furthermore, EDTA, when used as a capsular wash in rabbits prior to IOL insertion, was found to significantly reduce or delay PCO onset [46].

While two loading methods were initially sought for all three chelators, the PDMS curing method developed in previous work was found to be inappropriate for use with TPEN and EDTA as the PDMS did not cure in their presence. However, since PDMS disks were prepared following soaking in these chelators, their effect was analyzed especially in cell toxicity and cell migration studies as these tests are able to demonstrate the potential of these systems for inducing a significant effect on the LECs. Migration results were thought to provide the most relevant information and therefore were examined in this study. The results indicated that the 1-10 phenanthroline produced by the loading during preparation led to disks that were capable of significantly ($p<0.002$) reducing LEC migration in the first 24 hours. A reduction of more than 50% was observed. These results are dramatically better than those previously obtained the synthetic inhibitors GM6001 and MMP2/9 inhibitor II loaded into the PDMS disks using a similar method.

However, toxicity assays performed by measuring cell metabolic activity demonstrated that 1-10 phenanthroline accumulation may lead to significant decrease in cell viability in all ocular cell types tested. With all of the chelators, effects on cell detachment have to be clearly determined when using MTT assays to determine viability.

An alternative cell viability test may be necessary in these conditions to better determine cellular toxicity. Cell division studies using incorporation of labeled nucleotides (such as the Edu assay from Invitrogen, Burlington, ON) may also prove a good alternative for determining the effect of accumulating chelators on cell function. The rate of clearance and degradation of 1-10 phenanthroline from the eye has yet to be determined. EDTA showed very promising toxicity results, but no significant decrease in LEC migration. Higher loading amounts may be needed to produce the desired cellular effects. In contrast, TPEN showed high toxicity and no effect on cell migration. The specificity of this chelator might not be sufficient to balance its negative impact on cell viability, thus it will not be considered for further studies. Further animal testing is necessary to examine EDTA and 1-10 phenanthroline clearance and accumulation both following intraocular injection and following release from model lens materials.

5. Conclusions

In the current work, release of 1-10 phenanthroline was demonstrated to have the potential to be effective at decreasing the incidence of PCO, reducing LEC migration by more than 50% ($p<0.002$). Of the molecules tested, this chelator was the most versatile, as it could be incorporated into the PDMS by either loading during fabrication or loading post fabrication. The initial burst as well as the release rate were found to be somewhat altered by changing the loading method and solvent used. Furthermore, release of 1-10 phenanthroline was shown to significantly delay the migration of lens epithelial cells following the creation of a wound ($p<0.002$). However, further studies are necessary to

assess the efficacy of this system as this study also indicate a potential for cellular toxicity.

Acknowledgments: The funding support of NSERC is gratefully acknowledged.

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7.0 PAPER FIVE: SULFADIAZINE AS A WAY TO MITIGATE POSTERIOR CAPSULE OPACIFICATION BY IMPEDING LENS EPITHELIAL CELL MIGRATION

Authors: **Diana Morarescu**, Heather Sheardown

Objectives:

This work assessed the potential of the widely used antibiotic sulfadiazine for release from PDMS as a model IOL material, for activity against MMPs and for its ability to inhibit cellular changes consistent with PCO.

Main Scientific Contributions:

1. Sulfadiazine release can be modulated by controlling drug amount, solvent used to load and loading method.

2. Sulfadiazine can inhibit lens epithelial cell migration by up to 40% compared to untreated controls in the first 48 hours ($p<0.0005$). Of the various molecules tested, this molecule showed the greatest potential for reducing cell migration. Coupled with its long history of use and well known systemic effects, these results suggest that sulfadiazine may have significant potential for further development.

3. Sulfadiazine is stable when loaded in the PDMS matrix and stored in the dark at room temperature for over 18 months.

Abstract:

Posterior capsule opacification (PCO) remains the most significant complication of cataract surgery. Attempts to reduce its incidence have been somewhat successful, but not to completely mitigate the complication. Thus it is critical to find an effective, economical and feasible solution to this problem. Inhibitors of MMPs have been shown to have promise for minimizing the cellular changes associated with PCO. In this study, the common antibiotic sulfadiazine which has a similar chemical structure to commercially available synthetic MMP inhibitors, was delivered from PDMS as a model lens material. The released sulfadiazine was examined for its MMP inhibiting properties as well as its ability to inhibit migration of lens epithelial cells *in vitro*. Changing the solvent used to load sulfadiazine into the PDMS matrix proved to be an efficient method to manipulate initial burst and rate of release. Some preparations released inhibitor for up to 4 months. Both freshly prepared disks and disks stored for 18 months in the dark at room temperature, were able to significantly ($p<0.0005$) reduce lens epithelial cell migration in a scratch assay *in vitro*. Based on the known low systemic toxicity of this inhibitor as well as its low cost, delivery of this inhibitor from an IOL should be further investigated as a potentially feasible method for reducing the incidence of PCO.

Introduction:

Posterior capsule opacification (PCO) remains the main complication of cataract surgery. Despite the fact that its incidence has decreased dramatically in the past decade due to improvements to surgical technique and intraocular lens (IOL) design, the latest

study indicates that approximately 28% of patients still require secondary laser interventions to treat PCO within 4 years after surgery [1]. Lens epithelial cells (LEC) remaining in the capsule following insertion of the IOL undergo epithelial to mesenchymal (EMT) transformation, migrate to the posterior capsule and deposit aberrant extracellular matrix (ECM) components [1-3] which leads to the formation of the characteristic white opacities in the lens capsule [4,5].

While a number of factors contribute to PCO, lens epithelial cell migration is thought to play a crucial role in the remodeling of the lens capsule following cataract surgery [1]. Currently, the most effective method of reducing PCO to date is the square-edged IOL design, which is thought to physically block migration of the lens epithelial cells to the posterior of the capsule [6-8]. These new IOLs have led to a decrease in the incidence of PCO but a significant incidence of this complication still remains [1,9]. Studies have shown that it takes any LECs remaining in the capsule following extraction of the cataract 7-11 days after surgery to reach the posterior capsule [10-14]; however, it takes up to 1 month for the capsular bend to completely form a physical barrier a square-edged IOL [13,15]. Therefore a delay in cell migration, until the capsular bend seals, after a square-edged IOL is implanted, will theoretically stop the cells from reaching the posterior capsule, eliminating PCO.

Previous research in our lab has shown the potential of either synthetic MMP inhibitors or chelators released from PDMS as a model lens material in delaying LEC migration *in vitro*. The synthetic MMP inhibitors GM6001 and MMP 2/9 Inhibitor II were able to reduce migration rates by 20-30% relative to controls (submitted to

Biomaterials), while the chelators produce a more pronounced effect, with an up to 40% reduction using 1-10 phenanthroline (manuscript in preparation). However, the chelators proved to have a high potential for toxicity to adjacent ocular cell types. Therefore it remains of interest to find a molecule which can be released from an IOL material with sufficient potency to block migration during the first two weeks of surgery with minimal toxicity to surrounding ocular cells.

In the current work, the potential of sulfadiazine, a commonly used antibiotic, as an MMP-9 inhibitor was examined. This substance is commonly used as systemic therapy for the treatment of toxoplasmosis [16,17]. Its dose-related systemic effects on human cells have been studied in clinical trials at doses of up to 50-100 mg/kg per day in infants [16] and 1500 mg 4 times daily for adults [18]. Its use for treatment of ocular toxoplasmosis in particular is of interest, especially for patients who are intolerant of systemic treatments [19]. Chemically, its similar properties to those of the synthetic inhibitors suggest that it may be suitable for MMP inhibition (see Figure 1). Furthermore, this substance is cost effective when compared to the synthetic inhibitors used in previous studies, making it the ideal drug candidate from a pragmatic perspective.

Materials and Methods:

All reagents, unless otherwise specified, were purchased from Sigma Aldrich (Oakville ON). PDMS was prepared from Sylgard 184 from Dow Corning (Midland, MI), according to the manufacturer's instructions, using 10:1 ratio of elastomer base to curing agent, with and without the addition of solvent containing the drugs.

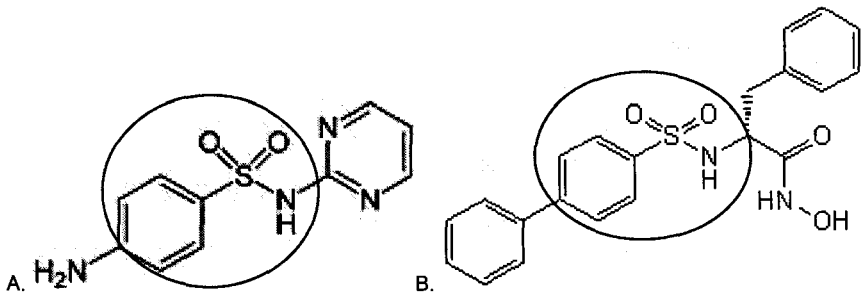

Figure 1: Chemical structure of Sulfadiazine (panel A) and MMP-2/MMP-9 Inhibitor II (panel B) from Calbiochem. Note the central sulfanilamide group bound to the aromatic ring that the two structures have in common (circled with blue).

The solvents were selected based on solubility of the PDMS base and the potential interactions in the system. An amount of 7 µl (0.8-1.0% v/v) for tetrahydrofurane (THF) and dimethyl sulfoxide (DMSO), or 40 µl (4.5-5.0% v/v) of dimethyl formamide (DMF), 1:1 (v/v) DMF:THF or water (pH 8.5 with NaOH) containing drug at a concentration of 100-300 µM was mixed with the PDMS elastomer base prior to the addition of the curing agent. This was subsequently mixed with an appropriate amount of curing agent and the films were cured for up to 7 days at 37°C.

Drug Release, Released Drug Activity

Disks of approximately 1/4" in size containing sulfadiazine were punched from the cured films. The samples were placed in 600 µl PBS at 37°C and the substance was released over a period of 120 days with regular sampling. The sulfadiazine concentration in PBS was determined by absorbance at 253 nm in comparison to a standard curve. Two individual batches of each sheet of PDMS and a total of 6 disks for each loading condition were used to construct the release curves.

Sulfadiazine activity against MMP-9 was measured using a protocol adapted after a modified version of the Invitrogen EnzCheck gelatinase activity kit (Invitrogen, Burlington, ON), using human active recombinant MMP-9 enzyme (Calbiochem, San Diego, CA), and DQ-gelatin substrate (Invitrogen, Burlington, ON). A total of 7.5 ng of enzyme was mixed with 5 μg DQ-gelatin substrate and 80 μl of sample. The reactions were performed in an aqueous solution containing 50 mM TrisHCl, 150 mM NaCl, 5 mM $CaCl_2$, and 1 μM ZnCl. The change in the relative fluorescence units (ΔRFU) was measured fluorimetrically using an excitation wavelength of 495 nm and an emission wavelength of 518 nm. The initial reading was made immediately after adding the substrate, and the final reading was performed after 2 hours of incubation, as per manufacturer's instructions (Invitrogen, Burlington, ON). Calibration was based on solutions of sulfadiazine of known concentration. All data was collected from sheets of PDMS loaded with drugs produced in 2 separate batches, with n=6.

Drug Toxicity to Ocular Cells

Fresh sulfadiazine (0.8 mM) was added to the *in vitro* cultures of various ocular cells in order to assess any potential for ocular toxicity. The cells were incubated with the sulfadiazine for periods of either 1 day or 5 days at which time the cells were analyzed using an MTT assay to determine cell viability following drug exposure. Human corneal epithelial cells were grown in KSFM media with complete supplement. Human corneal stromal fibroblasts were grown in M199 media with 1% ITS supplements. Human retinal pigment epithelial (RPE) cells were grown in DMEM:F12

media, with 5% FBS, 1% L-glutamine and 0.8% sodium bicarbonate. The human lens epithelial cell line, FHL 124, was grown in MEM media, with 1% L-glutamine, 10% FBS and 50μg/ml gentamycin. The human lens epithelial cell line B3 was grown in MEM:F15 media (Sigma, Burlington, ON), with 1% L-glutamine, 0.8% sodium bicarbonate, 20% FBS, 1% sodium pyruvate. All media preparations also contained 1% penicillin-streptomycin solution. All cell culture supplies were purchased from Invitrogen (Burlington, ON), with the exception of ITS supplement for HSFC, which was purchased from Becton Dickinson (Mississauga, ON).

Cell Migration Test

A scratch assay was used to test the effect of released sulfadiazine on FHL124 lens epithelial cells in culture, following a protocol previously described [20]. Briefly, cells were grown to confluence, then the monolayer was scratched with a cell scraper, creating an approximately 1-2 mm gap. The cells displaced by this procedure were removed from the plate by washing twice with PBS. The media added to the cells immediately following creation of the scratch was supplemented with 0.8 μM aphidicolin (Sigma, Burlington, ON), to prevent cell division [21], which allowed for monitoring of cell migration into the gap. The lack of cell division was confirmed by the Click-IT Edu assay (Invitrogen, Burlington, ON). Cell migration was measured microscopically, using a Zeiss Axiovert microscope.

Results:

Released Drug Activity and Concentration

As it has been previously shown that changing solvent during the curing/loading process can affect initial burst and rate of release, various solvents were examined as a means of controlling release parameters. The results demonstrated that sulfadiazine was released for periods of over 4 months, depending on solvent used to load. While this is far greater than the time for capsular bend formation to occur, the optimal release durations are as yet unknown and it was therefore of interest to determine that the inhibitor could be released for periods significantly longer than the anticipated time necessary. When the sulfadiazine dissolved in water was mixed with the curing PDMS, the result was a small burst and low sustained rate of release. However, the disks were translucent, making them inappropriate for use in ophthalmic applications. DMF, DMSO and a 1:1 (v/v) mixture of DMF/THF resulted in similar burst and release rates which were higher than those obtained for water but the disks were transparent even at relatively high loadings. Mixing the sulfadiazine with THF produced the highest burst and fastest rate of release. Figure 2 summarizes these results.

When looking at the inhibitory effect of sulfadiazine on MMP-9, an IC_{50} value was calculated to be approximately 26μM. However, more detailed analytical studies are needed to determine the exact value and the inhibition equilibrium constant, Ki (Figure 3).

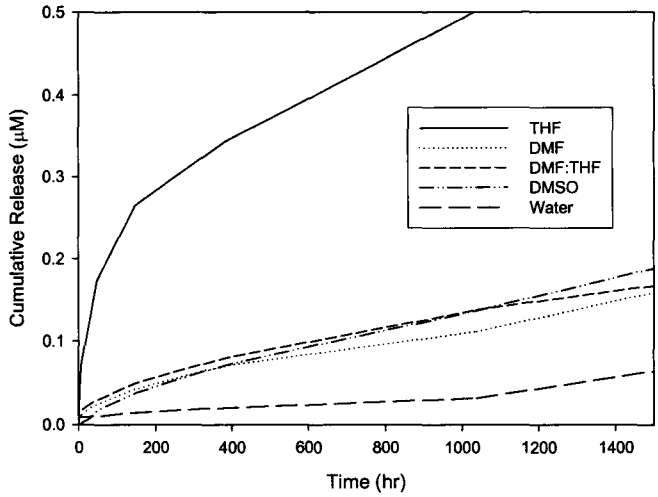

Figure 2: Sulfadiazine release can be somewhat altered by the change in solvent used to dissolve the drug prior to addition to the PDMS elastomer base. The use of THF as a solvent results in a larger burst and higher relative release rate, resulting in complete disk depletion in less than 45 days, while the use of other solvents increases the lifetime of the device for longer than 3 months.

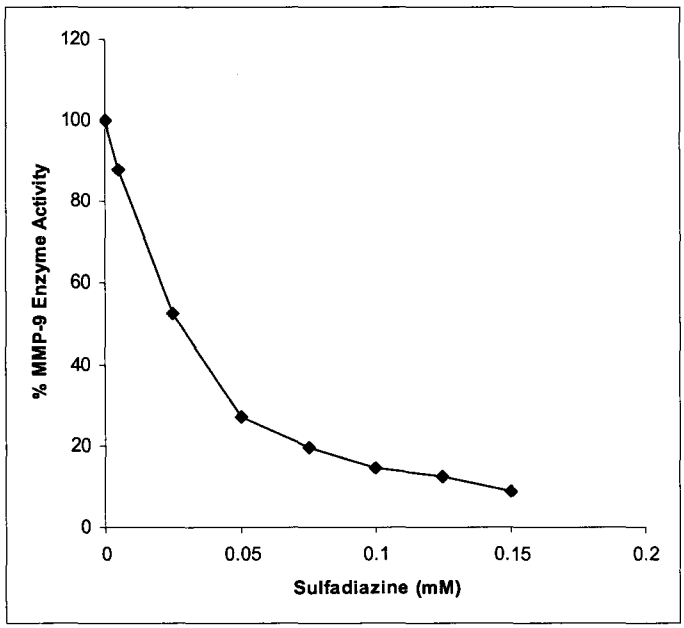

Figure 3: Sulfadiazine inhibits MMP-9 activity in a dose dependent manner. In this experiment, 7.5 ng of the enzyme was inhibited with an IC_{50} value of approximately 26μM.

As observed in tests using human MMP-9 recombinant enzyme, only a percentage of released sulfadiazine remains active after release. In Figure 4, it can be seen that after

only approximately 3 days of release, active sulfadiazine represents only a half of the total amount released, and that this percentage decreases with time. Only disks prepared with DMSO solvent were used for this test; a control prepared with DMSO without sulfadiazine was used. This control showed no activity against MMP-9.

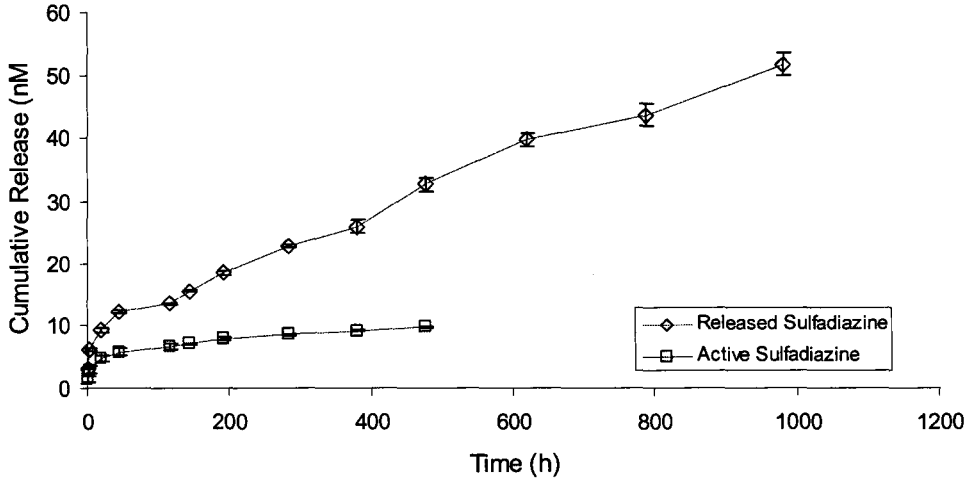

Figure 4: Active sulfadiazine against MMP-9 represents less that 50% of the total released amounts of the drug, starting 3 days after release.

Drug Toxicity to Ocular Cells

As the capsule is compromised by the surgical procedure, it is possible that drugs released from the lens inside the capsule have the potential to escape from the capsule through the opening left by the surgery. While systemic toxicity assays have shown that the drug is not toxic in quantities thousands of times larger than the doses proposed in this research [16-18], the specific effects of this drug on ocular cells has not been investigated. Exposure to sulfadiazine concentrations of 0.8 mM in DMSO for 1 or 5 days resulted in some adverse effects, with a significant ($p<0.04$) decrease of

approximately 15% in human corneal epithelial cell (HCEC) and retinal pigment epithelial cell numbers after 5 days, a 23% decrease in RPE cell numbers after just one day ($p<0.0004$) and a 35% decline in the numbers of HLE B3 cells, an infant derived lens epithelial cell line after 5 days ($p<0.003$), as shown in Figure 5. Exposure of cells to the same volume of DMSO without the drug resulted in no significant changes in viability.

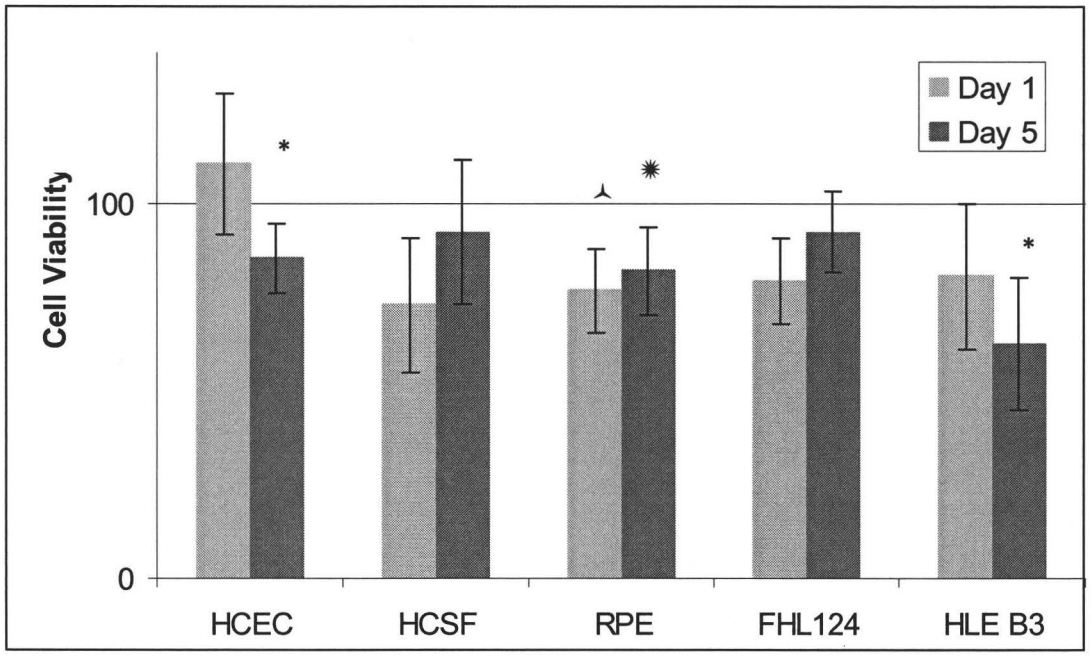

Figure 5: Exposure to sulfadiazine, at 0.8 mM, for 1 or 5 days, produces significant cell reduction in HCEC, RPE, FHL124 and HLE B3 cells. Results are presented with untreated controls for each cell line representing 100% viability. * $p<0.005$; ✳ $p<0.04$; ⅄ $p<0.0004$. Significance was calculated by ANOVA and bars represent standard deviation.

Cell Migration

Cell migration was monitored in 24-well plates (BD, Mississauga, ON), using a scratch assay as previously described [20]. The disks, prepared immediately prior to the

experiments as well as disks prepared 18 months in advance using DMSO as a solvent and stored in the dark at room temperature were used to assess drug stability in the solid matrix. There was no statistical difference between the freshly prepared disks and those which had been stored for 18 months ($p=0.67$). Furthermore, in both cases a significant ($p<0.0005$) reduction in migration of approximately 50% after 48 hours exposure to prepared disks was observed (Figure 6).

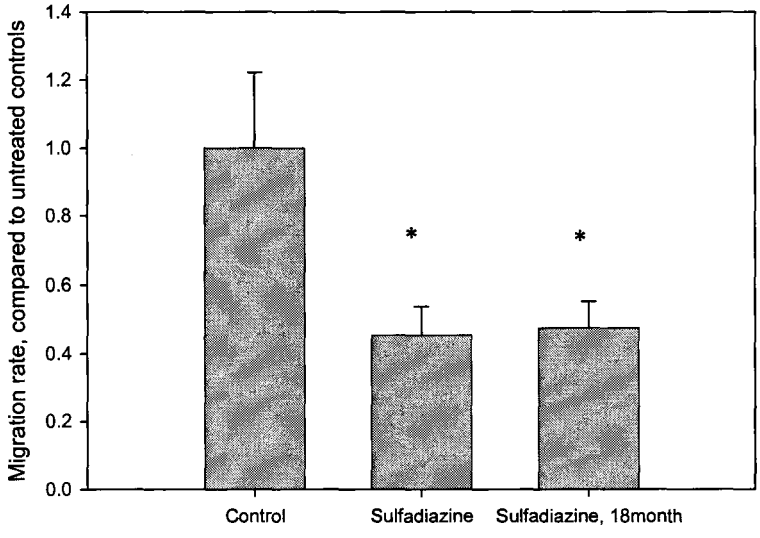

Figure 6: FHL124 cells migrate at approximately 50% slower rates (* $p<0.0005$), when exposed to sulfadiazine releasing disks, compared to untreated control. Migration of untreated cells is presented as 1, and migration of treated cells was calculated as a ratio relative to untreated controls. There is no significant difference ($p<0.67$) between freshly prepared disks and 18 month old disks.

Discussion:

PCO remains the main complication of cataract surgery, although the incidence has been reduced to some extent in the recent past, primarily due to improved surgical

technique and IOL design [1,8,22]. However, complete mitigation of this complication is still very desirable, both from a financial perspective, to ease the burden on the health system, and from a patient perspective, where a secondary intervention will no longer be necessary. The latter is especially important in developing countries, where facilities for laser treatment of secondary cataract might simply not be available [23,24].

While pharmacological approaches to treat PCO have become increasingly popular in research, none has been able to completely mitigate PCO [25,26], and only one pharmacological treatment, in the form of an injection in the capsular bag just after inserting the IOL and prior to closure of the surgery [27], is currently in human clinical trials.

Our laboratory has examined various pharmacological approaches to mitigating PCO based on inhibition of matrix metalloproteinases (MMPs), and has successfully developed a PDMS drug-release platform, with physical properties suitable for use as an IOL material [34, 35]. Using this drug-delivery platform, it was found that the release rate of various molecules could be manipulated and could presumably provide appropriate amounts of drug, as needed to control cellular changes associated with PCO. However, there is a lack of information about the specific concentrations which are required *in vivo* and therefore, further *ex vivo* and *in vivo* human and animal studies will be necessary.

Sulfadiazine, with chemical properties which are similar to the commercially available synthetic inhibitors, has numerous advantages compared with other inhibitors that have been previously tested. It has been successfully applied as systemic therapy in

humans for the past decade for the treatment of toxoplasmosis [17,18]. Ocular toxoplasmosis accounts for 20-30% of pediatric congenital toxoplasmosis cases [28]. Up to 10% of these infants are also born with congenital cataracts [29]. In post-natal acquired toxoplasmosis, ocular lesions can appear in up to 45% of patients [30]. In all cases, lesions are mainly cysts attached to the retina or the optic nerve, that can lead to vision loss when activated [18,30]. While sulfadiazine has been used in systemic therapy, with promising results of up to 71% of patients with ocular lesions having no retinal scarring after treatment [30], the levels of sulfadiazine in the eyes have not be measured. Thus the safe dose to ocular cells is unknown.

In this work we have shown that sulfadiazine can be released in a controlled manner for up to 4 months. It has been noted that some of the activity of the drug against MMP-9 is lost after less than 3 days from the start of the release. It is possible that the sulfadiazine is starting to degrade, as degradation products absorb at similar wavelengths as the original molecule [31-33]. Further testing should be performed using HLPC.

Notwithstanding the above, it is clear that sulfadiazine has the potential to reduce the rate of lens epithelial cell migration particularly during the first 48 hours. Due to the possible degradation noted, longer migration studies should be performed in order to determine whether the sulfadiazine will be able to maintain these inhibitory properties for the full 2-4 weeks that will presumably be necessary before the capsular bend closes [15]. It is also possible that only a short burst of sulfadiazine immediately following implantation of the device may be necessary to elicit cellular changes sufficient to inhibit PCO. Degradation products may also block migration as they maintain the sulfanilamide

group bound to the aromatic ring structure that is similar to the synthetic MMP 2/9 Inhibitor II molecule (see Figure 1) [32,33]. If that is the case, the effect of these degradation products on cell toxicity has to be assayed.

The more than 18 month stability of the sulfadiazine in the proposed PDMS drug-release matrix has promise for the development of systems for using this molecule for treating PCO. Other MMP inhibitors show much lower levels of stability after only 6 months of storage at 4°C.

It should be noted that the activity tests were performed against an MMP-9 enzyme. This substance has the potential to inhibit other enzymes, due to its small structure and similarity to other synthetic MMP inhibitors (see Figure 1). Therefore, tests to better assess potential targets of sulfadiazine related to its capacity to reduce lens epithelial cell migration *in vitro* should be performed.

The cumulative release profiles of the prepared disks demonstrates that the total amount of drug released is approximately 100 times lower than the experimentally determined IC_{50} value of 7.5 ng for MMP-9 recombinant enzyme. This combined with the low cell toxicity results suggests that this substance has potential for further development. However, information in the form of kinetic studies on this and other MMPs to determine *Ki* values will be useful in determining affinities of the various enzymes for the drug. A better understanding of the mechanism by which sulfadiazine is able to mitigate FHL124 migration would enable more efficacious systems to be prepared.

Conclusions

Sulfadiazine, an antibiotic drug commonly used in systemic human treatments, has demonstrated in this study to have significant potential in delaying lens cell migration *in vitro*. Its release profile from the PDMS matrix can be adjusted by changing dose and solvent. The stability of the sulfadiazine in the PDMS matrix is not significantly altered by 18 months of storage. However, further studies are necessary to completely evaluate its safety in the eye, as well as its long term ability to mitigate migration *in vitro*. Ultimately *in vivo* testing will be necessary to demonstrate efficacy in an appropriate model. Furthermore, due to the drug's efficacy against ocular toxoplasmosis, an IOL product delivering sulfadiazine might be ideal for cataract patients with ocular toxoplasmosis.

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8.0 SUMMARY, CONCLUSIONS AND FUTURE WORK

In this thesis, three different classes of MMP inhibiting molecules were loaded into and delivered from PDMS as a model IOL material, and the systems were investigated *in vitro* for their capacity to block changes in lens epithelial cells, consistent with those which are known to lead to PCO.

Two different methods of loading the molecules into the PDMS matrix were examined as well as material properties, their release rates and remnant activity. Loading drugs into PDMS by soaking pre-made PDMS in highly concentrated drug solutions in ethanol was found to be a feasible option, especially from a manufacturing perspective. However, shorter release times and a lower, limited amount of drug can be loaded by this method. Furthermore, some of the potential drugs were found to lose the majority of their activity when loaded by this method. Loading dissolved small molecular weight hydrophilic and hydrophobic molecules into the PDMS elastomer base prior to addition of curing agent proved to be a way to allow for adjustment of initial burst and subsequent release rate. It was found that the release rate could be controlled through the choice of solvent used to dissolve the drugs, and that this correlated with the ability of the solvent to swell PDMS. Furthermore, not unexpectedly, solvents such as ethanol, that have high PDMS swelling capacity [1], were found to result in a large initial burst followed by a rapid rate of release. Solvents which swell the PDMS matrix to a lesser extent including water, PBS, DMSO and DMF were shown to lead to release profiles characterized by small bursts and prolonged release at a slower rate. This loading method was also shown

to lead to more preferential release rates compared with those from samples prepared using traditional powder loading of drugs into the PDMS. Most importantly in ophthalmic applications, the loading of powdered drugs were shown to lead to a loss of polymer transparency.

While *in vivo* testing is ultimately necessary for demonstrating efficacy of any system under development, *in vitro* testing plays a valuable role in screening potential alternatives. However, for the screening of potential alternatives for minimization of PCO, there are not suitable *in vitro* models or tests. Therefore, two human lens epithelial cell lines were compared to assess their interaction with different materials as well as to assess their response to compounds known to stimulate cellular changes consistent with PCO. The results demonstrated that while it is possible to use cell lines for *in vitro* screening of potential PCO treatments, the limitations of these systems should be considered and the markers tested should be selected appropriately. Due to the difference in initial donor age and cell line preparation method, the two cell lines showed some significant differences in their response to stimuli and in their basal composition of commonly used PCO markers. However, further studies will be needed to determine which changes are due to donor age in particular, as this might be relevant in the future for examination of potential materials and other treatments aimed at use in the paediatric population. The results also demonstrated that, in the current work, examination of changes in cell migration may be useful for assessing the potential to mitigate PCO of the materials developed.

Synthetic MMP inhibitors including GM6001, a generic MMP inhibitor, and MMP 2/9 Inhibitor II, an inhibitor with specificity for MMP-9, and MMP-2, the two MMP enzymes most actively involved in PCO, were incorporated into PDMS, as a model lens material. Release profiles were manipulated by altering loading solvent. However, the released inhibitors were found to reduce cell migration by only 20-40% ($p<0.005$) over the first 48 hours. Furthermore, both of the inhibitors tested were found to lose activity after the prolonged periods of storage in aqueous buffers necessary to obtain measurable concentrations. However, this was thought to be primarily the result of the long storage periods rather than the material. Remnant activity *in vivo* would be expected as the inhibitors would be able to interact with the MMPs at the site of release. Further studies are needed to confirm this hypothesis.

As all MMP enzymes are Zn^{2+} dependent, three different chelators, EDTA, 1-10 phenanthroline and TPEN were tested. It was found that, using the same loading method as in the previous studies, none of the chelators was susceptible to hydrolysis. However, using several combinations of solvent and chelators, it was not possible to produce cured disks. Furthermore, in contrast with the synthetic MMP inhibitors and with the exception of EDTA, the chelators demonstrated a higher potential for toxicity to surrounding ocular cells. This is detrimental, since the released chelators can diffuse out of the lens capsule through the capsulorhexis and may reach other ocular cells. Notwithstanding the above, the 1-10 phenanthroline formulations were able to reduce lens epithelial cell migration by a promising 40-60% ($p<0.002$). Therefore, it may be possible to use chelators in this

application and to optimize the systems in order to obtain cellular changes consistent with decreased PCO.

As a result, a further alternative, sulfadiazine, was investigated. This molecule has both a small size and contains key functional groups which make it comparable to the chemical structure of synthetic MMP inhibitors. When tested against MMP-9 *in vitro*, this drug showed potent inhibitory activity at low concentrations. Low cell toxicity was also observed. When incorporated into the PDMS matrices, release rates and burst amounts could be adjusted with changes in solvent. Furthermore, in migration studies, formulations of sulfadiazine loaded PDMS were able to reduce lens epithelial cell migration by up to 60% in the first 24 hours. Therefore, the results suggest that, of the compounds tested, sulfadiazine has the greatest potential to impact cellular changes consistent with PCO. The effect of sulfadiazine on PCO markers and the EMT process should also be investigated. While reducing migration by a significant 60% ($p<0.0.0005$), it is not known whether this is sufficient to completely eradicate PCO. Therefore, drug combinations may provide increased efficacy. It may also be desirable to perform studies with IOL materials soaked in sulfadiazine solutions as this is a simple way to post modify a lens with little need to alter existing manufacturing processes. This treatment might be most beneficial in particular to patients with ocular toxoplasmosis and cataracts, as this molecule has the potential to treat toxoplasmosis and prevent secondary cataracts at the same time. Animal studies are needed to test this hypothesis. The delivery of sulfadiazine and other MMP inhibitors from a platform that allows for long and constant release rates

might be beneficial in other systems where MMP-9 activity in particular is a concern, such as breast cancer and other metastatic cancers [2,3] or coronary artery re-stenosis [4].

Testing using animal studies and studies of human lens epithelial cell migration in *ex vivo* lens capsules should ultimately be performed with the most promising formulations in order to further demonstrate efficacy in a system, where drug clearance and degradation might occur. Positive results from these tests would then precipitate IOL production and possible future animal and even human trials. The IOL production step is also crucial and with any successful system, manufacturing processes will need to be developed in order to fabricate devices.

8.1 References

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APPENDIX A: METHODS

A.1. Drug loading into PDMS

In order to study the effect of released drugs on cultured LECs, an appropriate drug-delivery platform had to be researched. Two methods of drug incorporation were designed and used in subsequent tests.

A.1.1 Drug Incorporation Prior to PDMS Curing

PDMS films were produced using the Sylgard 184 kit (Dow Corning, Midland, MI) and following manufacturer's instructions. A 1:10 curing agent to elastomer base ratio has been used throughout. For all films, after addition of curing agent, PDMS was first aerated under a fume hood for 1 hour (to remove any volatile solvents) then incubated for 1 hour under vacuum at room temperature, to remove air bubbles formed during mixing. Subsequently, films were incubated in a 37°C oven for 6 days to set.

Dissolved drug was incorporated in the elastomer base, prior to addition of curing agent, in order to obtain films with high transmittance. This method has been previously used successfully for production of other drug-delivery devices [1,2]. The most common solvents used for dissolved drug incorporation in literature are ethanol and hexanes [2-4], however, only one other report of water used as a solvent has been published [2]. In this work, the following solvents have been used to dissolve a variety of drugs prior to loading into PDMS: water, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), tetrahydrofurane (THF), isopropanol (IPA),

ethanol and some combinations of these solvents. In order to allow for curing within 6 days after film preparation, the volume/weight ratio of solvent to PDMS elastomer base has been maintained between 1% and 5%. Films that have not cured in this time interval have not been included in this research.

While the exact changes that occur in the PDMS network in the presence of these solvents have not been studied in detail, it has been documented that the solvent may have an effect in creating holes or channels in the network, allowing for differential burst and release rates of the incorporated drugs [1,5-7].

For all drugs used, a concentration close to saturation in each solvent was added to the PDMS film, to ensure maximum loading capacity. This resulted in film with unequal drug amounts, but constant solvent/elastomer ratios.

A.1.2 Drug Incorporation by Soaking

Several previous studies have incorporated drugs into prepared IOLs by soaking them into concentrated solutions of the desired drugs [8-12]. This method was used in this study in parallel to the method described above, since some drugs have failed to produce cured films. This drug loading method is not ideal, as it would follow a release pattern that is directly connected to the solvent used to soak [4,13], and on the concentration of the soaking solution [14]. The advantage of this method is that drugs can be incorporated in pre-fabricated IOLs, thus making it possibly easier to reach the market.

For PDMS disks, we have only used ethanol as the soaking solvent, since of all used solvents, it has the best PDMS swelling ability [13]. Soaking of disks was done with mild agitation, ensuring disks are completely submerged in solution. After incubation for 4 days, disks were rinsed briefly with water and air dried in the fume hood for 2 hours, then stored at 4°C.

A.2 PDMS Film Analysis

Film material properties are essential in designing a potential IOL material. To be compatible as an IOL, the material needs to meet specific transmittance and refractive index limits [15]. The material also needs to be easily manipulated, since silicone IOLs have been preferred by surgeons for their fast folding/unfolding times [16].

A.2.1 Microscopy for Film Properties

In initial study of the PDMS drug-delivery platform, studies of drug distribution and polymer morphology were performed. Transmission electron microscopy (TEM) is a method of discerning between material density or content, given that the electron density in the different phases is strong enough to be observed. TEM was necessary for these studies, as light microscopy is limited in resolution capacity by the wavelength of visible light. PDMS mixed with other polymers was previously shown to create interpenetrating networks of dimensions smaller than 100nm [5], thus TEM analysis was deemed necessary. TEM resolution is given by the capacity of the sample to absorb electrons. Thicker regions of the sample, or regions with a higher atomic number, appear darker.

Confocal microscopy was also used to determine fluorescent drug distribution for model PDMS materials. A Zeiss LSM 510 laser scanning microscope at 40x magnification was used. This technique allows image stacking in the z plane, and the creation of a three-dimensional image of the sample.

A.2.1 Transmittance Assay

Light transmittance is an important property of ophthalmic materials, as a high percentage of visible light (range of 360nm-700nm) has to reach the retina [17]. With age, the normal lens allows less and less light transmittance, with average values 70-80% between 15 and 60 years of age [18]. This measurement is important in order to ensure the material matches or improves the qualities of the normal lens. In order to determine light transmittance properties of the drug-loaded films, disks of 7/32" diameter were punched out and placed at the bottom of a well in a 96-well plate. The disks were then covered with 100µl water. Water was used as a control. Results were obtained from 2 different batches, with n=6-8. Light transmittance was measured as a percentage, at wavelengths in the visual spectrum: 405nm, 450nm, 540nm, 630nm, 700nm, on a BioRad plate-reader.

A.2.2 Refractive Index Measurement

The refractive index measurement provides an indication of how light is bent when passing through the material. The biconvex natural lens is able to bend light in order to focus it on the retina [17]. The range of refractive indices used commercially in

IOLs has varied from 1.38 to 1.55 [15,19]. To test whether these novel materials fit within the recommended values, ¼" disks were placed on a Digital Hand-held Pocket Refractometer, Model: PAL-RIA made by Atago. The measurements were done with disks originating from 2 or 3 different sheets of PDMS, with n=6-9.

A.3 Releasate Analysis

Release studies were performed using 6 disks, produced from the same sheet of PDMS, or soaked in the same inhibitor solution. Each drug-loaded silicone disk was incubated in a known volume (400 or 600µl) phosphate buffered saline (PBS, pH 7.4) or tris-buffered saline (TBS, pH 7.5) at 37°C with mild agitation. This relatively small release volume was selected to mimic the relatively low volume of fluid that would be expected in the lens capsule [20]. At regular time intervals, the disks were transferred to fresh buffer solution, and the releasates were analyzed.

A.3.1 Concentration Determination

The absorption spectra of each drug tested was measured on a Bio-Rad spectrometer machine, or obtained from literature. For each drug and each release condition, a standard curve was created, with freshly prepared drug solutions. Sulfadiazine measurements were done at 250nm, MMP 2/(iNhibitor II measurements were done at 260nm, while GM6001 measurements were done at 280nm.

Concentrations of the chelators 1-10 Phenanthroline and TPEN were determined with a colorimetric method adapted from an iron salt detection technique [21]. This

method is based on the ability of neighbouring aromatic hydroxyl groups to reduce iron ions, under acidic conditions. In this method, a constant ferrous salt was maintained, and incubated with varying concentrations of chelator, for 15 minutes at room temperature. The change in color was measured by reading absorbance at 450nm, with a reference at 630nm. A standard curve was established for each compound prior to testing releasate samples.

A.3.2 Inhibitory Activity Towards MMP-9

Activity of the released MMPIs from disks prepared with high concentrations of inhibitors (HC) was measured using a modified version of a MMP-9 activity assay [22]. The MMP Substrate III Fluorogenic (Calbiochem, San Diego, CA) was diluted to 12.5 mM in PBS. An 80µl volume of sample was placed into a 96 well plate and the substrate and enzyme added. At lower concentrations of inhibitor, activity was measured using a modified version of the Invitrogen EnzCheck gelatinase activity kit (Invitrogen, Burlington, ON), using human active recombinant MMP-9 enzyme (Calbiochem, San Diego, CA), and DQ-gelatin substrate (Invitrogen, Burlington, ON). A 250 µl volume of sample was used instead. The change in the relative fluorescence units (ΔRFU) was measured using an excitation wavelength of 340 nm and an emission wavelength of 485 nm. The initial reading was made immediately after adding substrate, and the final reading was performed after 18h. Calibration and quantification of the results was based on fresh inhibitors of known concentration.

A.4 Biological Effects of Drug-Loaded PDMS Disks

As the focus of this work was on changes induces by the drug-loaded materials on cells, several biological tests were used. Two different cell lines were studied, to determine the differences in response to the stimuli and the impact of the new materials on lens cell behaviour *in vitro*. Cells were examined every other day by inverted light microscopy, using a 10x, 20x or 40x objective. For quantification of cell growth, both visual counting and a Coulter Counter machine were used. All cells were detached using a trypsin-like protease in the Tryple-Express mix (Invitrogen, Burlington, ON).

A.4.1 Lens Epithelial Cell Culture

Two human epithelial lens cell lines were used in these studies. The differences between their responses to different drugs and surfaces were highlighted in Paper 2 (Chapter 5). HLE B3 cells are infant-derived, SV-40 transformed cells; they were grown in conditions recommended by supplier (ATCC, Manassas, VA) [23]. FHL 124 cells are adult-derived primary cells [24]; they were grown in MEM media with 10% FBS, 0.01mM L-glutamine for complete media conditions [25]. All media contained 1% antibiotic-antimycotic mix. Cells were washed in PBS prior to medium change, and the medium changed every 2-3 days.

A.4.2 Ocular Cell Lines in Culture

Human corneal epithelial cells were grown in KSFM media with complete supplement [26]. Human corneal stromal fibroblasts were grown in M199 media with 1%

ITS supplements (Becton Dickinson, Mississauga, ON). Human retinal pigment epithelial (RPE) cells were grown in DMEM:F12 media, with 5% FBS, 1% L-glutamine and 0.8% sodium bicarbonate [27]. All media preparations also contained 1% penicillin-streptomycin solution.

A.4.3 Immunostaining

Immunostaining experiments were done to assess the expression of both extracellular and intracellular markers. Fixed (using 4% formalin) and stained surfaces were observed for using fluorescence microscopy at 20x and 40x magnification. Antibody quantification was done using the Perkin-Elmer Victor 3 fluorometer and its software.

A.4.4 Colorimetric assays

Colorimetric assays are less sensitive, but more economical and often faster to perform than immunostaining. For quick assessments or for tests where antibodies are not readily available, colorimetric assays are ideal. Glycosaminoglycan (GAG) measurements in the extracellular matrix were done, as these levels are thought to be important in cytokine transport and retention through the ECM [28-31]. GAG staining is commonly done on histology specimens. A protocol using alizarin blue solutions was adapted and used in this study [32].

Another colorimetric assay was used to determine Collagen I/III amounts. The Sirius Red dye is commonly used in histological studies to differentially color the

different types of collagen, due to its birefringence, which is highly specific for collagen. Binding to different types of collagens will cause absorption at different wavelengths due to alignment and thickness of the different fibers [33-35]. For Collagen I/III, a wavelength of 630nm was recommended and used. Dye incubated with empty TCPS surfaces was used as a negative control.

A.4.5 Cell Number and Cell Viability Assays

In this study, the MTT assay was used to determine cell viability after treatment with the study drug. This assay is based on mitochondrial activity, more specifically on the activity of the mitochondrial succinic dehydrogenase, a reductase, capable of converting a tetrazole (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to formazan. This chemical reaction produces a color shift, from a pale yellow of the MTT, to a deep purple of the formazan [36].

As some cells can survive without mitochondrial activity, and the MTT product can be reduced by a variety of other factors in the media, especially if it contains FBS, a control of MTT in media with no cells has been used as a blank standard. The assays were done mostly after treatment with either drug solutions (positive controls) or drug-loaded PDMS disks. Cells were treated for a pre-determined period, then incubated with a 5mg/ml DMSO dissolved MTT solution. The volume of the MTT solution always represents 15% of the volume of the media. Cells were incubated with the MTT solution for 18 hours. Then the supernatant was carefully aspirated, without disturbing the purple formazan precipitate. The formazan precipitate was then dissolved in 300µl DMSO, by

incubating at room temperature for 15 minutes. The concentration was measured on BioRad machine, with absorbance set at 540nm, and a reference at 630nm. Viability was calculated as the ratio between the mean sample reading and the mean control reading.

For calculating cell numbers using this same assay, cells were exposed to different growth conditions, surfaces or drugs. A calibration curve has been produced in advance, showing the linear dependence of cell number and formazan production, for each cell line used. The number of cells in the experimental conditions was calculated using the standard curve.

Cell numbers were also calculated by using a Beckman Coulter cell and particle counter instrument. This machine uses a measurement of impedance between the electrodes to calculate particle number and to select a desired partiocle size by changing the aperture.

A.5 Cell Migration Assay

As cell migration is crucial to PCO development, assaying cell migration as influenced by the proposed drugs and drug-delivery systems, is important. FHL124 were grown to confluence, then the monolayer was scratched with a pipette tip and the gap was monitored by microscope (a 20x magnification, using a Zeiss Axiovert 200 microscope), following the scratch assay protocol previously described [24]. The cells were treated with an 800μM aphidicolin solution, to inhibit mitosis [37], so that the entire analysis is based on migration of already existing cells. Distance migrated by cells was measured after 48hr, using the AxioVision 3.1 software, and visualizing cells with a 20x objective.

The amount of aphidicolin necessary to inhibit mitosis in both HLEB3 and FHL124 was monitored by trying a variety of concentrations, ranging from 200 to 800μM aphidicolin solution. Cell division after aphidicolin treatment was monitored by using the ClickIT Edu (Invitrogen, Burlington, ON) kit, using manufacturer's instructions.

A.5.1 ELISA Assay for TGFβ2

As TGFβ2 is important in PCO etiology, it was necessary to ensure that its production was increased as a result of the scratch induced for the migration assay. An enzyme-linked immuno assay (ELSA) kit from R&D Systems (Minneapolis, MN) was used, as per manufacturer's instructions.

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APPENDIX B: PERMISSIONS

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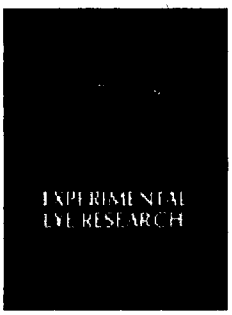

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