Contact Lenses and Tear Film Lipids

by

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The following served on the Examining Committee for this thesis. The decision of the Examining Committee is by majority vote.

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Authors's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

I would like to acknowledge the names of my co-authors who contributed to this thesis:

- Dr. Lyndon Jones (Chapters 3 – 7)
- Dr. Lakshman Subbaraman (Chapters 3 – 7)
- Dr. Chau-Minh Phan (Chapter 5)

Abstract

Introduction

Lipids are essential tear components that aid the stability of the tear film (TF) to protect it from excess evaporation. The composition, conformation, and function of TF lipids are jeopardized by external factors such as contact lens (CL) wear and environmental elements (i.e. UV radiation, oxidation). Specifically, silicone hydrogel (SiHy) CLs exhibit relatively high deposition of TF lipids that may be associated with visual disturbances and discomfort. Additionally, lipids are degraded by oxidation and may cause alterations of the TF lipid layer, which in turn might be a source for dry eye symptoms.

The overall goal of this thesis was to evaluate the quantity and location of lipid deposition on various CL materials over time and also to assess the impact lipid contamination may have on various care products and TF quality measurements.

The specific aims of each chapter of this thesis were as follows:

- Chapter 3: To determine the efficacy of multi-purpose solutions (MPS) on the removal of cholesterol deposits from SiHy lens materials.
- Chapter 4: To analyze the uptake of cholesterol on SiHy and conventional hydrogel (CH) daily disposable (DD) CL materials using an *in vitro* radiochemical detection method.

- Chapter 5: To evaluate the differences in lipid uptake and penetration in DD CL using the conventional “in-vial” method compared to a novel *in vitro* eye model.
- Chapter 6: To develop a novel *in vitro* model to determine pre-lens non-invasive break-up times (NIBUT) and to subsequently compare the break-up times over five contemporary DD lens materials.
- Chapter 7: To optimize and develop a method to determine and quantify lipid peroxidation by-products that indicates oxidative stress in tears.

Materials and Methods

- Chapter 3: Five contemporary SiHy lens materials were incubated for 7 days using a radiochemical experiment. Additionally, lenses were stored and cleaned in different MPSs using a rub and rinse technique. Lipids were then extracted from lenses with 2:1 chloroform:methanol, analyzed in a beta-particle radiation counter and µg/lens of cholesterol was determined.
- Chapter 4: Seven different commercially available DD CLs were incubated for 16 hours to determine the impact of material composition on cholesterol deposition. Subsequent to the incubation, lenses were extracted using 2:1 chloroform:methanol and the extracts were analyzed in a beta-particle radiation counter and (ng/lens) extrapolated from standard curves.
- Chapter 5: Seven DD CLs were incubated for 4 and 12 hours in an artificial tear solution (ATS) containing fluorescently-labelled cholesterol (7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol, or NBD-cholesterol). Additionally, CLs were incubated in an “in-vial”

condition and compared to a novel *in vitro* eye platform, designed to simulate physiological tear flow, tear volume, and 'simulated' blinking. After the incubation period, the CLs were analyzed using a laser scanning confocal microscope (LSCM), and quantitative analyses for penetration depth and relative fluorescence intensity values were carried out.

- Chapter 6: Five DD lens materials were incubated in an artificial tear solution using a model blink cell that mimics intermittent air exposure. CLs were incubated by repeatedly being submerged and exposed to air for up to 16 hours. A corneal topographer (Topcon CA-100) was used to illuminate the lens surfaces with placido rings, which were captured with a digital video camera and from which NIBUTs of the CL materials were determined.
- Chapter 7: Tear samples were collected using calibrated disposable capillary tubes and various assays that quantify lipid peroxidation by-products were compared against each other: thiobarbituric acid reactive substances (TBARS) assay, malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and oxidized low-density-lipoproteins (OxLDL) enzyme-linked immunosorbent assays (ELISAs). Pooled and individual tear samples were diluted in a wide range to determine the lowest volume of tears that could be used. Subsequently, the fluorescence was measured with a fluorescence spectrophotometer at 530 nm (excitation) and 590 nm (emission), as well as their absorbance at 450 nm.

Results

- Chapter 3: For all lens materials, only one of the multipurpose solutions removed more cholesterol than any other test solution; however, the amount of cholesterol removed from the individual CLs was statistically significant only for the two lens materials that deposited the most: balafilcon A (0.93±0.02µg/lens) and senofilcon A (0.95±0.01µg/lens). All of the other solutions evaluated showed no significant effect on lipid removal.
- Chapter 4: Cholesterol deposited significantly more on SiHy lenses than CHs. The uptake of cholesterol ranged from 22.63 ± 2.98 ng/lens to 97.94 ± 4.18 ng/lens for all lens materials, with narafilcon A accumulating the largest quantity of cholesterol. The accumulation of cholesterol was shown to be continuous throughout the 16 hours of incubation without reaching a plateau.
- Chapter 5: The depth of penetration of NBD-cholesterol varied between the vial and the eye-blink platform. In general, SiHy lenses showed higher intensities of NBD-cholesterol than CH materials and the fluorescence intensities also varied between the incubation methods as well as the lens materials.
- Chapter 6: Overall, NIBUTs ranged from 26.19 ± 5.79 s to 1.23 ± 0.13 s. After the initial (T_0) break-up times were determined, CH CLs revealed significantly longer NIBUTs than SiHy CLs. After 16 hours of incubation, the SiHy lens material delefilcon A had the longest break-up time. Significant changes of NIBUTs within the lens

materials varied between the examined time points. After 16 hours, all CLs showed significant reductions in NIBUTs in comparison to T_0.

- Chapter 7: After tear samples were pooled and concentrated, 0.056±0.004 µM of MDA could be measured using the TBARS assay. After optimizing various ELISAs, OxLDL in individual tear samples (2.5µL) ranged between 45.59 ± 2.95 ng/mL and 28.24 ± 4.66 ng/mL. All measurements using the MDA- and 4-HNE ELISA were below the assays limit of detection.

Conclusions

- Chapter 3: Lipid-removal efficacy varies depending on the combination of lens material and solution. Only one MPS showed a significant reduction of lipids for any of the tested lens materials.
- Chapter 4: For the periods of time that DD lens materials are worn, cholesterol deposits significantly more onto SiHy lenses than CHs. This could have implications for wearers who have higher levels of lipid in their tears that are fitted with SiHy DD materials.
- Chapter 5: This study provides a novel *in vitro* approach to evaluate deposition and penetration of lipids on CLs. We show that the traditional "in-vial" incubation method exposes the CLs to an excessively high amount of ATS, which results in an overestimation for cholesterol deposition. The novel eye-platform, provides a more natural environment for *in vitro* lens incubation studies which will consequently better elucidate the interactions between CLs and TF components.

- Chapter 6: NIBUT values reduced gradually over time and varying levels of deposition on different CLs may impact the measured pre-lens NIBUT of various lens materials. While NIBUT of CH materials are longer than that obtained with SiHy materials immediately out of the blister pack, it appears that after TF exposure, the NIBUTs determined between CH and SiHy DD materials are very similar.
- Chapter 7: Assays for oxidative stress were optimized and showed that oxidative stress is detectible in small quantities of tears (2.5 µL). These techniques could be employed to determine oxidative stress in TF lipids, which could potentially help to identify patients with dry eye and CL discomfort.

This thesis has provided previously unavailable information on lipid deposition on CLs and its effect on clinically relevant TF quality measurements. The results showed that current CL cleaning solutions fail to efficiently remove lipid contaminations and that DD SiHy lens materials provide options for clinicians to consider when patients experience complications and excess lipid uptake with daily wear lens materials. Furthermore, this thesis has presented novel *in vitro* methods that will be useful for other researchers and the CL industry to appropriately test and predict CL performance.

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Pursuing a PhD was not on my list of goals when I began my path as an optician in Germany, however, it will forever be one of the most profound achievements of my career. I now realize that one does not accomplish this in isolation, but only with the support of colleagues, friends and family. I would like to acknowledge and thank these individuals who have supported me in the completion of this endeavor.

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Dedication

To my parents and my beloved wife Carolyn who have supported me throughout this journey. This is as much your work as it is mine.

I love you!

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List of Abbreviations

%	percent
^{125}I	iodine-125
^{14}C	carbon-14
^{3}H	tritium
4-HNE	4-hydroxynonenal
ADDE	aqueous deficient dry eyes
ATS	artificial tear solution
BA	balafilcon A
CA	comfilcon A
CH	conventional hydrogel
CL	contact lens
CLD	contact lens discomfort
COD	coefficient of determination (R^2)
COETF	Canadian Optometric Education Trust Fund
CSS	complex salt solution
DD	daily disposable
DE	dry eyes
DED	dry eye disease
DEWS	Dry Eye WorkShop
Dk/t	oxygen transmissibility
DMA	N,N-dimethylacrylamide

DW	daily wear
EDE	evaporative dry eye
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOBO-41™	polyoxyethylene-polyoxybutylene
et al.	et alii (“and others”)
EW	extended wear
FDA	Food and Drug Administration
FMA	N-formylmethyl acrylamide
GC	Gas Chromatography
GLC	gas liquid chromatography
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HEMA	hydroxyethyl methacrylate
HPLC	high pressure liquid chromatography
i.e.	in essence (“in other words”)
IR	infrared
ISO	international organization of standardization
LA	lotrafilcon A
LB	lotrafilcon B
LCD	liquid-crystal display
LCMS	liquid chromatography mass-spectrometry

LOD	limit of detection
LOQ	limit of quantification
LSCM	laser scanning confocal microscope
m/z	mass/charge ratios
MA	methacrylic acid
MAPD	myristamidopropyl dimethylamine
MDA	malondialdehyde
MG	meibomian glands
mg	milligram
mL	millilitre
MPDMS	monofuncional polydimethylsiloxane
MPS	multi-purpose cleaning solution
MS	mass spectrometry
NBD-cholesterol	7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol
ng	nanogram
NIBUT	non-invasive break-up times
nm	nanometer
NMR	Nuclear Magnetic Resonance Spectroscopy
NVP	N-vinyl pyrrolidone
OAHFA	(O-acyl)-ω-hydroxy fatty acids
OxLDL	oxidized low-density-lipoproteins
P	statistical significance

PAPB	polyaminopropyl biguanide
PC	phosphatidylcholine
PC-ABS	polycarbonate-acrylonitrile-butadiene-styrene
PDMS	polydimethylsiloxane
PE	phosphatidylethanomaline
PEG	polyethylene glycol
pHEMA	poly(hydroxyethyl methacrylate)
PHMB	Polyhexamethylene biguanide
PMMA	poly(methyl methacrylate)
pmol	picomolar
PTFE	polytetrafluoroethylene (Teflon™)
PUFA	poly unsaturated fatty acids
PVA	polyvinyl alcohol
PVP	poly(vinylpyrollidone)
RIF	relative intensity of fluorescence
RM-ANOVA	repeated measures analysis of variance
SA	senofilcon A
SiHy	silicone hydrogel
$sPLA_2$	secretory phospholipase A_2
SPSS	Statistical Package for the Social Sciences
SS	Sjögren's Syndrome
TBA	thiobarbituric acid

TBARS	thiobarbituric acid reactive substances
TBUT	Tear break-up time
TEGDMA	tetraethyleneglycol dimethacrylate
TF	tear film
TLC	thin layer chromatography
USAN	United States Adopted Names
UV	ultra violet
µg	microgram
µL	microlitre
µm	micrometer
µM	micromolar

Chapter 1
Literature Review

1.1 The Meibomian Glands

1.1.1 Structure and Function

Meibomian glands (MG) are long, sebaceous, holocrine glands embedded within the tarsal plate of the eyelids that secrete lipids onto the ocular surface (Figure 1-1) and were first described in detail in the 17th century.[1, 2] Positioned in vertical rows within the eyelids,[2] their density varies, with approximately 20 to 30 individual glands in the lower lid and 25 to 40 glands in the upper lid.[3] The location of MGs determines their individual lengths, which can range between 5.5 mm in the upper eyelid and 2 mm in the lower eyelid.[2, 4]

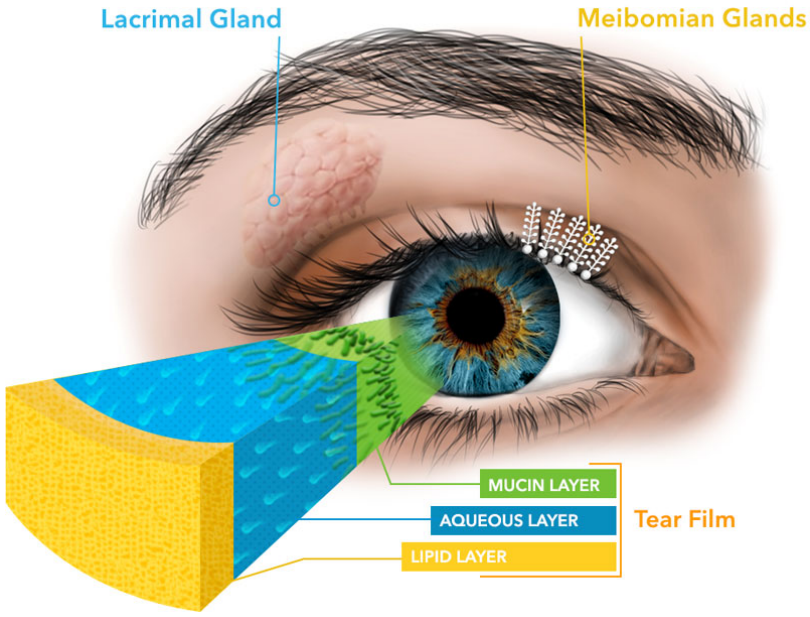

Figure 1-1. Tear producing structures and tear film structure of the eye.

Courtesy of http://visionsource-nfe2020.com/ocuslar-diseases-2/meibomian-gland-dysfunction/

Each gland is composed of a cluster of small acini that are connected to a central duct from which a mixture of various lipids (meibum) is secreted onto the tear meniscus through the orifices within the eyelid margins (Figure 1-2).[2, 3] The production of meibum begins within the small acini

units (with approximate diameters of 150 to 200 µm) of each MG.[2, 5, 6] The secretory cells within the acini (meibocytes) produce both polar and non-polar tear film (TF) lipids that are included in meibum.[2, 5, 6] As these meibocytes mature, they migrate from the periphery to the center within the acinus where they subsequently disintegrate.[2] The cell contents of the meibocytes are then released into the ductal system and are ultimately secreted as meibum through the main central duct (Figure 1-2).[2]

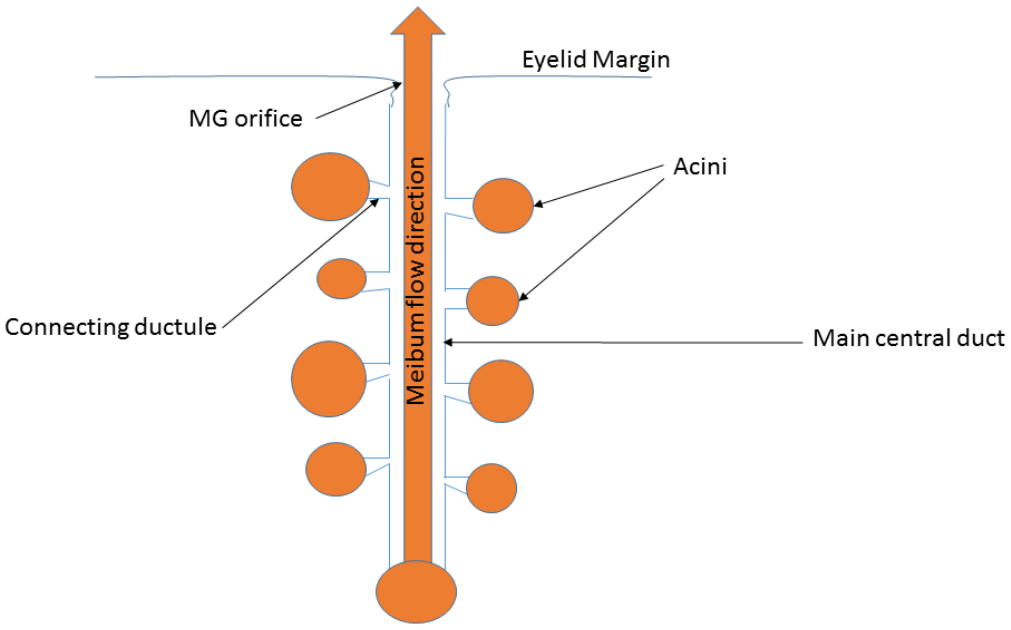

Figure 1-2. A MG functional unit with multiple acini units connected to a central duct. Meibum is produced at the acini units, which travels to the central duct, and eventually out of the MG orifice at the eyelid margin. Re-printed with the kind permission of Dr. William Ngo.

The exact processes that control and regulate the secretion of meibomian glands are currently not well understood. Sullivan et al., however, have published a significant amount of research that suggests that the expressed lipid pattern is influenced by hormones, such as androgen, estrogen, and progesterone, which regulate the meibomian gland secretion.[2, 3, 7-20] Other researchers have discussed neuronal control of the meibomian glands. [3]

The excretion of the meibum relies on two mechanisms that occur simultaneously: 1) the continuous production of meibum within the MGs; and 2) the mechanical expression from the glands with every blink.[3, 21] Once the lipids are secreted, they fulfill a variety of functions, including prevention of overflow of the tear meniscus by creating a hydrophobic barrier; formation of a seal between the eyelids during sleep; reduction of TF evaporation between blinks; lubrication during the blink; and potentially providing a protective layer against bacterial contamination of the ocular surface. [7, 22]

1.1.2 Meibum Composition

A significant amount of research has been conducted to classify and quantify the exact lipid compositions within meibum.[2, 21, 23-45] The application of different chromatography methods presented in these publications, however, makes it difficult to compare the results and be certain about the exact composition of lipids in meibum. Nevertheless, a variety of lipid categories have been identified and are listed in Table 1-1.[2] Although all of these lipids are usually found in meibum, the lipid composition varies greatly between individuals.[2, 45] Despite the inconsistency between subjects in terms of percentage present, the lipid types found in the meibomian glands remain fairly consistent in healthy eyes.

Table 1-1. The lipid composition of meibomian gland secretions [2, 45]

Lipid	% of Meibomian Gland Secretions	Lipid	% of Meibomian Gland Secretions
Cholesterol	1 - 14	Phospholipids	1 – 15
Cholesteryl Esters	2 – 34	Free Fatty Acids	0 - 24
Sterol Esters	27 - 39	Triacylglycerols	2 - 43
Wax Esters	13 - 69	Polar Lipids	0 - 15
Hydrocarbons	8 - 36		

Some of the common lipids found in meibum include cholesterol, cholesteryl oleate, cholesteryl linoleate, diacylglycerols, triolein, oleic acid methyl ester, oleic acid propyl ester, and triacylglycerols.[2, 21, 23-45] Additionally, a mixture of polar lipids has been detected in meibum, especially phospholipids, where phosphatidylethanomaline (PE) and phosphatidylcholine (PC) are most common, as well as sphingolipids, and ω-hydroxy fatty acids. [2, 6, 16, 25, 28, 29, 31, 35, 38, 46-53] Although the concentration of polar lipids in meibum is small, these amphiphilic lipids are thought to play a crucial role in the stability of the TF by functioning as mediators between their surrounding layers (see 1.2.2).[2, 29, 35, 45]

1.1.2.1 Cholesterol

Cholesterol and its esters are one of the major lipids found in meibum samples.[5, 23, 26, 28, 35, 54, 55] These non-polar lipids belong to the lipid group of isoprenoids, which are metabolically built up from five carbon units (isoprene). As shown in Figure 1-3, cholesterol is composed of 27 carbons that are arranged in four fused rings, a hydrocarbon division, two methyl groups, and a hydroxyl group. Based on its structure, it is an important component of cell membranes and aids their stability and rigidity.[56, 57] However, cholesterol's covalent bonds between its carbon and hydrogen atoms are of polar nature and, thus, render this lipid to be very hydrophobic and insoluble in water. Consequently, due to its hydrophobic characteristic, cholesterol is a common lipoidal contaminant on SiHy materials, [58-63] which is the reason why it was chosen to be the main lipid of interest for this thesis (see 1.4.2.2).

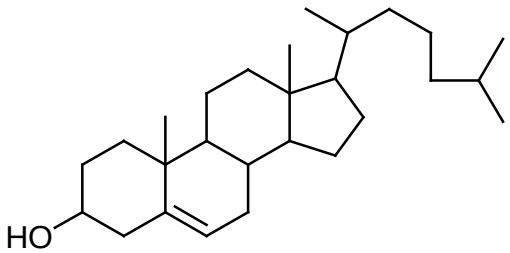

Figure 1-3. Chemical structure of cholesterol (386.65 g/mol)**.**

1.2 The Tear Film

1.2.1 Structure and Function

The TF is a complex layered structure that is historically believed to consist of three layers: the outermost lipid layer, accounting for approximately 1% to 1.5% of the total thickness of the TF ranging between < 60nm to 180nm; the middle aqueous layer, making up 98% (7 μm); and an inner mucin layer (0.5%; 0.02-0.05 μm) that lies closest to the ocular surface. [64-68] The outermost lipid layer mainly contains fatty secretions from the MGs (see section 1.1), which stabilizes the TF, aids lubrication and helps to avoid evaporation.[67-71] The aqueous layer is produced by the lacrimal glands found in the superior orbital cavity.[68] Among other components, it contains lysozyme, a protein that has antimicrobial properties.[72-74] The thin mucin layer is produced by membrane-bound and secreted mucins that are produced by goblet cells, located in the conjunctiva.[2, 75] It consists of fatty, glycosylated proteins that stay in direct contact with the cornea and penetrate the fine gaps between the corneal microvilli to create a smooth and even corneal surface, contribute to the stability of the TF, and provides lubricity for smooth blinks.[68, 76-78]

Several decades after the first TF model was proposed, researchers suggested a more complex structure of the TF, including a more gel-like mucin layer and a lipid layer far more complex than previously reported.[54, 79-81] Since then, researchers have investigated the TF intensively, resulting in further revisions and additional differentiation of the TF layers has been proposed.[2, 30] These changes include a superficial non-polar lipid layer, an inner polar lipid layer with intercalated proteins, an aqueous layer incorporating “gel-like” mucins and a mucoid layer (glycocalyx) on top of the ocular surface.[2, 30, 68] Figure 1-4 depicts a diagrammatic version of the three layer TF model, including the updated layers by Tiffany and McCulley et al.[54, 79, 80] Furthermore, current research looking at the total TF thickness of all layers estimates a thickness of 2 to 5.5 μm.[68, 82-90]

The TF is described as having five main functions: [91]

- it protects the cornea from debris and foreign materials by forcing them away from the central cornea upon blinking;
- it maintains the bulbar and palpebral conjunctival moisture and lubricates all surfaces which come into contact with air;
- it allows for a smooth optical surface by alleviating any small imperfections in the corneal epithelium;
- it contains various antibacterial and immunological agents to protect against ocular infection;
- it provides oxygen and nutrition to the underlying corneal epithelium.[91]

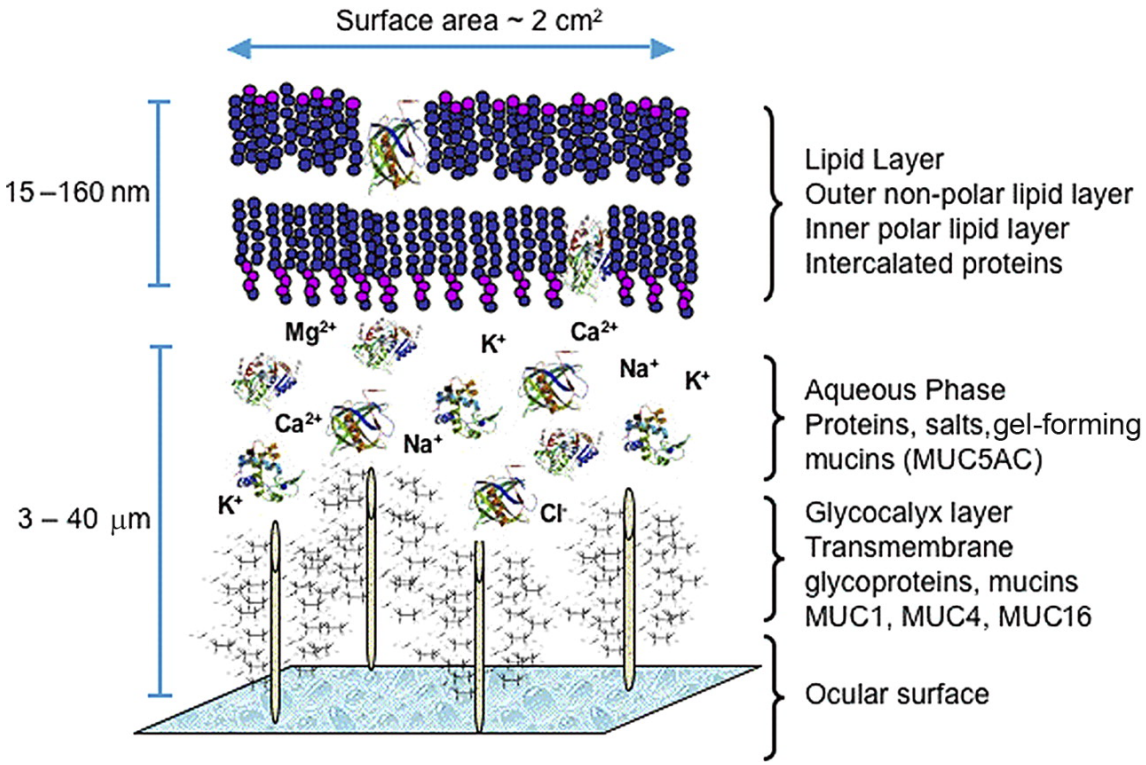

Figure 1-4. The structure of the tear film. Reprinted with permission from: Green-Church KB, Butovich I, Willcox M, Borchman D, Paulsen F, Barabino S, *et al*. The international workshop on meibomian gland dysfunction: report of the subcommittee on tear film lipids and lipid-protein interactions in health and disease. Invest Ophthalmol Vis Sci. 2011 Mar; 52(4):1979-93. © Association for Research in Vision and Ophthalmology 2017. [2]

1.2.2 Formation of the Lipid Layer

As mentioned in section 1.1, lipids originate from MGs and are found in several locations in the TF, including the base of the TF adjacent to the outermost corneal epithelium.[51, 52] However, the exact role that MG lipids play in the TF is complicated and not entirely understood.[51, 68]

After meibum is secreted, it mixes with tears on the ocular surface and the lipids form a thin sheet to stabilize and prevent evaporation of the TF.[70] It is believed that lipid conformation and composition may change after the meibum has mixed with tears due to the change of environment and reactions with other TF components. Several studies comparing lipid composition have shown varying lipid profiles and lipoidal components between the TF and meibum.[2, 24, 29, 31, 35, 53, 92] Many thousands of specific lipids are estimated to exist in meibum and tears and in spite of the differences in detected lipid profiles, the main types of lipids within meibum and the tears remain broadly the same: wax esters, cholesteryl esters, free fatty acids, fatty sterols, fatty alcohols, monoacylglycerols, diacylglycerols, and triglycerides. [2, 5, 9, 38, 43, 44, 93-96]

The TF lipid layer contains two different lipid phases: the outermost non-polar layer and an inner polar layer.[2, 54] The characteristics of each TF lipid layer are unique and provide imperative functions.[51]

1.2.2.1 Non-Polar Phase

The outermost non-polar lipid phase contains a large amount of non-polar lipids, including cholesterol, wax esters, cholesterol esters, triglycerides, fatty acids, and hydrocarbons.[54] The non-polar phase is believed to be thicker than the polar phase (85-95%) [30, 32, 52] and, therefore, it is those lipids that are found in the greatest quantities.[2, 29, 52, 54] The function of the non-polar phase is to regulate the transmission rate of water vapor, carbon dioxide, oxygen, and ions.[28, 48, 54]

Additionally, the non-polar layer is a storage unit for triglycerides, wax esters, other non-polar lipids, and surfactant proteins.[2, 30, 54]

1.2.2.2 Polar Phase

The polar phase of the TF lipid layer consists of a number of different types of polar lipids with the major ones being PE, PC, sphingomyelin, and ceramides, plus others.[25, 46, 54] More recently, (O-acyl)-ω-hydroxy fatty acids (OAHFAs) have been detected in meibum and the polar lipid phase in higher concentrations.[2, 33, 37, 42] Each of these polar lipids are thought to serve the purpose of acting as mediators between the hydrophobic non-polar layer and the aqueous layer to enhance the stabilization of the TF.[2]

Specifically, the amphiphilic nature of polar lipids – containing both polar and non-polar segments – is believed to contribute significantly to the TF stability by positioning their hydrophilic head towards the aqueous TF layer and submersing their hydrophobic tails within the non-polar lipid phase.[2]

1.2.2.3 Tear Film Lipid Concentration

In contrast to the previously listed lipid concentration in human meibum (Table 1-1), the lipid concentration of human TF samples have been found to consist of a more complex lipid configuration where the majority of identified lipids were polar lipids, such as phospholipids and sphingomyelin (Table 1-2).[92, 94, 97] These large quantities of polar lipids within the tear fluid emphasizes their important role as surfactants between the aqueous and lipid TF layer.

Table 1-2. Lipid concentration detected in human tears.[92, 94, 97]

Lipid	% of Tear Film Sample	Lipid	% of Tear Film Sample
Cholesterol esters	0 - 32	Phospholipids	45 – 70
Diesters	detected	Free Fatty Acids	15 - 18
Wax Esters	detected	Triacylglycerols	5.1 - 5.6
OAHFAs	detected	Sphingomyelin	3 - 40

1.2.3 Tear Film Stability

The stability of the TF is an important factor to fulfill its function in providing crisp vision and high comfort throughout the day.[65, 68, 70, 91, 98] TF stability represents itself as the duration during which the TF remains fully spread and covers the ocular surface entirely.[68, 98]

In healthy patients, research has shown that the TF may be stable for approximately 30 seconds between blinks until a break-up occurs and it has further been shown to be stable in the presence of disturbances within the TF itself (i.e. bubbles or particles).[68, 69, 99, 100] Furthermore, a stable TF is an indicator of a fully functioning lipid layer and is directly connected to the integrity and composition of TF lipids, mucin layers, the intercalated proteins within the lipid layer, and the surface tension of the TF lipids at the air interface.[68, 71, 98, 101-103] The TF stability, however, is very labile and can be affected by many factors such as age, medication, work environment, diet, ocular surface temperature, smoking, diurnal variations and contact lens (CL) wear.[68, 79, 98, 104-118] Additionally, various studies have shown increased TF evaporation rates and instability when the lipid layer is either absent, thinned, or has shown an unusual lipid arrangement within itself.[68, 70, 98, 119, 120]

The main causes of an unstable TF can be narrowed down to deficiencies in the quality and quantity of tears, which are directly correlated to the two types of dry eyes (DE): evaporative (EDE) and aqueous deficient (ADDE).[121-124] While ADDE is caused by a deficiency of the lacrimal glands to produce sufficient quantities of tear volume, EDE can be triggered by various factors, such as ocular surface disorders, blink disorders, TF disorders, or meibomian gland dysfunction (MGD).[124] As defined within the recently released DEWS II report, both EDE and ADDE coexist and may cause damage to the inter-palpebral ocular surface and are associated with symptoms of ocular discomfort.[124]

TF stability may further be affected by lipid degradation caused by oxidation or enzymatic lipolysis (through secretory phospholipase A_2 [$sPLA_2$]), which degrades lipids and may cause deterioration of the native lipid structure.[125-131] Lipids that are highly amphiphilic, such as OAHFAs within the polar phase, may be affected by oxidative degradation, which can lead to loss of functionality that can subsequently cause tissue or cell damage due to the formation of toxic species, which in turn could result in an unstable TF.[56, 97, 125, 126, 128, 129, 132-134] Phospholipase A_2 is an enzyme that specifically degrades phospholipids such as PC and PE that are present in the TF (see section 1.2.2.2). By-products and biomarkers of both degradation processes have been associated with decreased comfort ratings in intolerant CL wearers.[125] Some of the external stress factors that cause lipid oxidation are wind, extreme temperature, UV radiation, pollutants, irritants, and smoke.[108, 132, 135-137] Lipid peroxidation is known to be influential in the development of Alzheimer's disease, Parkinson's,[138, 139] and cancer,[135] and in the eye specifically, lipid oxidation is associated with age-related macular degeneration,[140, 141] DE,[137, 142, 143] uveitis,[144] cataract,[145-148] and keratitis.[135, 149]

Lipid oxidation mostly affects unsaturated fatty acids that contain one or more carbon double bonds that are the main target.[129, 150] Such lipids are prone to highly reactive oxygen free radicals that initiate an extraction of allylic hydrogen molecules to form lipid allylic radicals. Thereafter, the lipid radical reacts with oxygen to form peroxide radicals that are then able to form other lipid allylic radicals or degrade a lipid further to form highly reactive aldehydes.[128-130, 150] Major lipid peroxidation by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are detectable in blood, serum, and tear samples.[106, 125, 128, 129, 133, 142, 145, 150-163]

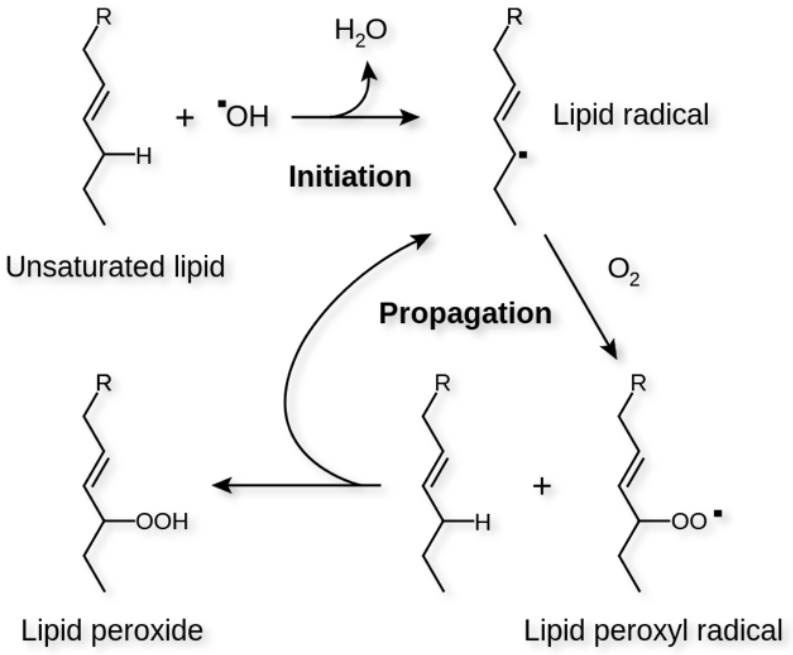

Figure 1-5. Lipid peroxidation cycle.[150] Illustration is courtesy of https://en.wikipedia.org/wiki/File:Lipid_peroxidation.svg.

1.3 Contact Lens Materials

CLs have shown a steady increase since their introduction and remain a popular medical device for an estimated 140 million people worldwide to correct refractive errors.[164-166] Their history dates back more than 500 years, when Leonardo da Vinci first showed the principle of altering the eyes refractive power by placing his head into a bowl of water.[167, 168] The first recorded use of a CL, however, had to wait until late in the 19th century when the ophthalmologists Fick and Kalt fitted glass scleral lenses on rabbits, patients, and themselves to correct the optical power of eyes.[168, 169] However, the choice of glass as the first material for CLs failed to succeed as it is uncomfortable to wear and is impermeable to oxygen.[168, 169] Another century passed until the very first biocompatible lens material was developed and rigid CLs were made out of polymethylmethacrylate (PMMA) that could be worn successfully.[168, 169] Since then, the

development of CL materials has had an impressive journey, which led to the success they have today. The most prescribed lens materials to-date are soft hydrogels, that can be further categorized into conventional hydrogels (CH) and silicone hydrogels (SiHy).[166, 168, 169]

1.3.1 Conventional Hydrogels

In the 1960s, the Czech chemists Otto Wichterle and Drahoslav Lim developed the basis for the first soft CL materials that were made out of poly(hydroxyethyl methacrylate) (pHEMA).[168-172] The main benefits of the pHEMA lens material were its biocompatibility and its ability to absorb and retain water, which significantly increased their flexibility and comfort during lens wear. In the early 1970's spin-casting technology for the mass production of pHEMA lenses was sufficiently developed to permit the commercialization of soft CLs as a device to correct refractive errors.[168, 173]

Despite the supremacy of pHEMA-based CL materials over many years, these materials presented a shortcoming in providing sufficient amounts of oxygen to the eye.[168, 172] The main source of oxygen to the eye is through its outermost TF layer that is in direct contact with the surrounding air interface and the circle of blood vessels within the limbus that circle the cornea. The placement of a CL onto the ocular surface introduces an additional obstacle (Figure 1-6) that limits the amount of oxygen reaching the cornea and leads to undernourishment of the cornea when pHEMA CLs are worn.[168, 169, 174, 175] Extended wear of these lens materials eventually leads to increased hypoxic exposure, which limits the metabolic capabilities of the corneal endothelium to regulate the hydration of the cornea, and may subsequently manifest in edema within the cornea, increased inflammatory responses (i.e. infectious keratitis), neovascularisation, changes in endothelial morphology and eventually in potential compromise of corneal function.[175-178]

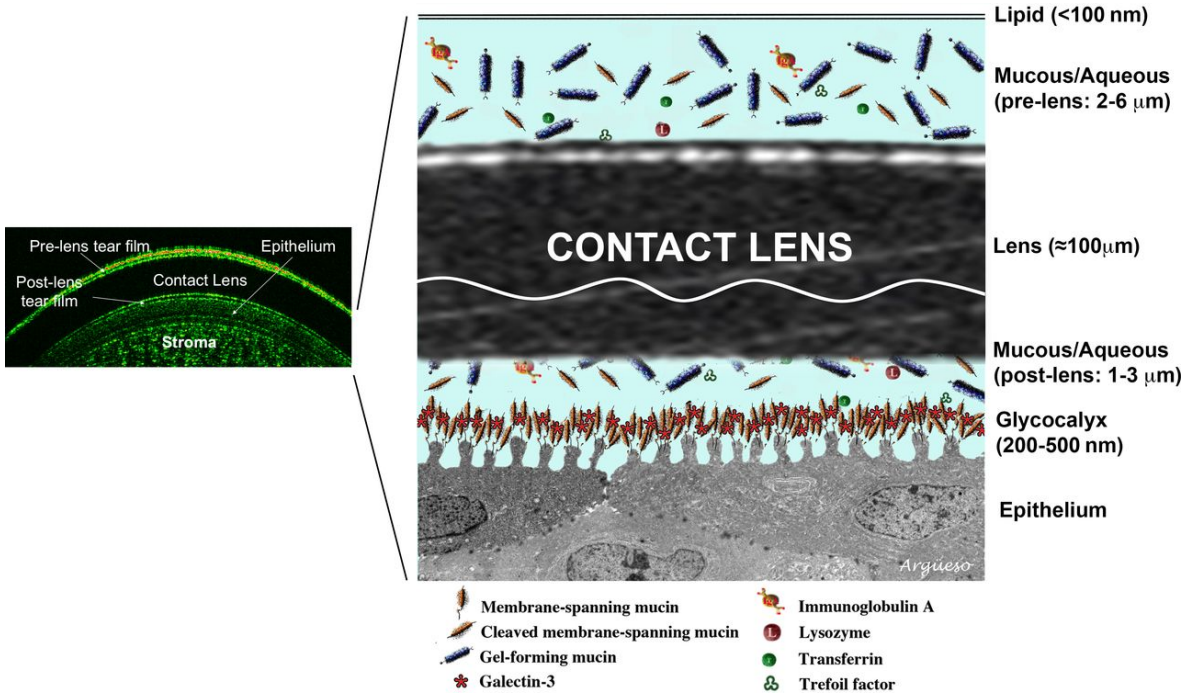

Figure 1-6. Disruption of the tear film layer when a contact lens is placed onto the ocular surface. Reprinted with permission from: Craig JP, Willcox MD, Argueso P, Maissa C, Stahl U, Tomlinson A, et al. The TFOS International Workshop on Contact Lens Discomfort: report of the contact lens interactions with the tear film subcommittee. Invest Ophthalmol Vis Sci. 2013;54(11):TFOS123-56. © Association for Research in Vision and Ophthalmology 2017.[175]

1.3.2 Silicone Hydrogels

To eliminate and/or reduce complications associated with hypoxia that were triggered by pHEMA based CL materials, manufacturers had to find a way to increase the amount of oxygen that was able to pass through a CL (also known as the oxygen transmissibility (Dk/t) of a CL). The water within CH lens materials is the dictating - and simultaneously limiting - factor in determining how much oxygen can flow through the lens materials.[168, 174] A previous material known to have high oxygen transport capabilities were silicone-based. In fact, the first lenses with high oxygen transmissibility were made of silicone elastomer, used to treat infants after congenital cataract surgery.[168, 179-183] However, silicone elastomers were not successful mainly due to their tendency to adhere to the eye, poor surface wettability, high lipid deposition and poor comfort during wear.[168, 184]

Silicone-based soft lens materials became a commercial reality with the introduction of SiHy CLs in the late 1990s, when Bausch & Lomb and CIBA Vision presented the 1st generation SiHy lens materials, balafilcon A (Pure Vision) and lotrafilcon B (Focus Night & Day).[168, 185] Combining both components provided CLs with the required oxygen transmissibility through the silicone backbone and the necessary ion and water permeation capabilities via the hydrogel that are crucial for lens movement and comfort.[171, 185-187] However, including siloxy monomers into CL materials simultaneously led to an increase in hydrophobicity of SiHy CLs, which creates less wettable lens surfaces which may jeopardize CL comfort during lens wear.[187] Both, oxygen transmissibility and hydrophobicity of SiHy lens materials is attributed to the silicon-oxygen bonds these CL are composed of [187] and to enhance the hydrophilicity of SiHy lens materials, manufacturers applied surface modifications. For the balafilcon A lens material, Bausch & Lomb applied a plasma oxidation process that created wettable silicate "islands" on the lens surfaces.[171, 187-189] On the surface of the lotrafilcon B lens materials, CIBA Vision added a thin and continuous wettable non-siloxy plasma coating (25nm) enclosing the entire CL.[171, 187-189] The 2nd generation of SiHy lens materials was released by Johnson & Johnson, with the introduction of galyfilcon A (Acuvue Advance) and senofilcon A (Acuvue Oasys). Instead of applying an additional surface modification, Johnson & Johnson opted to incorporate the internal wetting agent poly(vinylpyrollidone) (PVP) to improve the hydrophilicity of these CLs.[186, 187] The 3rd generation was introduced shortly after this through the release of CooperVision's comfilcon A (Biofinity) lens material. In contrast to the previous two generations of SiHy CLs, comfilcon A lenses did not require any surface modification nor the incorporation of any internal wetting agents. Instead, CooperVision made use of incorporated inherently wettable moieties to improve comfort.[168, 186, 187] More recently, daily disposable (DD) lens materials were introduced to the portfolio of contemporary SiHy lenses. The newest members include Alcon's delefilcon A (Dailies Total 1),

CooperVision's somofilcon A (Clariti 1 Day), and Johnson & Johnson's narafilcon B (1-Day Acuvue TruEye) lens materials. Whilst somofilcon A CLs do not include any surface modification or internal wetting agents,[190] the internal wetting agent PVP is incorporated into narafilcon B CLs,[191, 192] and Alcon developed a unique design strategy for its delefilcon A lens materials to enhance comfort.[187] Delefilcon A CLs consist of a silicone core that is enclosed by a 5µm thick hydrogel layer. This design introduced a "water gradient" concept, where the CL surface has a very high water content (WC; >80%) material compared with the CL core SiHy material (33%).[193-196] Although SiHy lens materials have undergone extensive improvements to increase lens comfort and wettability, the main reason for patient "dropout" remains CL discomfort even with contemporary materials.[197-201]

1.3.3 Contact Lens Classifications

To initially differentiate the performance of a growing variety of contemporary CH lens materials and their interaction with TF proteins and various lens care products, researchers [202] proposed a system to categorize these lenses into four groups based on the materials WC and ionic content, which was later adopted and implemented by the US Food and Drug Administration (FDA).[203] Since then, the chemically more complex SiHy lens materials have been introduced to the market and showed different interaction patterns with TF components and lens care products, mainly due to their relatively hydrophobic lens material characteristics. To address these differences, researchers proposed the introduction of a fifth group [204-206] that is now implemented into the most current conventional group system shown in Table 1-3.[207]

Table 1-3. FDA and ISO approved Contact Lens Classifications.[207]

FDA Group	Subgroup	Water Content	Ionic Charge	Surface Treatment
I	-	< 50%	No	-
II	-	> 50%	No	-
III	-	< 50%	Yes	-
IV	-	> 50%	Yes	-
V	**A**	< 50%	No	Yes
	B1 & B2	< 50%	No	No Contains a hydrophilic monomer & Semi-interpenetrating network
	C	> 50%	No	-
	D	No specification	Yes	-

1.3.4 Contact Lens Distribution

For almost two decades, an international consortium of clinicians, researchers, and industry partners has been conducting valuable information about the distribution of CLs in over 62 different countries across the world.[208] These annual reports highlight the steady increase in popularity of SiHy lens materials since their introduction to the CL market.[166, 208-213] From the most recent market evaluation (Figure 1-7), it is apparent that within the Canadian market patients wear significantly more SiHy lens materials than CHs, which presents additional incentives for this thesis to investigate the interactions between current CLs and TF lipids.

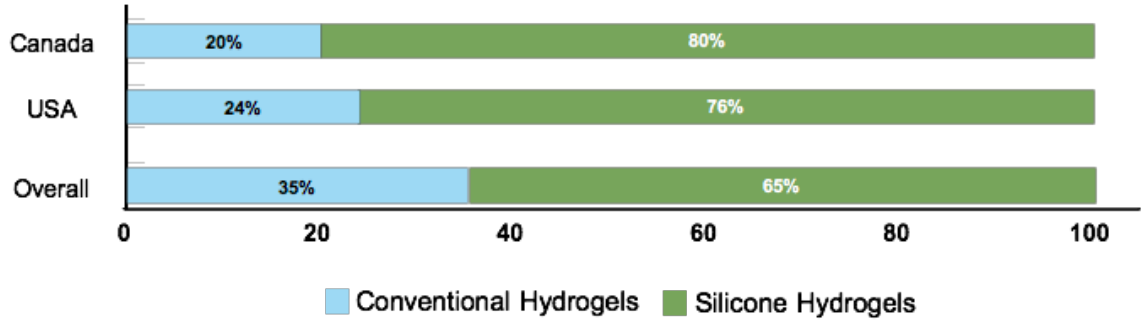

Figure 1-7. Distribution of conventional vs. silicone hydrogel contact lenses in 2017, comparing between the world wide distribution vs. Canada vs. the USA.[208]

1.4 Tear Film Lipid and Contact Lens Interactions

1.4.1 Contact Lenses and Tear Film Stability

Inserting a CL onto the ocular surface disturbs and alters the general structure and integrity of the TF.[91, 175] Figure 1-6 depicts how a CL separates the TF into post- and pre-lens films as it lies within the aqueous TF layer,[175] which in turn creates a significantly thinner aqueous layer for TF lipids to adhere to. Furthermore, CLs present a hurdle that impacts the smoothness of a blink and the redistribution of a stable TF layer over their surfaces.[175, 214, 215] For CLs to be worn comfortably until the end of the day and be considered biocompatible with the ocular surface, it is crucial to have a lens material that allows the TF to rebuild its intrinsic structure.

CL wear has a notable impact on TF stability as TF lipids may be scarcely present over the CL surface or not at all. If they are present, TF lipids come into direct contact with the CLs that can lead to undesirable interactions with the lens materials. Lipid deposition may occur that could subsequently alter CL properties, impact the lens comfort during wear, cause symptoms of dryness, and affect visual acuity.[216-219] The permeable characteristic of soft lenses allows lipids to both adsorb and absorb, which has a direct effect on the amount of detected lipid deposition.[186] Protein deposition on CH lens materials and their impact on lens comfort and CL-related complications has been documented extensively over the past decades. They are mainly triggered by the attraction of TF proteins to the ionic charge of CHs; i.e. negatively charged CLs deposit higher amounts of positively charged proteins (i.e. lactoferrin or lysozyme) than positively or neutrally charged CHs.[220-239] It was not until the appearance of silicone-based lens materials and the tendency of hydrophobic TF lipids to adhere to their hydrophobic sites that the topic of lipid deposition became an issue, leading to a shift of focus and increased interest by researchers and practitioners alike.

1.4.2 Contact Lens Deposition

1.4.2.1 Lipid Deposition on Conventional Hydrogel Contact Lenses

One of the earliest observations of the interaction of TF lipids with CHs was made by Hart et al.[240, 241] who first detected "Jelly Bumps" that mainly consist of cholesteryl esters that are also found in meibum and tear samples.[240, 241] The extended wear (EW) lens materials examined showed varying levels of lipid deposition, which led to the conclusion that the extent of lipid deposition on these CL is highly subject-dependent.[240, 241] By using histochemical staining analysis and microscopy, Hart and colleagues were able to determine that the major component of these deposits were lipids, particularly cholesteryl esters, wax esters, and triglycerides.[240, 241] Furthermore, Hart et al. found that subjects with a lipid-rich TF, taking certain medications, and subjects with a diet rich in alcohol, fats, and proteins tended to be heavier TF lipid depositors.[112, 241] Moreover, the researchers found that once these lipids adhered to the CL surfaces, they could not be removed by cleaning regimens.[241]

Later on, Bowers and Tighe et al. published further work on lipids, showing that "white surface films" are different than "elevated white spot deposits" [242, 243] and that these films mainly consist of cholesterol and their esters. Through the use of various microscopy technologies (i.e. scanning electron, light and dark-phase, phase contrast, and stereo microscopy), the researchers were able to determine that white spot deposits formed a three-layered structure, with unsaturated lipids forming the base layer and cholesterol and their esters creating the second and third layer.[242, 243] The morphology of these white spot deposits was consistent in spite of differences in CL wear routine and type of worn lens material; however, the rate of accumulation differed and was influenced by subject variability and CL material.[242, 243] From these studies,[242, 243] Bowers and Tighe concluded that the biocompatibility and the characteristics of CL surfaces was mainly

affected by the unsaturated lipids that form the base layer of white spots.[242, 243] In contrast, "white surface films" on CL materials showed a different morphology than "elevated white spot deposits", in that the amount of calcium that the CLs were exposed to determined how much lipid deposition was detected. The more calcium that was present, the lower the number of white spots found on these lens materials.[244]

Further research by Tighe and colleagues found lipids in the matrix of CHs [245, 246] and showed that lipid deposition is influenced by lifestyle choices of the CL wearer, their TF composition, the surrounding environment, and that various cleaning regimens were only moderately efficient in removing lipid deposition from CH CLs.[245, 247, 248] Of particular note was the finding that the effectiveness of surfactant-containing, traditional chlorine-, and peroxide-based cleaning solutions was highly influenced by the composition of the lipid deposition and lens material, making them only moderately helpful; disinfectants removed virtually no lipids at all.[248] Usually, the benefits of these cleaners are to be observed only in the first few days of wear/use, as the lipid layer of the TF is constantly replenished.[245]

Rapp and colleagues conducted extensive *in vitro* and *ex vivo* research to determine lipid deposition on various lens materials using a wide variety of techniques, including thin layer chromatography (TLC), high pressure liquid chromatography (HPLC) and gas liquid chromatography (GLC).[224, 249-252] The researchers were able to show that certain TF lipids (cholesterol, cholesteryl esters and triglycerides) were not detectable on CLs, as opposed to fatty acids, alcohols, sterols, wax esters, and diglycerides. Moreover, Rapp et al. concluded that polar TF lipids deposit favourably on CH lens materials compared to non-polar lipids.[249] Additionally, Bontempo and Rapp confirmed that the amount of lipid deposition found on CLs is highly material and lipid-type dependent.[224, 250, 252] Specifically, the researchers were able to conclude that FDA

group II CLs deposit the most TF lipids and FDA group III lens materials the least, and showed that non-ionic CLs and those with a high WC deposit significantly more TF lipids.[224, 250, 252]

More recent publications by Jones and Tighe et al. showed that lipid deposition was significantly higher on FDA II group lens materials and presented further proof that lipid uptake was primarily driven by the lens material characteristics and inter-subject differences.[253, 254] FDA group II lens materials containing N-vinyl pyrrolidone (NVP) showed high quantities of lipid deposition.[246, 255] Furthermore, Jones et al. were amongst the first to find that the amount of lipid deposition found on CLs strongly correlates with the replacement frequency, showing an increase of 44% more lipid uptake on lenses worn for three months compared to one month.[255]

1.4.2.2 Lipid Deposition on Silicone Hydrogels

With the introduction of SiHy lens materials, studying lipid deposition has become more important. Adding siloxane monomers has brought major improvements in eliminating hypoxic clinical findings, allowing patients to wear their CL for far longer than before.[256] The downside, however, is the increased hydrophobicity of CLs due to the presence of the siloxane moieties, which has produced new areas for material research and development due to the fact that these materials deposit greater amounts of lipid than CHs.[58, 62, 257]

The very first study examining the type and quantity of lipid deposition onto worn SiHy lens materials was that conducted by Jones et al.[227] In this study, the researchers found significantly greater amounts of cholesterol, oleic acid methyl esters, and oleic acid deposits on SiHy lens materials compared to CH CLs.[227] Since then, additional studies conducted by other researchers confirmed these findings and further determined cholesterol to be the highest depositing TF lipid on SiHy lens materials.[58, 59, 258-261]

Several other studies have looked at other factors that could impact lipid deposition and the impact lipids may have on CL wear. [60, 62, 63, 258, 260, 262-268] Lorentz et al., for example, examined the effect of TF lipids on CL surface wettability and unexpectedly found an initial improvement in wettability on surface treated SiHy CLs, which could explain higher comfort ratings experienced with such lens materials after the first few hours of wear.[269] An *in vitro* study by Carney et al. examined the kinetic uptake of various TF lipids over 20 days and showed varying lipid uptake patterns between different contemporary SiHy lens materials and was the first to show deposition differences between polar and non-polar lipids.[260] In total, Carney and colleagues detected ~20 µg/lens of non-polar cholesterol and ~5 µg/lens of the polar PE.[260] Iwata et al. reported similar trends of lipid deposition on SiHy lenses, however, they found slightly lower amounts of cholesterol (between 1 to 8 µg/lens) by using gas chromatography mass spectrometry (GC-MS).[259] Since then, other studies examining the kinetic uptake rates of TF lipids on various SiHy lens materials showed significant variations between different lens types and continuous lipid uptake between 1 to 28 days of simulated CL wear.[58, 59, 63] Furthermore, proportional relationships were found between the quantity of lipid deposition, the lipid concentration within an artificial tear solution (ATS), and the renewal of the test solution.[63] Bearing this in mind, more recent publications have been conducted that try to use more advanced *in vitro* models to simulate lipid contamination on various lens materials.[266, 270] In particular, a study by Lorentz et al. showed the importance of a more advanced model when the researchers found increased lipid contamination on various SiHy lens materials by simulating intermittent air exposure between blinks.[266]

Only a few studies to-date have investigated the impact of lens care products on lipid deposition on SiHy lens materials, showing that lens cleaning efficiency varied between different lens care products.[60, 263, 265] Particularly, Polyquad/Aldox- and polyhexanide-based lens care products

performed significantly better than one-step hydrogen peroxide cleaning systems in reducing the amount of accumulated TF lipids.[60, 263, 265]

Research relating the impact of cholesterol deposition on microbial contamination on SiHy lens materials was conducted by Omali et al.[264] The researchers were able to show that the amount of extracted cholesterol from worn CLs did not affect the adhesion of the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[264]

Thus far, the number of studies that have been conducted that examined the clinical impact of lipid contamination on SiHy are very few.[261, 271, 272] In an *in vivo* study, Cheung et al. examined the effects of lipid deposition on comfort, visual acuity, and ocular integrity but found no detrimental correlation.[261] In a later study by Zhao et al., cholesterol deposits only showed a small influence on CL-induced adverse events.[271] A more recent study, however, was able to show increased levels of lipid contamination in tolerant CL wearers, which could be an indicator that lipid uptake may be associated with CL comfort.[272]

Even though current research has helped tremendously to better our understanding of the ways TF lipids deposit on various lens materials, the factors affecting lipid contamination, and the impact lipid deposition may have on lens wear, further research is required that elucidates the effects that lipid deposits have on CL discomfort and the ocular environment. Specifically, future studies need to investigate the performance of new lens care products and CL materials to provide up to date knowledge to the CL industry, practitioners, and patients.

1.5 Lens Care Products

Lens care products are an essential part of a daily CL wear routine. Two of the main goals all lens care products should accomplish is to provide disinfection to prevent contamination and infection with any pathogenic micro-organisms and to remove accumulated TF deposits, prior to

re-insertion of a CL.[273-275] Further requirements include enhancing CL comfort and that they are easy to use, convenient, affordable, provide relatively quick disinfection, and that they can be safely used by anyone and in combination with any contemporary CL material on the market.[273]

The following sections will provide an overview of available lens care regimens and portray their efficacy in removing TF deposits to potentially enhance CL comfort.

1.5.1 Hydrogen Peroxide-Base Systems

The use of lens care products based on hydrogen peroxide (H_2O_2) has a long history and for many, is considered to be the most efficient cleaning regimen for CLs. However, the use of these lens care products requires good compliance by patients as H_2O_2 requires a minimum amount of time to be neutralized to be harmless to the ocular surface.[273, 276-278] Traditionally, H_2O_2 lens care regimens are differentiated into two- and one-step systems. The main difference between these two systems is that when using a two-step cleaning regimen the H_2O_2 continuously disinfects the CL until the hydrogen peroxide reaction is neutralized by applying an appropriate method, usually after an overnight soak.[273] In contrast, a one-step regimen combines the disinfection and neutralization step such that neutralisation occurs promptly, resulting in a system that is far more convenient and encourages compliance.[273] One-step systems however present a greater risk of potential bacterial regrowth and infection if lenses are stored for longer periods of time after the H_2O_2 is neutralized as the lens is essentialy left sitting in saline.[273]

In terms of lipid deposition removal, however, H_2O_2 lens care systems were not able to show significant reduction in lipid contamination nor were they able to enhance CL comfort for patients.[265, 273, 275, 279-281]

1.5.2 Multipurpose Solutions

Multipurpose solutions (MPS) are the most commonly used lens care products [166, 282] and combine a wide range of components that allow CLs to be disinfected and stored at the same time.[273] Therefore, all MPS products mainly contain a buffer, a preservative/biocide, and in many cases, additionally include lubrication-improving agents, chelating agents, and surfactants.[273]

Disinfectants can be broadly categorized into two groups: the ones that damage (indicated by the term "-static") or the ones that destroy ("-cidal") microbial cells.[273] The first disinfectants included in lens care products were chlorhexidine and thimerosal; however, these agents caused severe ocular reactions due to allergy and induced both corneal infiltrates and diffuse staining.[273, 283-286] Modern day MPS use a variety of preservatives that are far more effective and less toxic to the ocular surface, including polyhexamethylene biguanide (PHMB), polyquaternium-1 (Polyquad), alexidine, and myristamidopropyl dimethylamine (MAPD). Other than in lens care products, PHMB is a common antimicrobial that is used as a disinfectant in hand wipes and swimming pool sanitizers. By binding to exposed phospholipids of microbial cell membranes, PHMB causes cell membrane disruption and destruction. The disinfecting strategy of polyquaternium-1 is the same as PHMB, in that it binds to microbial cell membranes. Polyquaternium-1 however, is a substantially higher molecular weight molecule than PHMB, which prevents it from being absorbed into CL materials, lowering solution induced toxic signs and symptoms after CL wear and providing higher efficacy.[273, 287, 288] Alexidine has commonly been used in mouthwashes and is a fast acting antimicrobial and fungicidal agent that has a comparable structure to the first generation disinfectant chlorhexidine.[289-291] However, the industry stopped using alexidine in lens care products for quite some time due to an association of a product containing alexidine with *Fusarium* keratitis, although they might have been more linked to non-

compliance rather than the disinfectant itself.[273, 292-296] Alexidine has recently been re-introduced in a contemporary solution (AMO RevitaLens OcuTec).

Chelating agents are additives to MPSs that enhance the efficacy of a preservative and/or support the removal of deposited TF proteins. Commonly used chelating agents are ethylenediamine tetraacetic acid (EDTA), hydroxyalkylphosphonate (Hydranate™), and citrate.[273] While EDTA is synergistic with biocides and aids the destruction of microbial cells, Hydranate and citrate are sequestering agents that remove proteins by reacting with calcium and breaking the bonds between TF deposits on CL materials.[273, 297]

Besides biocides and chelating agents, buffers are included to either stabilize the pH and osmolality of the MPSs or to enhance the efficacy and functionality of incorporated preservatives or chelating agents.[273, 274] Common buffers in lens care products included to maintain and stabilize osmolality and pH may include tromethamine, taurine, citrate buffers, phosphate buffers, borate buffers, salts, and others.[273, 274] Sorbitol and dexpanthenol however, are additives to some MPSs to enhance lubricity and wettability of CLs in combination with other ingredients. [273, 274]

1.5.3 Surfactants and Lipid Removal

Surface active agents (surfactants) are important ingredients in MPS products, particularly in those used with SiHy lens materials. Similar to phospholipids, surfactants are amphiphilic, which allows them to interact at both the air-water interface and between the liquid-liquid interface of water and lipids.[273, 274] Therefore, surfactants fulfill two main functions in lens care products: the binding and subsequent removal of loose deposits and debris, and the augmentation of surface wettability by decreasing the CL materials' surface tension, which is especially important for SiHy lens materials.[273, 274, 298] The two most common copolymers for surfactants used in MPS are

poloxamer (Pluronic) and poloxamine (Tetronic) that function as backbones for various surfactant derivatives.[273, 274]

To remove loosely bound components from CL surfaces, surfactants create bonds with the targeted substances to form micelles that are then easier to remove when applying a rub and rinse to the lens material before CL disinfection.[273, 274, 299-302] Furthermore, their amphiphilic structure provides surfactants with the ability to increase the wettability of a lens surface, especially of SiHy lens materials. The surfactant's hydrophobic segment binds and occupies any hydrophobic silicone monomer of a CL, leaving its hydrophilic segment exposed, which in turn, increases the wettability and may simultaneously prevent or decrease lipid deposition.[273, 274, 303-306] Based on these functions, surfactants were also added to H_2O_2 lens care products.[273, 274, 298]

Various studies examining the efficacy of different lens care products have shown surfactants to successfully reduce surface deposition but have yet to proof their capabilities to reduce lipid contamination effectively. [60, 265, 279, 307-310] Because of the industry's relentless approach to improve CL performances and the efficacy of lens care products to improve lens wear comfort, it is of great importance to consistently investigate the interaction between the newest lens material/care product combinations.

1.5.4 Patient Compliance

Patient compliance can play an important role in the success of lens care products.[274, 311-313] Many studies have examined patient compliance in the past and have shown that approximately 50% of CL wearers fail to follow recommendations relating to cleaning procedures or appropriate hygiene.[274, 313-320] Among the most common recommendations patients neglect are: allowing enough disinfection time; to apply a rub and rinse technique to aid with cleaning the lens; and to always use fresh lens care solution every time a CL is disinfected.[311, 313, 321]

1.6 Methods to Detect and Analyse TF Lipids

Sufficient collection and subsequent analytical methods are required to study MG lipids, TF lipids, and lipid deposition on CLs. To analyse lipid deposition in various lens materials, CLs can either be incubated in an ATS or be extracted from CLs post-wear (*ex vivo*).[45, 58, 60-63, 227, 249, 250, 252, 253, 259, 263, 265, 266, 279, 322-325]

The following section outlines the main collection and analytical methods used to measure TF lipids.

1.6.1 Lipid Collection Methods

The collection of MG and TF lipid samples uses a variety of methods described below, which are categorized into soft and hard expressions of MGs, microcapillary collection, and Schirmer test strips.[2, 23, 29-31, 39, 45, 53] For further analysis and to avoid sample contamination, MG and TF lipid samples require the same appropriate methods of handling and storage.[2, 45] Ideally, it is recommended that lipid samples are stored in a dark place at -80°C, in an oxygen-free environment, and that they are handled and stored in containers free of silicone or plastic.[2, 45]

1.6.1.1 Meibum Collection

When applying “soft expression” for meibum collection, the eyelid is first cleansed using a sterile swap before applying gentle pressure only to the outer portion of the lid.[2, 29, 31, 45, 326] To perform a “hard expression” meibum collection, the lid is compressed between a sterile lid conformer, while applying pressure to the lid using a finger or another device.[2, 23, 28, 29, 31, 40, 43, 45, 119, 326-328] After the MGs have been expressed, meibum can be collected from the lid margin with a curette or spatula.[28, 29, 31, 40, 43, 45, 326-328]

Meibum samples may also be collected directly from the MG orifices using microcapillary tubes, which are thin-walled glass tubes that use capillary forces to draw in fluid when a tube is placed in contact with the orifices.[2, 31, 45] Despite being less invasive, the use of microcapillary tubes for meibum collection has limitations: they yield smaller sample quantities, meibum samples will solidify almost immediately within the tubes, and meibum samples might be contaminated with small tear volumes.[2, 21, 30, 32, 39, 45]

1.6.1.2 Tear Collection

Microcapillary tubes are the most commonly used tool to collect TF samples.[2, 45, 71, 112, 125] To collect tears, the tubes are gently placed on the lower lid margin at the lateral canthus of the eye to collect tears from the lower tear meniscus.[45, 71] Researchers typically prefer to collect basal tears (un-stimulated), but in certain circumstances researchers may also collect reflex tears (stimulated).[71, 125, 329, 330] Once TF samples are collected, the fluid is removed using a thin wire or solvents and analyzed.[2, 45]

The use of absorbent filter paper (Schirmer) strips is another way to collect tears, however, they are usually applied to confirm the presence of very dry eyes due to aqueous tear deficiency.[24, 241] To collect tears, the Schirmer strips are placed in the lower conjunctival sac of the eye where they absorb tear fluid.[241] Using Schirmer strips is a non-specific method, in that all components of the TF are absorbed.[30] Furthermore, this test is usually conducted without anesthetic and may be less comfortable than collecting tears with microcapillary tubes, resulting in tear stimulation and altering the composition of the TF collected from that which is usually present. After tear fluids are collected, the TF components can then be extracted from the filter strips and analysed further.

1.6.2 Analytical Methods of Lipid Deposition

To remove TF lipids from CLs, researchers traditionally expose them to extraction solutions that commonly include the solvents chloroform and methanol. These lipids are then analyzed using a variety of methods.[58, 61-63, 250, 259, 263, 265, 266, 279] Several techniques are highlighted below that have been used to study meibum and TF composition and provide valuable information on the composition and conformation of lipids.

1.6.2.1 Radiochemical Experiments

Radiochemical methods are used to analyze the uptake of TF lipids and proteins on CL materials in *in vitro* experiments.[61-63, 229, 231, 234, 266, 323, 331-335] Trace amounts of radioactive elements of interest are incorporated into an ATS mixture or "doping solution". In one of the first radiochemical lipid studies, Prager et al.[323] added the radiolabelled lipids tritium (^{3}H)-cholesteryl oleate and carbon-14 (^{14}C)-dioleoyl-PC to a complex ATS. Various CL materials were then exposed to the ATS and the amount of radiolabelled lipid taken up was quantified using scintillation beta counting.[323] In more recent studies, radiochemical experiments have been used to determine the impact of a variety of testing conditions on the deposition of lipid [61-63, 266, 279] and protein [229, 231, 234, 331-333] on SiHy and CH lens materials. The two main advantages of using radiolabeling for deposition detection relates to its degree of sensitivity and that the radioactive isotope tracers (i.e. iodine-125 (^{125}I), ^{14}C, or ^{3}H) are very small and thus only modify the original size, structure, or functions of the molecules' of interest by a small degree.

1.6.2.2 Chromatography

Initially, lipids could only be quantified as being present or absent on CLs through histo-chemical staining, in conjunction with the use of electron and light microscopy.[322] In more recent years, various chromatography techniques have been applied to detect the components of the MGs

and the TF and to analyse individual lipid conformation. A range of the most commonly used chromatography techniques are highlighted below: thin-layer chromatography (TLC), gas chromatography (GC), and high-pressure liquid chromatography (HPLC).[2, 45]

1.6.2.2.1 Thin-Layer Chromatography (TLC)

TLC can be used to both analyse and separate different lipid types, which can then be further analysed using additional and more advanced technologies.[2, 45, 336, 337] Samples are applied at the base of a stationary phase, which is applied on a solid plate (plastic, aluminum, or glass) that is coated with a thin layer of an absorbent silica gel. Further, samples are applied into a solvent (commonly chloroform), which is known as the mobile phase. Capillary forces then draw the lipids of interest dissolved in the mobile phase across the plate.[2, 45, 336, 337] During this process, lipids will travel through the solid phase at different rates, separate and settle at different heights due to different affinities of the sample molecules to the solid and mobile phases, solubility, and polarity.[2, 45, 336, 337] After separation, the sample composition can be further identified visually (by UV light) or without additional equipment when charred.[2, 45, 336, 337]

While TLC was often used for early deposition studies, it is not sensitive enough to detect differences between some major TF lipids and requires large sample quantities. Despite this, early studies used this method to detect and analyze lipids from various locations in the eye. [2, 43, 45, 241, 336-338]

1.6.2.3 Gas Chromatography (GC)

Similar to TLC, the separation of sample compounds via GC is performed through a mobile and stationary phase. When using GC, however, the samples are in a gaseous mobile phase that is sent through either a liquid (gas-liquid chromatography) or solid (gas-solid chromatography) stationary phase.[2, 45, 337, 339] Through the use of a carrier gas (methane, nitrogen, or helium) and

heat vaporization, the samples are sent through the stationary phase within a chromatography column. Sample separation occurs based on the affinity of the individual compounds to the mobile and stationary phase. After the compound separation, detectors measure the different retention times to identify individual lipid types.[2, 45, 337, 339] The separation capabilities of GC can be used in combination with a variety of detectors, including UV absorption, infrared, and mass spectrometers (MS).[2, 45, 337, 339] However, the heating process involved with GC can induce high temperatures that could potentially change sample structures, which in turn may lead to false interpretation/readings of sample components. Despite this, GC has been used in various studies to determine meibum and TF composition.[2, 30, 35, 36, 40, 45, 52, 326]

1.6.2.4 High-Pressure Liquid Chromatography (HPLC)

Instead of a gaseous mobile phase, liquid chromatography (LC) uses a liquid mobile phase to separate sample mixtures into its components.[2, 45, 336, 337] In HPLC, the liquid mobile phase is additionally pushed through a column with high pressure to enhance sample separation and once the sample component separation occurs the individual compounds can be analyzed with additional detectors.[2, 45, 336, 337] A broad variety of usable solvents, reusable columns, and detectors make HPLC a very flexible and powerful method to use and it has thus been applied in several studies to analyze polar and non-polar lipids. [2, 23, 45, 109, 249, 265, 336, 337, 340]

1.6.2.5 Spectrophotometry

Spectrophotometry (spectroscopy) includes sensitive analytical techniques to detect and determine any type of molecules within small sample mixtures.[2, 45, 336, 337] The main principle of these methods entails the excitation of a sample with a specific wavelength and the detection of the subsequent transmittance or absorbance, which are then compared to known standards.[2, 45, 336, 337]

1.6.2.5.1 Mass Spectroscopy (MS)

In mass spectroscopy (MS), an ion source “shoots” high-energy electrons at sample molecules to form ions with specific mass/charge ratios (m/z).[2, 45, 341, 342] These ions are sent through a mass analyzer that induces an electronic or magnetic field to separate the ions based on their different m/z ratios.[2, 45, 341] Once the ions are detected, software plots a mass spectrum of distinctive m/z ratio peaks of each ion of a sample as a function of the relative abundance.[45, 341, 342]

Several different variations/combinations of MS have been used to analyse lipids extracted from CLs and within meibum samples, including GC-MS, LC-MS, matrix assisted-laser desorption ionization (MALDI), atmospheric pressure chemical ionization (APCI-MS), and electrospray ionization mass spectroscopy (ESI-MS).[2, 16, 29, 32, 33, 35, 36, 43, 45, 54, 60, 95, 96, 249, 259, 343, 344] Although MS allows for the analysis of lipidomes of small sample quantities, it requires standards to efficiently identify and quantify lipids in meibum or TF samples, which can make exact lipid determination difficult to achieve.

1.6.2.5.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

In nuclear magnetic resonance (NMR) spectroscopy the nucleus of a molecule is excited into a higher energy level through radiofrequency radiations that are induced by a magnetic field.[45, 342, 345] The molecules can then be characterized and identified based on re-emitted radiation that is detected at a unique frequency.[45, 342, 345]

NMR is one of the most powerful analytical tools to analyse molecular structures because it is non-destructive and has been used in several studies to analyze lipids in meibum and TF samples.[2, 25, 45, 46, 345-349] NMR is considered to have a relatively low sensitivity that generally requires large volumes (mg) and pooling of samples.[2, 45, 342] However, in a recent study researchers were able to show high sensitivities with small sample quantities (µg) when using a 600mHz NMR.[350]

1.6.2.5.3 Raman and Infrared Spectroscopy

Raman and infrared spectroscopy (IR) both detect vibrational changes in molecules to determine conformational and compositional information, but use different energy wavenlengths.[2, 45] In Raman spectroscopy, a sample is excited with a monochromatic light/laser, the light scatters at varying frequencies, which are then detected as a Raman spectrum, which provides unique information about the molecules within a sample. In IR spectroscopy, infrared radiation is projected through a sample mixture to then measure its absorbance and transmittance and create an interferogram. When a sample is energized, the molecules absorb the radiation, which excites them into a vibrational and rotational state, which is detected by IR spectroscopy and provides information about the molecular characteristic.[2, 45] Both methods have disadvantages which can, however, complement each other to create a broad knowledge of molecular structures, [2, 45, 351, 352] e.g. water within a sample interferes with IR but not with Raman spectroscopy. Several researchers have successfully used both technologies to determine differences in meibum lipids.[2, 24, 45, 52, 351-356]

1.6.2.5.4 Colorimetric Assays

Colorimetric assays are spectroscopic methods that have been used to detect lipid deposition on CLs.[45, 58, 59, 126, 127, 233, 253-255, 260, 357-359] These methods make use of colorimetric changes of a sample mixture after a reagent has been added, measuring the absorbance or fluorescence with either spectrophotometers or confocal microscopes. However, most of these methods have low specificities and only allow for the detection of lipid classes. Various methods have been developed and used to analyse the quantities of total lipids,[127, 253-255] phospholipids,[126, 127] cholesterol,[58, 59] and differences in the amount of accumulated on CLs.[358]

A special colorimetric method employs fluorescently-labeled lipids [260] or proteins. [233, 357, 359, 360] By using these fluorescently-labeled TF components, researchers have been able to analyze the penetration patterns of specific lipids or proteins of interest that provide further knowledge about the interaction between the TF and various CL materials.[45, 233, 260, 357, 359, 360] To measure the penetration of TF deposits, fluorescently labeled molecules of interest are added to a test solution in which various CL are incubated over varying periods of time. Using this methodology, Carney et al.[260] measured the absorbance of various CLs after 14 hours and 20 days of lens incubation and tracked the penetration of lipids. Luensmann et al. used a similar incubation method to determine the penetration pattern of different TF proteins (albumin and lysozyme) on various lens materials, but instead used LSCM for detection.[233, 357, 359, 360] LSCM can be differentiated from traditional microscopic techniques in that it makes use of a spatial pinhole phenomenon that eliminates any light rays that are out-of-focus on the focal plane, allowing for high resolution images. Additionally, an adjustable focal plane allows for scanning the medium of interest at different depths to create 2D penetration profiles.

Two additional colorimetric assays of interest for this thesis are thiobarbituric reactive substances (TBARS) and enzyme-linked immunosorbent assays (ELISA). Both assays are well established assays used to detect and monitor lipid peroxidation by-products in blood, serum, or TF samples.[106, 128, 133, 142, 152, 153, 161, 163, 361-365] TBARS is solely used to detect the oxidation by-product MDA, whereas ELISAs can be used to detect various by-products (e.g. MDA, 4-HNE, or oxidized low-density lipoproteins[ox-LDL]). To determine the amount of MDA in a sample, the TBARS assay forms an adduct between thiobarbituric acid (TBA) and MDA through heat and acidic conditions to subsequently measure the presence of the bond colorimetrically at specific wavelengths (Figure 1-7).[133, 153, 366, 367] However, the efficacy and specificity of the TBARS assay is still questioned, as TBA is known to react with other components within samples.[133, 366, 367]

ELISAs use antibodies that are bound to micro-titre plates (e.g. polystyrene) that may react with a protein within a sample mixture that is of interest.[368] Thereafter, protein quantities are detected through fluorescent or chemiluminescent reactions and wavelengths are measured using a spectrophotometer [368] and have traditionally been used to determine TF proteins on CLs.[221, 236, 238, 253, 369-371]

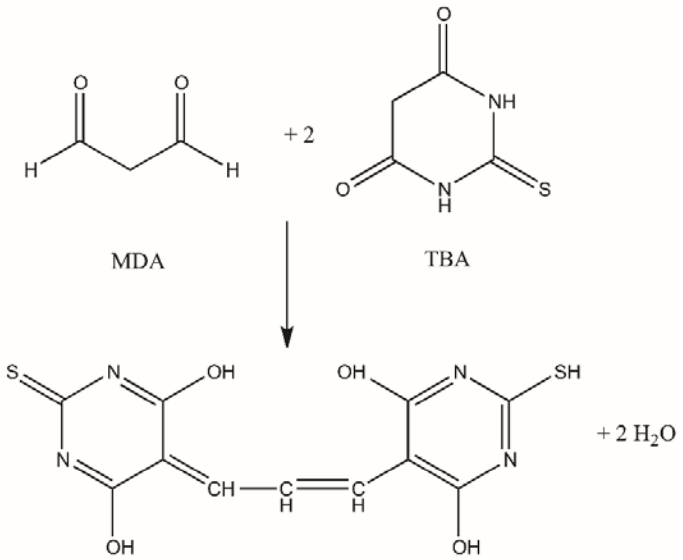

Figure 1-8. MDA-TBA bond principle in the TBARS assay.

In summary, the analysis of MG, TF, and CLs lipids is a very complex research field. The outermost ocular surface layer is composed of a large variety of lipids that all have an important role in discomfort, the performance of CLs on the eye, DE, and CL discontinuation. Various analytical methods are available to determine the impact of these lipids, but no method can be singled out to be the single "correct" one to use. It is therefore necessary to further explore these and other methods to continue to narrow down the impact various lipids may have on the comfort of the eye, CL wear, and DE disease.

Chapter 2
Objectives and Rationale

The introduction of silicone hydrogel (SiHy) lens materials gained significant interest amongst practitioners, patients, and researchers alike. It was initially believed that these lens materials would resolve a number of issues that occurred with conventional hydrogel lenses, particularly those associated with hypoxia. While SiHy lenses did indeed live up to their potential in respect to the management of hypoxia, an unforeseen issue related to excessive lipid deposition in certain wearers appeared with these lens materials. [187, 227, 252, 257, 268, 372-374]

During my master thesis, the deposition of cholesterol onto daily wear (DW) lens materials over a period of 28 days was studied.[63] This *in vitro* study addressed the effects of incubation time, contact lens (CL) material, lipid concentration, and tear film (TF) replenishment on the amount of cholesterol deposition.[63] This work showed that cholesterol accumulates significantly more onto SiHy lens materials and continuously builds up on CLs over time, without reaching a plateau. It also showed that the quantity of cholesterol deposition was proportional to the lipid concentration in the doping solution and that replenishing the incubation solution led to an increase in deposition on all lens materials. Our results led us to theorize that wearers of reusable SiHy CL materials with excessive lipid in their tears might experience lens discomfort linked to an increase in lipid deposition.

Lens materials that are worn on a DW basis and reused following daily removal require the use of a nightly solution routine to ensure that the lens is appropriately disinfected prior to reinsertion. Many different lens care products are available, and in addition to disinfection these solutions incorporate various components that are designed to remove TF deposits (mainly proteins and lipids) to attempt to optimise the performance of CLs during wear. Despite the

importance of this function, few studies to-date have reported on the efficacy of solutions at removing lipid deposition from lens materials.[307, 308] To further our knowledge in this area, Chapter 3 is an *in vitro* exploration of the efficacy of a variety of contemporary multi-purpose solutions (MPS) on the removal of a TF lipid from current DW lens materials.

A different approach to reducing discomfort for patients that present increased rates of lipid uptake on CLs might be to recommend a switch into lenses with shorter wear modalities, such as daily disposables (DDs), which have gained a steady increase in popularity in recent years. [166] At the time of my previous work,[63] DD SiHy lens materials were not available and to-date no published studies have determined the degree of lipid contamination on such materials. Chapter 4 provides the results of an *in vitro* study to deepen our insight into the lipid deposition behaviour of contemporary DD lens materials.

When undertaking *in vitro* deposition studies, the vast majority of previous studies have exposed the lens materials to the deposit type of interest by merely soaking the lens in a solution containing the protein or lipid being studied. However, recent work has shown that the static vial incubation method used in such studies is not optimal and has limitations when we want to compare the results to on-eye lens wear conditions.[375-380] *In vitro* models that mimic blinking, air exposure, tear replenishment, and that decrease the amount of incubation solution that the CLs are exposed to appear far better at predicting on eye performance and show uptake results that are more similar to those found in *ex vivo* samples.[62, 266, 270, 375-380] With this in mind, our laboratory developed a novel *in vitro* platform (OcuFlow) that simulates blinking, air exposure between blinks, and physiological tear volumes and flow rates.[381] This advanced *in vitro* eye model has previously been used for studies to examine drug release [382, 383] and protein uptake,[384] and has been shown to be a versatile method to provide new insights into CL material performance. After determining the quantities of lipid contamination on various lens materials and modalities, and in consideration of

the results from Chapter 3, the question of where lipid deposition is located within a CL material remained unanswered. By using our advanced eye model, Chapter 5 examines the penetration of a TF lipid on commercially available DD lens materials.

A major interest persists in the clinical value of understanding the role of lipid deposition in contact lens discomfort (CLD) and the involvement of lipids in causing dry eye disease (DED). Increased lipid deposition on SiHy lens materials may increase discomfort.[63, 257, 268] When deposited on CLs, lipids may exacerbate poor lens wettability by adding an additional hydrophobic layer over the lens surface, which may play a significant role in impacting on-eye performance and comfort ratings.[372-374] Tear break-up time (TBUT) measurements are a method to determine and predict CL performance *in vivo* over a lens surface.[338, 385-387] Although it appears that denatured proteins on CLs may impact lens comfort, a recent study has shown that protein deposition on lens materials does not influence TBUT measurements over the surface of lenses.[238] To-date, no studies have been published that examine the impact lipid deposition on TBUT measurements. Thus, Chapter 6 investigates the potential use of TBUT technology and compares contemporary DD CLs, *in vitro.*

Finally, lipid degradation through oxidation is thought to be influential in the development of DED and discomfort.[106, 125, 160] Lipids can "break down", losing their functionality, which may potentially destabilize the TF lipid layer and could ultimately cause tissue damage.[56, 129, 132, 135] To our knowledge, only a few studies have been able to measure lipid degradation in tears or CLs.[125, 142, 156, 163, 365] However, there is no sensitive analytical method currently available to determine lipid oxidation in small volumes of individual tear samples. To help determine if lipid oxidation in tears might be a cause for CLD and DED, Chapter 7 investigates various contemporary lipid oxidation assays to develop a new sensitive test method to test for oxidative by-products in tears.

The concluding Chapter 8 provides a summary of the presented work and suggestions for future research endeavours in this area of study.

In the following chapter, the uptake rate of tear film lipids on various commercial contact lenses will be measured over time using a radiochemical methodology, and the capabilities of various contemporary multipurpose cleaning solutions to remove lipid deposition will be assessed.

Chapter 3

Efficacy of Contact Lens Care Solutions in Removing Cholesterol Deposits from Silicone Hydrogel Contact Lenses

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Author	Concept/Design	Data Acquisition	Analysis	Write Up /Publication
Walther	Y	Y	Y	Y
Subbaraman	Y			Y
Jones	Y			Y

3.1 Outline

3.1.1 Purpose

To determine the efficacy of multi-purpose solutions (MPS) on the removal of cholesterol deposits from silicone hydrogel (SiHy) contact lens materials using an *in vitro* model.

3.1.2 Materials & Methods

Five SiHy lens materials: senofilcon A, comfilcon A, balafilcon A, lotrafilcon A and lotrafilcon B were removed from the blister pack (n=4 for each lens type), incubated for 7 days at 37°C in an artificial tear solution (ATS) containing ^{14}C radiolabeled cholesterol. Thereafter, lenses were stored in a preserved saline solution control (Sensitive Eyes Saline Plus) or cleaned with one of five MPSs incorporating different preservatives (Polyquad®/Aldox®, polyquaternium-1/alexidine, polyquaternium-1/PHMB and 2 based on PHMB alone) using a rub and rinse technique, according to the manufacturer recommendations, and stored in the MPS for eight hours. Lenses were then extracted with 2:1 chloroform:methanol, analyzed in a beta counter and µg/lens of cholesterol was determined.

3.1.3 Results

Balafilcon A and senofilcon A showed the highest amounts of accumulated cholesterol (0.93±0.02µg/lens and 0.95±0.01µg/lens, respectively), while lotrafilcon A and lotrafilcon B deposited the lowest amounts (0.37±0.03; 0.47±0.12). For all lens materials, the MPS preserved with Polyquad®/Aldox® removed more cholesterol than any other test solution; however, the amount of cholesterol removed from the individual CLs was statistically significant only for balafilcon A and senofilcon A ($P = 0.006$ and $P = 0.042$, respectively). Sensitive Eyes and the other MPSs evaluated showed no significant effect on lipid removal ($P > 0.05$).

3.1.4 Conclusion

Lipid-removal efficacy varies depending on the combination of lens material and solution. Only one MPS showed a significant reduction of lipids for any of the tested lens materials.

3.1.5 Keywords

Lipid deposition; silicone hydrogel; contact lenses; cholesterol; cleaning; multi purpose solution

3.2 Introduction

Soft contact lenses (CL) interact with the ocular surface and rapidly sorb tear film (TF) components, particularly proteins [220, 221, 233, 239, 253, 268, 388-390] and lipids.[58, 63, 224, 227, 246, 249, 252, 253, 255, 257, 258, 265, 268] These deposits can influence the on-eye performance of lenses and potentially influence dryness and discomfort associated with lens wear.[216, 238, 255] In severe cases, they can lead to inflammatory responses resulting in contact lens associated papillary conjunctivitis [226, 391, 392] and may also increase bacterial adhesion to the lens materials.[324, 393-395]

Today, the majority of contact lens wearers are fitted with silicone hydrogel (SiHy) lens materials, which are worn most commonly on a reusable basis, utilizing replacement periods of two or four weeks.[396] Lens materials used as frequent replacement lenses require a suitable lens care solution system for overnight disinfection and to remove TF and extraneous deposits. The majority of patients use preserved multipurpose solutions (MPS) to care for their soft contact lenses.[396] MPS combine many different ingredients, including biocides to disinfect the lenses, wetting agents to reduce the hydrophobicity of the lens surface and surfactants to remove TF deposits that have accumulated during wear.[274, 397]

Silicone hydrogel lens materials accumulate greater amounts of lipid compared to conventional hydrogel (CH) lenses.[58, 63, 227, 252, 258, 266, 268] One TF lipid that has been found to fairly consistently deposit on SiHy lens materials is the non-polar lipid cholesterol, and its esters.[58-61, 63, 258, 260, 263, 265, 325, 398-400] The efficacy of various MPS in removing deposits from lenses depends upon the composition of the MPS and the lens material properties. Previous *in vitro* [279, 333, 401] and *ex vivo* [60, 228, 263, 265, 297, 402] studies demonstrated that cleaning solutions have varying abilities to remove TF deposits on SiHy lens materials.

The purpose of this study was to determine the efficacy of several contemporary MPS in removing cholesterol deposits from various SiHy CL materials, using an *in vitro* radiolabeling model.

3.3 Material & Methods

3.3.1 Lens Materials

Five different SiHy lens materials were investigated; senofilcon A ([SA] Acuvue® Oasys™; Johnson & Johnson, Jacksonville, FL), balafilcon A ([BA] PureVision® 2; Bausch+Lomb, Rochester, NY), comfilcon A ([CA] Biofinity®; CooperVision, Pleasanton, CA), lotrafilcon A ([LA] Air Optix® Night & Day® Aqua; Alcon, Fort Worth, TX) and lotrafilcon B ([LB] Air Optix® Aqua; Alcon, Fort Worth, TX). The lens care solutions that were evaluated included five contemporary MPS of varying compositions: polyquaternium-1/PHMB based system [Biotrue® MPS; Bausch+Lomb, Rochester, NY]; PHMB-1 based solution [renu® fresh™; Bausch+Lomb, Rochester, NY], polyquaternium-1/alexidine based system [Blink RevitaLens® MPS; Johnson & Johnson, Jacksonville, FL], Polyquad®/Aldox® based cleaning solution [OptiFree® PureMoist® MPS; Alcon Inc., Fort Worth, TX], PHMB-2 based system [Solo-Care Aqua® MPS; Menicon Ltd., Nagoya, Japan]); and a saline (Sensitive Eyes® Plus Saline, Bausch+Lomb, Rochester, NY) that was used as a control. Table 3-1 and Table 3-2 provide further information on the lenses and solutions used in this study.

Table 3-1. Properties of contact lens materials evaluated in the study**.**

USAN	senofilcon A	balafilcon A	comfilcon A	lotrafilcon A	lotrafilcon B
Lens Type	SiHy	SiHy	SiHy	SiHy	SiHy
Trade Name	Acuvue® OASYS™ with HYDRACLEAR™ Plus	PureVision® 2 HD	Biofinity®	Air Optix® Night&Day® Aqua	Air Optix® Aqua
Water Content	38%	36%	48%	24%	33%
Oxygen permeability (Dk) ($\times 10^{-11}$)	147	91	116	175	138
Surface Modification	None. Internal wetting agent	Plasma oxidation	None	Plasma surface treatment	Plasma surface treatment
Manufacturer	Johnson & Johnson	Bausch + Lomb	Cooper Vision	Alcon	Alcon

USAN: United States Adopted names, SiHy: silicone hydrogel, FDA: Food and Drug Administration

Table 3-2. Saline and multi-purpose solution information.

	Sensitive Eyes® Plus Saline	Biotrue® MPS	renu® fresh™	Blink RevitaLens® MPS	OptiFree® PureMoist® MPS	SoloCare Aqua® MPS
Surfactants / Wetting agents	N/A	Poloxamer	Poloxamer	TETRONIC® 904	TETRONIC® 1304, EOBO-41™	Dexpanthenol, Poloxamer, Sorbitol
Buffer	Sodium borate, sodium chloride	Sodium borate, sodium chloride	Hydroxyalkylphosphonate, sodium borate, sodium chloride	Sodium chloride, sodium citrate, sodium borate	Sodium citrate, sodium chloride,	N/A
Other Ingredients	Disodium edetate, Boric acid	Disodium edetate, Hyaluronic acid, boric acid	Disodium edetate, Poloxamine hydrate, boric acid	Disodium edetate, Boric acid	Boric acid, sorbitol, disodium edetate	Disodium edetate, pluronic F127
Preservatives	PAPB	PHMB, HCl, polyquaternium-1	PAPB	Alexidine dihydrochloride, poliquaternium-1	POLYQUAD®, Aldox®	N/A
Manufacturer	Bausch+Lomb	Bausch+Lomb	Bausch+Lomb	Johnson&Johnson	Alcon	Menicon America

EOBO-41™: polyoxyethylene-polyoxybutylene; HCl: hydrogen chloride; PAPB: polyaminopropyl biguanide; PHMB: Polyhexamethylene biguanide

3.3.2 Artificial Tear Solution

In an effort to mimic the complexity of human tears, our laboratory has developed an artificial tear solution (ATS) that has been utilized for the *in vitro* incubation of various lens materials in several previous studies.[62, 63, 237, 266, 279, 325, 331] The full composition of the ATS has been previously published. [61]

3.3.3 Cholesterol and ^{14}C Radioactivity

To quantify the deposited cholesterol, a trace amount of radioactive ^{14}C labeled cholesterol (Perkin Elmer, Waltham, MA) was added to the ATS. The concentration of ^{14}C-cholesterol was 3% of the total non-radioactive concentration of cholesterol, as previously described.[61-63, 279, 325]

3.3.4 Vial pre-treatment

The porosity of the borosilicate vials (6 ml) interferes with the degree of lipid uptake onto contact lens materials, as the lipid adheres to the inner walls (unpublished internal data). To overcome this issue the vials were pre-treated with 3.5 ml of non-radioactive ATS to saturate the inner surface of the vial with lipid and protein to minimize the uptake of lipids onto the inner vial surface, prior to radioactive incubation. Subsequently, the vials were emptied of the pre-treatment solution, rinsed with a complex salt solution (CSS) [61] and refilled with the same quantity of ATS that contains the ^{14}C-cholesterol.

3.3.5 Lens Incubation and Extraction

The lens materials tested (n=4 for each lens–solution combination, in addition to uncleaned control lenses) were soaked in a CSS for 24 hours on a shaker to remove any residual blister pack solution. Thereafter, the lenses were rinsed twice in CSS, gently blotted on a lens paper, and placed

in a vial containing fresh radioactive ATS. The vials were capped, sealed with Parafilm®, and incubated at 37°C with shaking for 1 week. At the end of the one-week incubation period, each lens was rinsed twice in CSS and blotted on a lens paper. Thereafter, each test lens was rubbed and/or rinsed with the appropriate MPS as per the manufacturer's instructions and then stored in the MPS or saline for eight hours. Subsequently, lenses were placed in 20 mL glass scintillation vials with 2 mL of 2:1 chloroform:methanol extraction solution and shaken at 1600 rpm for 30 minutes at room temperature. Each lens was extracted in this manner at two separate times and both extracts were pooled in the same vial. The extract vials were dried down completely using air or nitrogen in a water bath at 35° ± 5°C. All vials were re-suspended in 1 mL of chloroform, then sonicated for one minute, and 10 mL of Ultima Gold™ F (Perkin Elmer, Waltham, MA) scintillation fluor was added. The vials were subjected to liquid scintillation beta counting using a L6500 Beckman Coulter Beta Counter (Beckmann Coulter Inc., Brea, CA, U.S.). Standard radioactive counts were prepared and counted to determine the total radioactive counts in the system. The uncleaned control lenses were subjected to radioactive counting (as detailed previously) and did not undergo the cleaning procedure. The efficacy of a given MPS for different lens materials was determined by calculating the difference in deposition between "uncleaned" and "cleaned" lenses. Figure 3-1 provides a schematic of the study design.

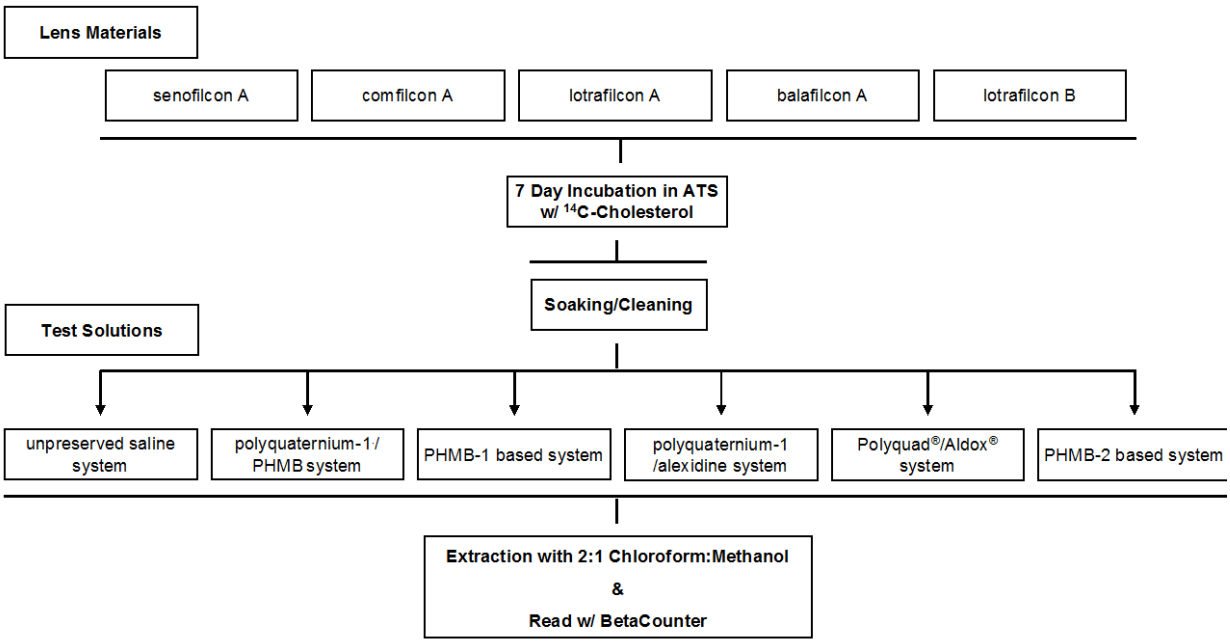

Figure 3-1. Study design.

3.3.6 Statistical Analysis

Statistical analysis was performed using repeated measures analysis of variance (RM-ANOVA) and Tukey post-hoc analysis was performed when required. Analysis was performed using SPSS 20 (IBM Corporation, Armonk, NY, U.S.) and statistical significance was considered at a *P* value of <0.05.

3.4 Results

Figure 3-2 and Figure 3-3 and Table 3-3 detail the results. Overall, we found that both variables (lens care solution and CL material) were statistically significant ($P \leq 0.001$), as well as the interaction between both ($P = 0.001$).

Table 3-3. Repeated measures ANOVA statistical results for cholesterol uptake.

Variable	Sum of Squares	Degrees of Freedom	Mean Square	*F*	*P* value
Lens Care Solution	0.133	5	0.027	11.958	<0.001
Contact Lens Material	4.512	4	1.128	1334.35	<0.001
Lens Care Solution * Contact Lens Material	0.134	20	0.007	3.022	0.001
Error	0.111	50	0.002		

3.4.1 Total uptake of lipids onto lens materials

Figure 3-2 illustrates cholesterol deposition on all the control (uncleaned) lenses. Balafilcon A and senofilcon A lens materials deposited the most cholesterol (0.93±0.02µg/lens; 0.95±0.01µg/lens respectively), lotrafilcon B and lotrafilcon A the least amount of cholesterol (0.47±0.12µg/lens; 0.37±0.03µg/lens, respectively). Post-hoc analysis revealed that comfilcon A lenses (0.76±0.06µg/lens) deposited significantly less cholesterol than senofilcon A and balafilcon A ($P < 0.05$), but significantly more than the lotrafilcon lens materials ($P < 0.05$). No significant differences were found between senofilcon A and balafilcon A ($P = 0.97$) or between lotrafilcon A and lotrafilcon B ($P = 0.131$).

Figure 3-3 depicts the average cholesterol reduction post-cleaning for the saline solution and each MPS product compared to uncleaned lenses. The average reduction of cholesterol varies greatly between the test solutions, where Polyquad®/Aldox® (0.086 µg/lens; $P \leq 0.001$) and PHMB-1 (0/054 µg/lens; $P = 0.035$) removed the most. None of the other MPS or the saline showed significant removal of cholesterol ($P \geq 0.111$).

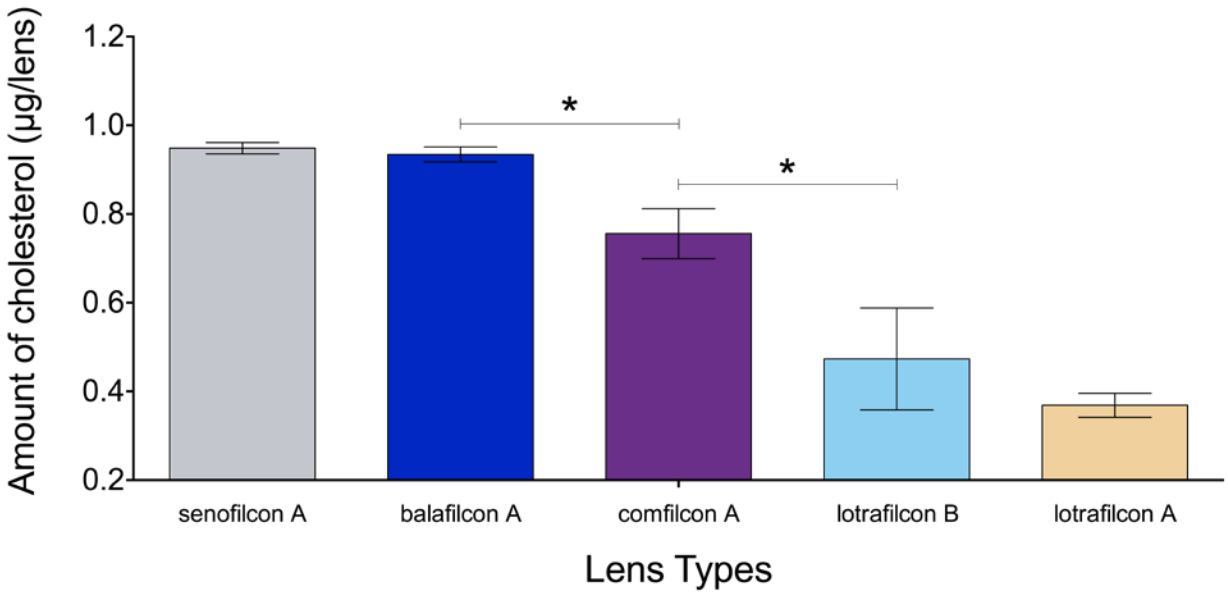

Figure 3-2. The graph shows the Mean (± SD) total cholesterol uptake on the contact lens materials after 7 days of incubation in a complex tear solution containing radiolabeled ^{14}C-cholesterol. (*P < 0.05 for differences in uptake between CLs).

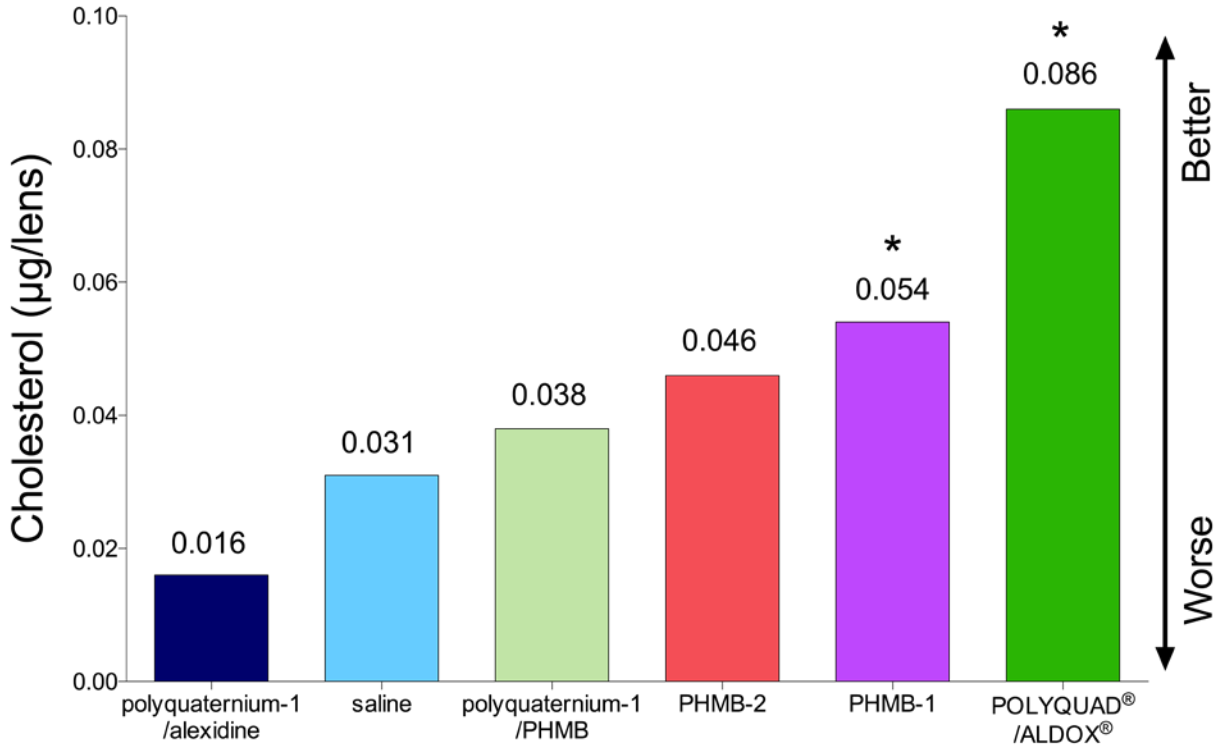

Figure 3-3. Average reduction in cholesterol deposits (in µg/lens) when compared to uncleaned lenses by combining data for all the lens materials. (*P < 0.05 for differences in lipid reduction vs. uncleaned lenses).

3.4.2 Lens Care Product Performance on Lipid Removal

Figure 3-4 shows the comparison of cholesterol remaining on each CL material, after storing them in saline and cleaning with each test MPS. In addition, Table 3-4 provides a comparison chart (%-difference) of the amount of cholesterol that was removed from each CL material by various test solutions.

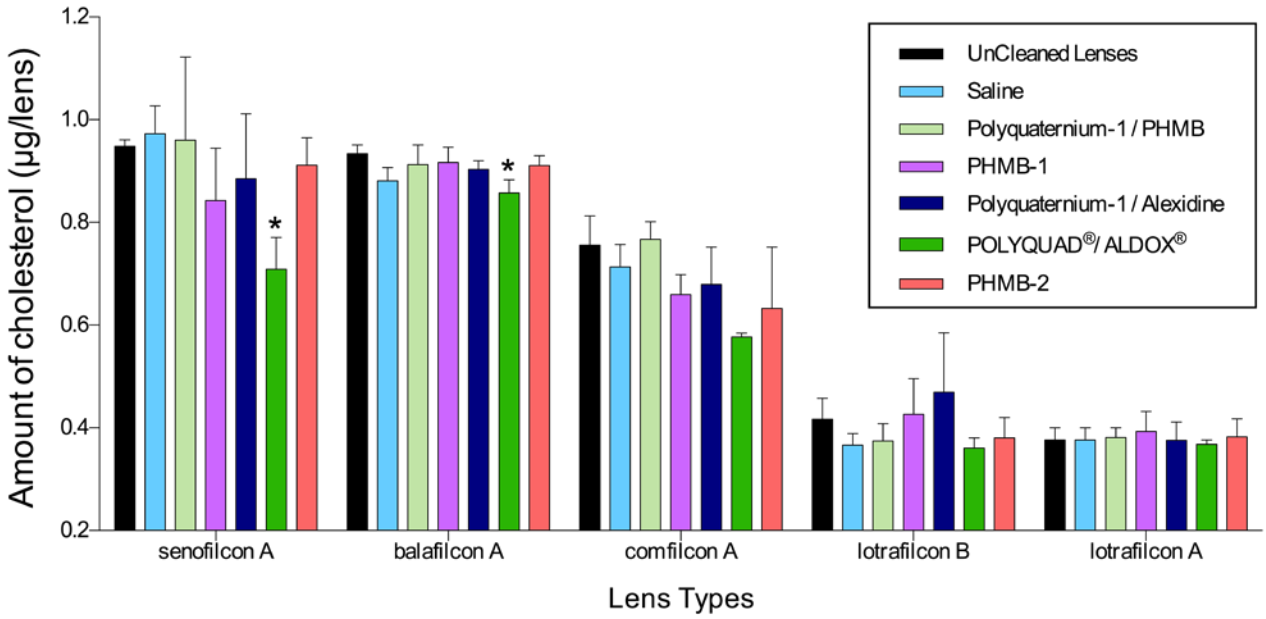

Figure 3-4. The graph shows the Mean (± SD) total cholesterol uptake on various lens material when soaked and cleaned in saline and 5 different MPS after 7 days of incubation in a complex tear solution containing radiolabeled ^{14}C-cholesterol. (*P < 0.05 for cleaned vs. uncleaned lenses).

For the senofilcon A lens material, Polyquad®/Aldox® removed the most cholesterol when compared to the control lenses (25% reduction, p=0.042 vs. uncleaned lenses). No significant lipid reduction (P > 0,05) was found when the lenses were cleaned with the PHMB-1 solution (11% reduction), the polyquaternium-1/alexidine-based MPS (7% reduction), PHMB-2 (4% reduction), and with saline and the polyquaternium-1/PHMB solution (0% reduction).

For balafilcon A, the Polyquad®/Aldox® based MPS (P=0.006) was the only solution to show a significant amount of cholesterol removal, with 8% removal compared to the control, respectively. The percentage removal of cholesterol by saline was 6% and by the other MPS products were 3% (polyquaternium-1/alexidine and PHMB-2), and 2% (polyquaternium-1/PHMB, PHMB-1), which were all statistically irrelevant (Tukey HSD, $P > 0.05$).

Although Figure 3-4 shows the highest reduction of cholesterol on comfilcon A lenses after they were cleaned with the Polyquad®/Aldox® solution (24% reduction), post-hoc analysis did not reveal a significant reduction for any of the tested lens care products (Tukey HSD, $P > 0.05$). The products PHMB-2, PHMB-1, polyquaternium-1/alexidine, saline, and polyquaternium-1/PHMB reduced the amount of lipid by 16%, 13%, 10%, 6%, and 0% respectively.

Figure 3-4 shows a reduction of cholesterol on the lotrafilcon B lens material when cleaned with Polyquad®/Aldox®, polyquaternium-1/PHMB-based, PHMB-2 solution to be 24%, 21%, and 20%, respectively. These results were not statistically significant ($P \geq 0.330$). The reduction of cholesterol using saline and PHMB-1 were 12% and 10% respectively, and polyquaternium-1/alexidine showed minimal reduction (1%) in comparison to the control lenses. None of these were significant ($P > 0.05$).

For the lotrafilcon A lens material, none of the tested MPS solutions removed the low levels of cholesterol from the lens material (0%). A post-hoc analysis revealed no significant differences between the uncleaned controls and the tested products (P>0.05).

Table 3-4. Percent difference of cholesterol removal in comparison to uncleaned lens materials. (highlighted values in red represent statistically significant differences [$P \leq 0.05$])

	Sensitive Eyes	Biotrue	RevitaLens OcuTec	Renu Fresh	OptiFree PureMoist	Solo-Care Aqua
Comfilcon A	6%	0%	13%	10%	24%	16%
Senofilcon A	0%	0%	11%	7%	**25%**	4%
Lotrafilcon A	2%	0%	0%	0%	0%	0%
Balafilcon A	6%	2%	2%	3%	**8%**	2%
Lotrafilcon B	9%	2%	4%	6%	11%	6%
Preservative		**polyquaternium-1/ PHMB**	**polyquaternium-1/ alexidine**	**PHMB-1**	**POLYQUAD®/ ALDOX®**	**PHMB-2**
Surfactant/ Wetting Agent		**poloxamine**	**Tetronic® 904**	**poloxamine 1107**	**Tetronic® 1304**	**poloxamer 407**

3.5 Discussion

These results showed that among the SiHy materials evaluated, after 7 days of incubation in an artificial tear solution, that senofilcon A and balafilcon A deposited the highest amounts of cholesterol, whereas the lotrafilcon A and B lenses accumulated the least. The quantity of deposited cholesterol determined from this study is in broad agreement with previously measured quantities, determined after the same time span [62, 63, 260] and *ex vivo* studies.[60, 263, 265] Furthermore, we found that the Polyquad®/Aldox® based MPS removed more cholesterol than the other MPS systems evaluated, with the results for balafilcon A and senofilcon A being statistically significant. In comparison, all the other MPSs tested were not efficient in removing cholesterol from any of the CL materials.

The commercialization of SiHy materials in the late 1990's produced materials with significantly improved oxygen transmissibility [185, 403] and these materials now account for some 70-80% of all new fits.[396] However, the relatively hydrophobic nature of silicone results in the

materials being more likely to deposit lipids from the TF [58, 60, 62, 63, 260, 265, 268, 325, 399] and the materials to be less wettable.[187, 404-406]

To create a hydrophilic and more wettable surface, some manufacturers modify their SiHy materials, using a wide variety of methods. A plasma oxidation procedure is applied to balafilcon A lens materials, creating silicate islands that spread discontinuously over the lens.[185, 189, 407] The comfilcon A and senofilcon A materials are not surface modified, relying on internal wetting agents or monomer characteristics to augment wettability.[407-411] A 25nm thick permanent plasma surface treatment surrounds the lotrafilcon A and B lens materials and conceals the hydrophobic moieties, to provide a hydrophilic outer layer.[185, 189, 407] In previous studies using similar procedures to those reported in this study we also found balafilcon A materials to be one of the highest depositors of cholesterol.[61, 63] The distribution of the silicate islands and a relatively porous internal structure [412] allow more lipids to penetrate into and onto the lens material, thus, showing higher uptake rates than most other lens materials. In comparison, the plasma surface treatment of the lotrafilcon A and B lens materials prevents lipid uptake more effectively, which is evident by the low levels of cholesterol sorption demonstrated in this study.

Contact lens care systems play a major role in disinfecting, removing, and preventing the accumulation of TF components on lens materials. MPS consist of a wide variety of components, to ensure adequate disinfection, cleaning and to aid wettability.[274, 397] Removal of deposited TF components is undertaken through the incorporation of amphiphilic surfactants (1.5.3), such as poloxamer and poloxamine (Tetronic). These surface-active agents consist of hydrophobic tails and hydrophilic heads that have the capability to chemically adhere and remove lipids, proteins and other debris.[413] Furthermore, wetting agents that are incorporated in MPS also have surfactant properties and, thus, may have a supportive effect on the removal of TF components on CLs. Table 3-2 provides a list of the various surfactants and wetting agents that are included in the MPS

evaluated in this study. Our results show that the combination of components found in the Polyquad®/Aldox® solution was the most efficacious in reducing the amount of cholesterol. When examining the comparison chart Table 3-4, it is apparent that the type of surfactant that is being used in a care regimen may be a driving factor for higher cleaning efficiency of a MPS, with Tetronic® 1304 performing better in removing cholesterol deposition than using "simple" poloxamine as in the polyquaternium-1/PHMB cleaning solution. In addition, the lipid-cleaning efficacy of the MPSs varied between the lens materials, which shows that the cleaning performance is highly dependent on the combination of lens material and lens care product. Of note, is that the highest amounts of lipid were removed from the higher depositing CL materials.

This is the first study to evaluate the cleaning efficacy of different MPSs in removing cholesterol deposits from SiHy contact lens materials using an *in vitro* radiolabeling technique. The results from this study are in agreement with previously published work from our laboratory,[27, 37, 46] demonstrating that radiolabeling is a reproducible and reliable technique to quantify cholesterol deposition on and into silicone hydrogel contact lenses.

"To rub or not to rub", has been a common question relating to contact lens care. Manufacturers introduced solutions with "no rub" instructions to improve patient convenience and such an approach was well received by patients. However, various research groups have reported that MPS products were more effective at removing microorganisms, TF constituents and make-up components when a "rub and rinse" step was applied [414-419] and such data has resulted in calls for all such products to be used with a rub step included.[420] While previous work has been equivocal regarding the removal of TF deposits, with some reports suggesting that protein removal was not different when comparing "rub and rinse" with "no-rub", [333] we believe that a gentle rub and rinse step should increase the efficacy of lipid removal. This is borne out by studies by Tam *et al,* [307, 308] who determined lipid sorption before and after pre-soaking test lenses in one of two MPSs,[307] and

applying a “rub and soak” or “soak” only test procedure. [308] Tam and co-workers found significant removal of lipids only when the MPS was used in a gentle rub and rinse regimen.[308] This further shows that incorporating a rubbing step into the cleaning procedure increases the efficiency of MPS to remove sorbed lipids from CLs. However, despite these findings, many CL patients still fail to apply a rubbing step to their cleaning routine,[314, 321] obviating any benefit that rubbing may provide in removing lipids.

In conclusion, this experiment shows that the efficiency of MPS to remove lipids from SiHy lens materials is highly dependent on the lens material/solution combination. Herein, we found that the Polyquad/Aldox based solution demonstrated the best performance in removing cholesterol from the tested lens materials; however, this was statistically significant only for balafilcon A and senofilcon A lens materials. It is also worth noting that this study only evaluated lipid deposition over 7 days, and most SiHy lenses are replaced after one month.[396] Lipid deposition is cumulative over time [58, 63, 227, 255, 260, 325] and so another management option to manage lipid deposition would be to replace lenses after shorter periods of wear. These results would suggest that if lipid deposition is an issue for patients wearing SiHy materials on a reusable basis that trying differing MPS products would only marginally enhance lipid removal. In such cases where lipid deposition is an issue, switching to another SiHy material, switching to CH materials or increasing the frequency of replacement of the SiHy material would also be appropriate clinical management options.

3.6 Acknowledgement

This study was s*p*onsored by Alcon Research Ltd.

In the next chapter, the degree of lipid uptake on commercially available DD CLs using a radiochemical experiment will be measured, and comparisons between the different lens types will be made.

Chapter 4

In Vitro Cholesterol Deposition on Daily Disposable Contact Lens Materials

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Author	Concept/Design	Data Acquisition	Analysis	Write Up / Publication
Walther	Y	Y	Y	Y
Subbaraman	Y			Y
Jones	Y			Y

4.1 Outline

4.1.1 Objective

The goal of this study was to analyze how various incubation times affect the uptake of cholesterol on silicone hydrogel (SiHy) and conventional hydrogel (CH) daily disposable (DD) contact lens materials using an *in vitro* radiochemical detection method.

4.1.2 Materials & Methods

Three SiHy (somofilcon A, delefilcon A, and narafilcon A) and four CH (etafilcon A, nesofilcon A, ocufilcon A, and nelfilcon A) contact lenses were incubated in an artificial tear solution (ATS) that contained major TF components, and a portion of radioactive ^{14}C-cholesterol. Lenses (n=4) were incubated for four incubation times (2, 6, 12 or 16 hours) to assess the effects on cholesterol deposition. Subsequent to the incubation, the lenses were extracted using 2:1 chloroform: methanol and the extracts were analyzed in a beta counter and (ng/lens) extrapolated from standard curves.

4.1.3 Results

In general, cholesterol deposited statistically significantly more on SiHy lenses than CHs ($P \leq 0.033$), with the exception of somofilcon A and nesolfilcon A materials ($P = 0.067$). Within the SiHy materials, narafilcon A accumulated the largest quantity of cholesterol ($P < 0.05$) and somofilcon A the lowest ($P < 0.05$). The uptake of cholesterol ranged from 22.63 ± 2.98 ng/lens to 97.94 ± 4.18 ng/lens for all lens materials. The accumulation of cholesterol was shown to be continuous throughout the 16 hours of incubation, without reaching a plateau ($P < 0.001$).

4.1.4 Conclusion

For the periods of time that DD lens materials are worn, cholesterol deposits significantly more onto SiHy contact lenses than CHs. This could have implications for wearers who have higher levels of lipid in their tears that are fitted with SiHy DD materials.

4.2 Introduction

The introduction of frequent replacement daily wear soft lenses in the late 1980's resulted in a significant reduction in complications that were due to lens deposition and ageing and resulted in increased patient satisfaction with lens wear. [421-423] The ultimate option for daily wear frequent replacement lenses, daily disposable (DD) soft lenses that are worn once and then discarded, became a commercial reality in 1995.[424, 425] These lenses provide the ultimate in convenience for wearers, have high levels of compliance with replacement frequency [426, 427] and many studies have shown their benefits in terms of clinical performance and reduction in complications. [428-438]

Over the past decade, silicone hydrogel (SiHy) contact lenses have become the predominant material of choice for practitioners and lens wearers, [211] primarily due to their high oxygen transmissibility. [177, 439, 440] Until recently, practitioners were unable to offer DD lenses manufactured from SiHy materials, with the majority of DD lenses being manufactured from conventional hydrogel (CH) materials. While SiHy materials will increase oxygen transport, the presence of siloxane groups within these materials results in relatively hydrophobic surfaces that may impact wettability. [404, 405, 441] The hydrophobicity may also result in increased deposition of certain lipid species, [58, 227, 257, 260, 266] which might enhance the prevalence of lens-induced dryness [70, 373, 374, 442] and discomfort [443] during lens wear if the inappropriate type or quantity of lipids were deposited.

To-date, no studies have examined the degree to which DD lens materials may deposit lipid, in particular cholesterol, which is a common lipoidal contaminant on SiHy materials. [58-63] Patients who have an oily TF may notice rapid deposition of lipid onto SiHy materials (Figure 4-1). The purpose of this study was to quantify the amount of cholesterol contamination on hydrogel and SiHy DD lens materials over typical periods of wear, using an in vitro deposition model.

Figure 4-1. Female patient aged 23 with significant oily contamination of the tear film was fitted with a silicone hydrogel daily disposable lens and complained of "smeary vision" after 3-4 hours of wear. The image shows significant deposition with what appears to be a lipid-like film after only 3 hours of wear. Refitting the patient with an FDA group IV hydrogel daily disposable resulted in no visible deposition and comfortable wear for 12 hours.

4.3 Materials and Methods

4.3.1 Lens Materials

The study included seven commercially available, unworn DD lens materials (n=4 per type), including three SiHy materials (delefilcon A [Alcon, Ft Worth, TX]; somofilcon A [CooperVision, Pleasanton, CA]; narafilcon A [Johnson & Johnson, Jacksonville, FL]), and four CH lens materials (ocufilcon B and nesofilcon A [CooperVision, Pleasanton, CA]; nelfilcon A [Alcon, Ft Worth, TX]; etafilcon A [Johnson & Johnson, Jacksonville, FL]). The properties of these lens materials are listed in Table 4-1 and all lenses had an optical power of -3.00 diopters.

Table 4-1. Contact lens material information

USAN	Trade Name	Water Content	FDA Group	Oxygen Permeability (Dk) (x 10^{-11})	Lens Type
Delefilcon A	DAILIES TOTAL1	33%	V	156	SiHy
Somofilcon A	clariti 1day	56%	V	86	SiHy
Narafilcon A	1-Day ACUVUE TrueEye	46%	V	118	SiHy
Etafilcon A	1-Day ACUVUE MOIST	58%	IV	25.5	CH
Ocufilcon B	Biomedics 1Day	52%	IV	24	CH
Nelfilcon A	DAILIES AquaComfort Plus	69%	II	26	CH
Nesofilcon A	Biotrue ONEday	78%	II	42	CH

USAN: United States Adopted Names, CH: conventional hydrogel, SiHy: silicone hydrogel, FDA: Food and Drug Administration

4.3.2 Contact Lens Incubation and Extraction

The methods used to expose the lenses to lipid and to extract the lipid post-doping are similar to those previously published by our laboratory.[61-63]

4.3.3 Vial pre-treatment

Initial in-house studies showed that the vials used to store the lenses during the incubation process adsorbed some of the cholesterol and impacted the deposition data. To avoid this, every vial was initially pre-treated with a previously characterised artificial tear solution (ATS) at 37°C for seven days prior to the experiment.[61] This was demonstrated to minimize lipid uptake from the incubation solution and negate this issue, as previously reported.[63]

4.3.4 Lens incubation and extraction

To quantify the deposited cholesterol, a trace amount of radioactive ^{14}C labeled cholesterol (Perkin Elmer, Waltham, MA) was added to the ATS, as previously described. [63] This ATS included six lipids commonly found in the tears, at physiologically relevant concentrations. [61] The concentration of ^{14}C-cholesterol was 3% of the total non-radioactive concentration of cholesterol. Following the addition of radioactivity, the solution was sonicated for 5 minutes and the ATS was ready to use as the incubation solution. The lens materials were taken directly out of their blister packages, rinsed in phosphate buffer saline (PBS), and blotted dry on lens paper before placing them into the incubation vials. Following vial pre-treatment, the vials were filled with 3.5mL of the prepared radioactive ATS and the pre-soaked lenses were placed in the vials. All vials were sealed with Parafilm® and incubated at 37°C while shaking at 60 RPM for their specified incubation periods.

The study design involved four different incubation times that simulate different wearing times that are typical for DD lenses, ranging from very short periods of wear (to simulate that often seen

for use for sports activities), through to an entire day. Lenses were incubated in the doping solution for 2, 6, 12 and 16 hours.

Following incubation, each lens was removed from the vial and rinsed twice in PBS to remove excess incubating ATS. The contact lenses were then transferred to 20 ml glass scintillation vials and extracted twice with 2mL of 2:1 chloroform: methanol for three hours. During extraction, the vials were incubated at 37°C with orbital shaking. Once extraction was complete the extraction solution was dried completely using nitrogen gas and the dried samples were re-suspended with 1 ml of chloroform. Each vial was then sonicated, filled with 10 ml of Ultima Gold F (Perkin Elmer, Waltham, MA) scintillation fluid and counted using the L6500 Beckman Coulter Beta Counter (Beckmann Coulter Inc., Brea, CA). The radioactive counts per minute were then converted to masses of cholesterol (µg) using standard lipid calibration curves.

4.3.5 Data Analysis

Repeated measures ANOVA, univariate and Tukey post-hoc analysis were performed when required. The factors analysed were lens material and incubation time. The statistical program was IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY, U.S.). Statistical significance was considered at a P value of < 0.05.

4.4 Results

Table 4-2 and Figure 4-2 report the amount of cholesterol accumulated by the lens materials over the times investigated. Table 4-3 shows that cholesterol deposition was dependent on both the contact lens type and length of incubation ($P < 0.001$), as was the interaction between them ($P < 0.001$). Increasing time of incubation resulted in increased cholesterol deposition, regardless of the lens material.

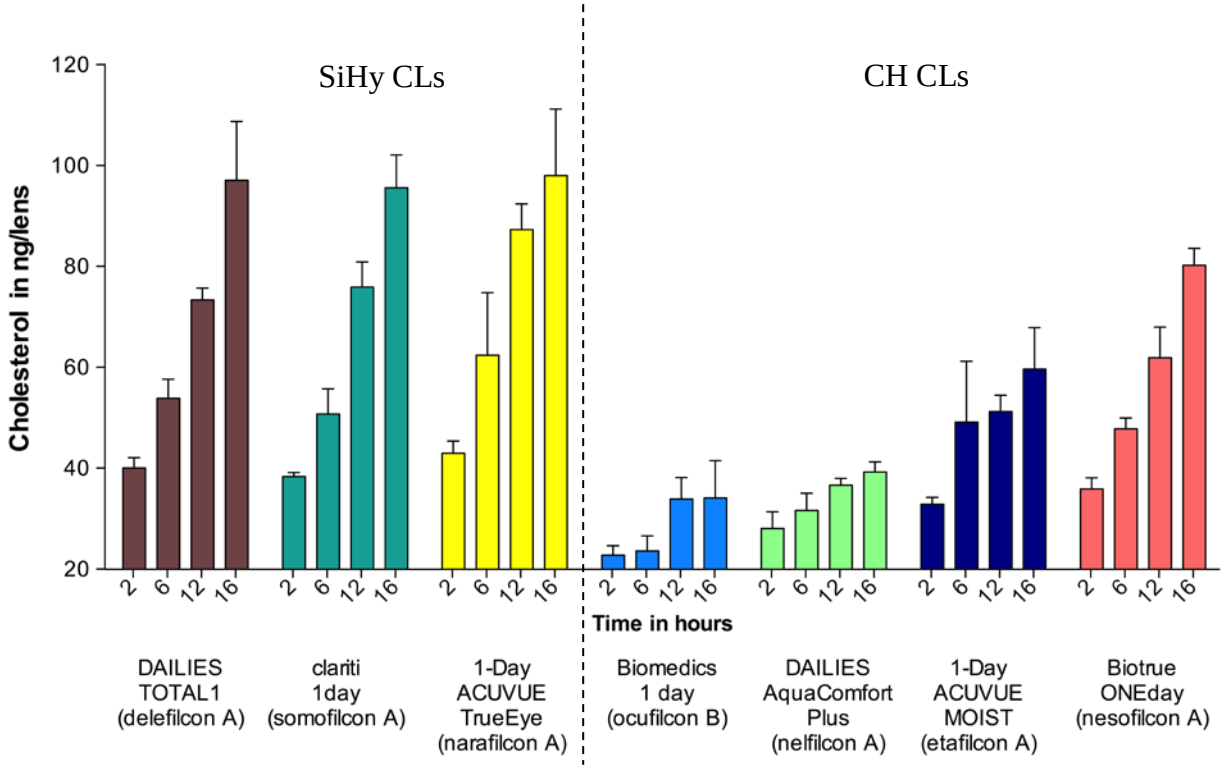

Figure 4-2. Mean (± SD) total cholesterol uptake on various daily disposable contact lens materials over 16 hours. Lipid quantities were measured using a radiolabel method in which cholesterol was labelled within an artificial tear solution containing a variety of proteins, lipids and mucin.

Table 4-2. Cholesterol deposition on lenses in ng/lens (Mean ± SD)

USAN	2 hours	6 hours	12 hours	16 hours
Delefilcon A	39.91 ± 2.06	53.76 ± 3.76	73.25 ± 2.35	96.97 ± 11.66
Somofilcon A	38.23 ± 0.82	50.62 ± 5.01	75.79 ± 4.99	95.52 ± 6.51
Narafilcon A	42.86 ± 2.44	62.30 ± 12.40	87.21 ± 5.10	97.94 ± 13.18
Ocufilcon B	25.34 ± 14.77	49.03 ± 12.09	51.12 ± 3.26	59.52 ± 8.22
Nelfilcon A	22.63 ± 1.94	23.51 ± 2.96	33.79 ± 4.28	33.98 ± 7.43
Etafilcon A	27.95 ± 3.31	31.51 ± 3.46	36.54 ± 1.34	39.13 ± 2.01
Nesofilcon A	35.76 ± 2.21	47.73 ± 2.15	61.76 ± 6.13	80.11 ± 3.35

USAN: United States Adopted Names, CH: conventional hydrogel, SiHy: silicone hydrogel, FDA: Food and Drug Administration

Table 4-3. Repeated measures ANOVA statistical results for cholesterol uptake onto various DD contact lens materials.

Repeated Measures ANOVA statistical results for cholesterol uptake over time					
Variable	Sum of Squares	Degrees of Freedom	Mean Square	F	*P* value
Length of Incubation	23819.11	3	7939.71	211.29	< 0.001
Lens Material	27565.38	6	4594.23	73.28	< 0.001
Length of Incubation * Lens Material	6536.26	18	363.13	9.66	< 0.001
Error	2367.42	63	37.58		

Within the SiHy materials, there was no significant difference in the amount of cholesterol deposited ($P \geq 0.149$). Within the CH materials, a difference did exist. In terms of order of cholesterol deposited, nesofilcon A deposited the most, followed by ocufilcon B ($P = 0.024$), and then etafilcon A and nelfilcon A deposited the least (these latter two were not statistically different; $P = 0.504$). Ocufilcon B deposited more cholesterol than both etafilcon A ($P = 0.004$) and nelfilcon A ($P < 0.001$).

Overall, SiHy lens materials deposited significantly more cholesterol than CH materials ($P \leq 0.033$). The exception to this was that there was no statistically significant difference ($P = 0.067$) between the lowest depositing SiHy material (somofilcon A) and the highest depositing CH (nesofilcon A).

4.5 Discussion

Daily disposable lenses have many advantages over reusable lenses, including convenience and high levels of wearer satisfaction,[429] in addition to providing a more cost-effective option for the occasional wearer. In comparison to re-usable hydrogel frequent replacement modalities, DD lenses provide improved comfort and vision,[429, 436, 444] better visual acuity,[444] reduced lens deposition[430] and a low rate of complications.[445, 446] As a result of these benefits, practitioners have continued to adopt the use of this modality and some markets now see almost 50% of new fits with DD lenses.[211, 447]

Recently, manufacturers have started to introduce DD materials manufactured in SiHy materials. Several previous studies have shown that cholesterol deposits significantly more onto silicone-based lens materials than CHs. [58, 62, 63, 227, 258, 260, 263] However, these studies were conducted on reusable lens materials and this in vitro study is the first to investigate the sorption of a TF lipid (cholesterol) onto different DD lens materials, using an established radiolabeling method. Previous studies examined the accumulation of lipids over timespans of days or weeks, whereas, the DD materials in this study were only incubated for a maximum time of 16 hours. As shown in Figure 4-2, regardless of the time that the lens materials were exposed to the lipid-containing solution, the SiHy materials deposited more cholesterol than the CH materials.

In contrast to several DW SiHy contact lenses, SiHy DDs are not surface treated. Narafilcon A is a silicone hydrogel in which an internal wetting agent poly(vinyl-pyrrolidone) (PVP) is incorporated into the material, [191, 192] to lower lens dehydration, decrease friction, and to prevent deposits on the lens materials. Somofilcon A-based lenses do not feature an internal wetting agent or a surface treatment. [190] The latest DD SiHy material, delefilcon A, features a 'water-gradient' design, with a silicone core that is surrounded by a hydrogel layer that is approximately 5µm thick. [193-196] This strategy provides a higher amount of water (>80%) on the lens surface, whereas, the bulk material has a water content of 33%. Despite these obvious differences in approach to the development of SiHy DD lenses, there were minimal differences in cholesterol uptake after 16-hours of incubation, suggesting that these differences in material composition have a relatively minimal impact on overall cholesterol accumulated by the SiHy DD materials examined, over the period of time that they were tested. However, it should be borne in mind that we did not examine the uptake of other lipids by these materials or indeed whether these lipids were oxidised or not, which may have clinical relevance [132] and warrants further evaluation.

In a recent study using similar methods to this report, [63] we examined uptake rates of cholesterol on reusable SiHy materials, with accumulations of ≥0.21µg/lens (210ng/lens) after one day of incubation. The SiHy DDs examined in this study accumulated far less cholesterol after 16 hours of incubation than their DW counterparts, depositing approximately 96 ng/lens, which is less than half the amount of cholesterol uptake found on DW lenses, suggesting that SiHy DD lenses may be a good option for patients who tend to have deposition problems with reusable SiHy lenses.

Conventional hydrogel DD materials do not include any siloxane moieties and thus require no surface modification. Figure 4-2 demonstrates that the CH material with the highest water content (nesofilcon A; 78%) deposited the highest amount of cholesterol, followed by the material with the lowest water content (ocufilcon B; 52%). This demonstrates that water content is clearly not a driver for cholesterol deposition within CH materials. Nesofilcon is a co-polymer of polyHEMA and N-vinylpyrrolidone (NVP) and a number of previous studies have shown that NVP has a tendency to increase lipid deposition, [132, 246, 253, 254] which is further confirmed by the data from this current study, even for NVP-containing materials that are exposed to lipid for short periods of time. This known tendency for pyrrolidone-derivatives to selectively adsorb lipids is one of the reasons it has been used as a transdermal penetration enhancer. [448] Ocufilcon B and etafilcon A are both FDA group IV (high water content, ionic charge) copolymers of polyHEMA and methacrylic acid. Examination of Figure 4-2 shows that etafilcon A deposited less cholesterol than ocufilcon B at all points in time, and the reason for this difference is not obvious from their composition.

The nelfilcon A material is an FDA group II (high water content, neutral charge) material that is composed of polyvinyl alcohol (PVA) and exhibited similarly low levels of cholesterol uptake to that seen with etafilcon A. For patients who wish to use DD lenses and have a relatively heavy contamination of lipid within their tears, potentially due to the presence of meibomian gland

dysfunction, these latter two CH materials may be excellent choices. In wearers who require higher levels of oxygen transport but who exhibit oily tears, switching them into a SiHy DD lens may potentially result in some surface wetting issues (see Figure 4-1) and this is worthy of consideration, particularly if long wearing periods are required, as this cholesterol uptake is cumulative with time of wear, as clearly shown in Figure 4-2. This variability in lipid deposition between individuals being greater than that seen with protein deposition (which is far more mediated by lens material composition) has been shown in a number of previous studies. [246, 254, 255, 449, 450]

To our knowledge, this is the only study to-date that elucidates the uptake rate of TF lipids on commercially available SiHy and CH DD lens materials, albeit using an in vitro model. A recent *ex vivo* study by Maissa et al. [449] determined the accumulation of various TF lipids onto SiHy (balafilcon A) and CH (etafilcon A) materials, in which the lenses were worn on a daily disposable basis for 10 hrs and an overnight basis for 7 consecutive days. Maissa and co-workers confirmed that SiHy materials deposit more lipid than CH materials and also determined that lipid uptake was cumulative, which supports previous studies [62, 63, 254, 255] investigating kinetic uptake of lipid and also the results from this current study. After 10 hours of lens wear, Maissa and colleagues measured 0.03 µg/lens (30 ng/lens) of cholesterol on the etafilcon A material, which is very similar to the values we report in this manuscript for the same material. This suggests that the technique utilized in this experiment is a sensitive *in vitro* method to determine lipid uptake on CL materials and that the data reported should be relatively reflective of that seen *in vivo.* However, one issue that is worthy of discussion is that this current study was conducted in a static vial format, preventing the TF rupture that often occurs during the inter-blink period. Our group has previously shown that incorporating such a break in the surrounding fluid does tend to increase lipid deposition, due to the increased hydrophobicity that can occur. [266] Thus, it is plausible that the

values reported in this manuscript may be slightly lower than that seen with lenses collected from an in vivo study, particularly for more hydrophobic materials in which dehydration and surface drying may be more relevant than in more hydrophilic materials such as etafilcon A.

It is possible that this higher lipid uptake may cause some surface wetting issues and may result in in-eye de-wetting and potential reduced comfort. However, results to date have shown that DD SiHy materials appear to be well received by the majority of wearers and provide excellent in-eye comfort. [451-453]

In conclusion, the SiHy DD lens materials demonstrated significantly higher rates of cholesterol deposition than the CH DD materials and this information is valuable for practitioners to consider when considering using such materials in patients with TFs that may contain excess lipid. This in vitro study is the first to investigate lipid uptake onto various DD lens materials and provides hitherto unavailable information to practitioners, patients, and contact lens manufacturers. Further studies are required to determine if these levels of lipid may prove deleterious during wear and follow-up clinical studies are warranted, particularly in patients with meibomian gland dysfunction.

4.6 Acknowledgment

This study was funded by the Canadian Optometric Educational Trust Fund (COETF).

In the following chapter, it will be determined where lipids are located after depositing on contemporary DD CL materials by using fluorescently labeled cholesterol and detecting its position with a confocal microscope. Comparisons of the lipid penetration will be made between the different test lenses and between a traditional and a novel *in vitro* lens incubation method.

Chapter 5

Differential Deposition of Fluorescently Tagged Cholesterol on Commercial Contact Lenses Using a Novel *In Vitro* Eye Model

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Author	Concept/Design	Data Acquisition	Analysis	Write Up / Publication
Walther	Y	Y	Y	Y
Phan	Y		Y	Y
Subbaraman	Y			Y
Jones	Y			Y

5.1 Outline

5.1.1 Purpose

Current *in vitro* models to evaluate tear film (TF) deposition on various contact lenses (CLs) do not adequately simulate physiological ocular conditions. The aim of this study was to evaluate the differences in lipid uptake and penetration in daily disposable (DD) CLs using the conventional "in-vial" method compared to a novel *in vitro* eye model.

5.1.2 Methods

The penetration of NBD-cholesterol (7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol) on three silicone hydrogel (SiHy) (delefilcon A, somofilcon A, narafilcon A) and four conventional hydrogel (CH) (etafilcon A, ocufilcon B, nesofilcon A, nelfilcon A) DD CLs were investigated. The CLs were incubated for 4 and 12 hours (h) in an artificial tear solution (ATS) containing fluorescently labelled NBD-cholesterol at room temperature (21^{o}C). For the vial condition, the CLs were incubated in a vial containing 3.5 mL of ATS. In the *in vitro* eye model, the CLs were mounted on our eye-blink platform designed to simulate physiological tear flow (2 mL/24 h), tear volume and 'simulated' blinking. After the incubation period, the CLs were analyzed using laser scanning confocal microscopy (LSCM). Quantitative analysis for penetration depth and relative fluorescence intensity values was determined using ImageJ.

5.1.3 Results

The depth of penetration of NBD-cholesterol varied between the vial and the eye-blink platform. Using the traditional vial incubation method, NBD-cholesterol uptake occurred

equally on both sides of all lens materials. However, employing our eye-blink model, cholesterol penetration was observed primarily on the anterior surface of the CLs. In general, SiHy lenses showed higher intensities of NBD-cholesterol than CH materials. Fluorescence intensities also varied between the incubation methods as well as the lens materials.

5.1.4 Conclusions

This study provides a novel *in vitro* approach to evaluating deposition and penetration of lipids on CLs. We show that the traditional "in-vial" incubation method exposes the CLs to an excessively high amount of ATS on both the front and back surface of the lens, which results in an overestimation for cholesterol deposition. Our model, which incorporates important ocular factors such as intermittent air exposure, small tear volume, and physiological tear flow between blinks, provides a more natural environment for *in vitro* lens incubation. Consequently, this will better elucidate the interactions between CLs and TF components.

5.1.5 Keywords

cholesterol, lipid, contact lens, daily disposable, eye model, deposition, laser scanning confocal microscopy, conventional hydrogel, silicone hydrogel

5.2 Introduction

Contact lens (CL) dropout remains a pressing concern for the CL industry, with discomfort being a primary factor.[197, 198, 454] Consequently, there is an increasing demand on manufacturers to continually produce safer and more comfortable CLs.[171, 185, 186] The first generation of soft CLs, consisting of poly(2-hydroxyethyl methacrylate) (pHEMA) and its derivatives, were relatively comfortable, but unfortunately did not permit adequate oxygen transmission for the cornea to function optimally.[176, 455] This problem was addressed in the late 1990s with the introduction of silicone hydrogel (SiHy) CL [177, 439, 440] materials that provided relatively high oxygen transmissibility. [177, 439, 440] However, due to the hydrophobic siloxane moieties within SiHy CLs, these materials suffered from reduced surface wettability,[404, 405, 441] and increased lipid deposition.[58, 62, 257, 260, 267, 456] As a result, these lenses were not as comfortable as initially expected.

Despite extensive research over the past five decades, the paradigm for CL discomfort remains unclear, likely due to the multifactorial nature of comfort. One potential hypothesis suggests that discomfort manifests from the deposition of tear components, such as lipids, on the lens over time, which leads to changes within and on the surface of the lens.[63, 257, 268] One strategy to overcome the complications associated with long-term lipid deposition is to switch to daily disposable (DD) lens wear. Even then, lipid deposition from short term or daily wear modality could still lead to end of day discomfort.[325]

To investigate this phenomenon, studies have historically systematically investigated factors which can influence lipid deposition on CLs.[61-63, 325] Important elements to consider are

TF lipid concentration, exposure time, properties of the lens material, and interactions between various TF components with the lens.[62, 63, 224, 259, 260, 263] Previously, we have also shown that intermittent air exposure from a simulated blinking motion is also a crucial factor in influencing the degree of lipid deposition.[266]

The challenge in elucidating the mechanisms of TF deposition *in vivo* is to adequately model a similar scenario *in vitro*. In the past, for CL deposition studies, researchers employed simplistic models by immersing lenses in a vial containing 3.5 mL of simulated tear fluid containing the component of interest.[62, 63, 325] However, on the ocular surface, the tear volume is estimated to be only 7 ± 2 µL,[457] with a tear exchange rate of 0.95-1.55µL/min.[458] Thus, it is apparent that the previous models are too rudimentary, lacking not only the tear flow component, but the incubation volume also far exceeds physiological levels. Thus, to further our understanding of TF deposition, a better *in vitro* model is necessary.

Studies evaluating lipid deposition on CLs traditionally have focused on quantifying the amount of lipids deposited on the lens.[58, 61, 62, 257, 260, 266] In order to gain further insights on tear deposition, it is also of interest to evaluate the patterns of lipid deposition and penetration through the lens over time. The use of laser scanning confocal microscopy (LSCM) has been used previously to map protein penetration on the surface and matrix of CLs using fluorescently labeled proteins.[233, 357] The ability to visualize lipid penetration in different CL materials may help explain the variations in comfort experienced between different CLs. The aim of this study was to characterize the penetration of fluorescently-tagged cholesterol on

commercially available DD CLs using a novel ocular model [459] which simulates tear volume, tear flow, and blinking and to compare the results to the standard vial incubation method.

5.3 Materials & Methods

5.3.1 Contact lenses and pre-treatment

Four commercially available conventional hydrogel (CH) DD CLs [etafilcon A (Johnson & Johnson), ocufilcon B (CooperVision), nesofilcon A (Bausch+Lomb), nelfilcon A (Alcon)] and three SiHy lenses [delefilcon A (Alcon), somofilcon A (CooperVision), narafilcon A (Johnson & Johnson)] were evaluated in the study. All lenses had a dioptric power of -3.00 and base curve of 8.5 or 8.6 mm, obtained from the manufacturer in the original packaging. Table 5-1 and Table 5-2 detail the properties of the CH and SiHy CLs respectively. Prior to all incubation studies, all CLs were removed from their packaging solutions and soaked in 5mL of PBS for 24 hours (h) while shaking at 30 RPM to remove excess packaging solution. After the 24h soaking, the CLs were removed from the pre-treatment solution and were blotted on lens paper to remove any excess liquid.

Table 5-1. Properties of conventional hydrogels (CH) used in the study

	1-DAY ACUVUE MOIST	BioMedics 1Day	Biotrue 1Day	DAILIES AquaComfort Plus
United States adopted name (USAN)	etafilcon A	ocufilcon B	nesolfilcon A	nelfilcon A
Manufacturer	Johnson & Johnson	CooperVision	Bausch+Lomb	Alcon
Water content (%)	58%	52%	78%	69%
FDA group	IV	IV	II	II
Centre thickness (mm)	0.08	0.07	0.05	0.10
Oxygen transmissibility ($x10^{-9}$)	25.5	24	24	26
Principal monomers	HEMA, MA	HEMA, PVP, MA	HEMA, NVP	FMA, PVA, PEG

FMA, N-formylmethyl acrylamide; HEMA, hydroxyethyl methacrylate; MA, methacrylic acid; PEG, polyethylene glycol; PVA, polyvinyl alcohol; PVP, polyvinyl pyrrolidone; NVP, N-vinylpyrrolidone

Table 5-2. Properties of silicone hydrogels (SiHy) used in the study

	DAILIES TOTAL1®	clariti™ 1day	1-DAY ACUVUE® TruEye®
United States adopted name (USAN)	delefilcon A	somofilcon A	narafilcon A
Manufacturer	Alcon	CooperVision	Johnson & Johnson
Water content (%)	33% (surface >80%)	56%	46%
FDA group	V	V	V
Centre thickness (mm)	0.09	0.07	0.09
Oxygen transmissibility ($x10^{-9}$)	156.0	86	118.0
Principal monomers	Not disclosed	Not disclosed	MPDMS, DMA, HEMA, siloxane macromer, TEGDMA, PVP

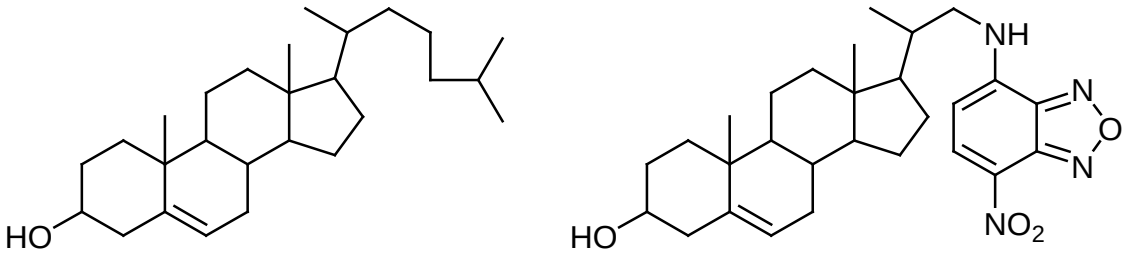
DMA, N,N-dimethylacrylamide; HEMA, hydroxyethyl methacrylate; MPDMS, monofunctional polydimethylsiloxane; PVP, polyvinyl pyrrolidone; TEGDMA, tetraethyleneglycol dimethacrylate

5.3.2 Artificial Tear Solution

The composition of the artificial tear solution (ATS) has been previously reported by our group.[61] Briefly, it contains various mucins, urea, salts, glucose, proteins (lysozyme and albumin), and various lipids (oleic acid methyl ester, cholesterol, triolein, phosphatidylcholine, cholesteryl oleate, and oleic acid).[61]

5.3.3 Fluorescently tagged cholesterol

Cholesterol and its derivatives are one of the primary lipid deposits found on CLs, and thus it was chosen as the representative lipid for this study.[58, 60, 258, 263, 265] Fluorescently-tagged NBD-cholesterol [22-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3ß-ol-cholesterol) (Figure 5-1), obtained from Avanti Polar Lipids Inc. (Alabama), was used to visualize the deposition and penetration of cholesterol into CLs. For this study, NBD-cholesterol was dissolved at a physiological concentration of 1.9 mg/mL in a cholesterol-free solution of ATS.



Cholesterol NBD-Cholesterol

Figure 5-1. Chemical structure of Cholesterol (386.65 g/mol) and NBD-cholesterol (494.63 g/mol) [460]

5.3.4 Ocular flow model

Our *in vitro* model (OcuFlow) consists of a two-piece model that includes a "corneal/scleral" piece and an "eyelid" component, spaced 250 µm apart. The templates for the eye models were designed using a computer aided drawing software (Solid Works 2013), and printed using 3-D printing technology.[459] The resulting 3-D printed molds (PC-ABS, polycarbonate-acrylonitrile-butadiene-styrene) were filled with polydimethylsiloxane (PDMS) and cured at 75˚C for 1 h. The corneal and the eyelid pieces were then mounted on a special clip, which attaches to our blink platform.

The platform consists of two mechanical actuators. The first motor moves the eyelid laterally to simulate the closing of the eye, spread of the TF, and intermittent air exposure (Figure 5-2A). The second motor rotates the corneal piece circularly when the two eye pieces come together to simulate the rubbing action produced during blinking (Figure 5-2B). The system is connected to a microfluidic syringe pump (PHD UltraTM, Harvard Apparatus), which injects ATS into the eye models at a physiological flow rate at 1.3 µL/min (Figure 5-2C). The general set up of the model with attachment to the microfluidic system is shown in Figure 5-2D.[459]

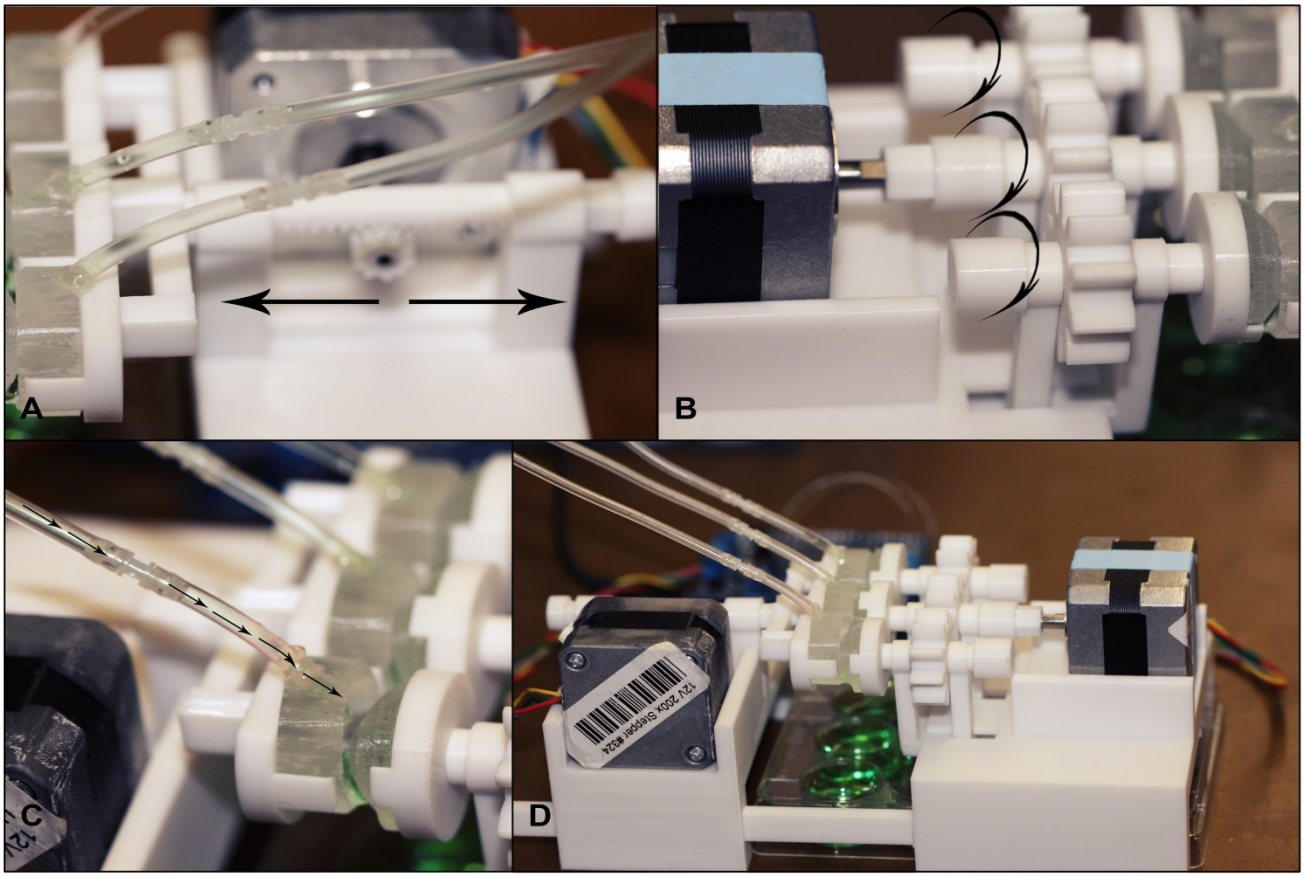


Figure 5-2. Lateral motion produces intermittent air exposure (A); Circular motion simulates rubbing action during blinking (B); tear fluid infusion into eyelid (C); OcuFlow platform (D).

5.3.5 Experimental Outline

For the vial incubation condition (1), six lenses of each type were immersed in a vial containing 3.5 mL of ATS with NBD-cholesterol for 12h at room temperature with shaking. For the eye model condition (2), six lenses of each type were placed on the OcuFlow model and allowed to run for 12h at room temperature with a flow rate at 1.3µL/min (2 mL/24h).

At 4h and 12h, three lenses of each type were removed from each experimental condition, blot dried on lens paper, and prepared for imaging. These time intervals were chosen to

correspond to typical short wearing times found in part-time wearers of DD lenses and an all-day daily CL wear time period. Using a hole-punch, 5 mm diameter discs were punched out from the centre of the CLs. The lens discs were then carefully mounted onto a piece of 22 x 40 x 1 mm Fisherbrand® microscope glass cover slip (Fisher Scientific, Pittsburgh, PA). 40 µL of PBS was then carefully pipetted onto the lens disc, and a second glass cover slip was carefully placed on top. To secure the cover slip onto the microscope slide, a small amount of clear nail polish was applied to the sides of the cover glass using a pipette tip.

5.3.6 Confocal Microscopy

To image the slides, a Zeiss LSM 510 Meta LSCM (ZEISS Inc., Toronto, Canada) was used to both excite the NBD-cholesterol with an argon laser at 488 nm and to capture the emitted fluorescence at its peak wavelength of 528 nm by using a band pass filter of 505nm-530nm. The LSCM captured a series of consecutive images spaced 0.5 µm apart. The resulting images were rendered into a two-dimensional cross section using the ZEN 2009 light software (Zeiss). The fluorescence was recorded for every fourth image per sample using ImageJ (National Institute of Health, United States) and the subsequent data were averaged and corrected for the auto-fluorescence from the control lenses soaked in PBS and plotted on a histogram. Based on this plot, the depth of cholesterol penetration into the CL material over time was determined. By sustaining the identical laser settings for all CLs, a direct relationship can be drawn between an increase of relative intensity of fluorescence (RIF) values and NBD-cholesterol sorption on the CLs.

5.3.7 Statistical Analysis

IBM SPSS Statistics 23 for Macintosh (IBM Corp., Armonk, NY) software was used to conduct repeated measures analysis of variance (RM-ANOVA) and *post-hoc* Tukey's multiple comparisons to test the impact of the incubation methods, CL materials, and incubation times on the lipid penetration. Statistical differences were considered significant for a P value of < 0.05. The graphs were plotted using GraphPad Prism version 6.0h for Macintosh (GraphPad Software, La Jolla, CA).

5.4 Results

The RIF of accumulated NBD-cholesterol varied greatly between the tested lens materials and the tested incubation methods. A collage of the penetration patterns and a graphical illustration of the results are shown in Figure 5-3 and Figure 5-4, respectively. The results of the performed RM-ANOVA are shown in Table 5-3 and reveal that all three test variables were statistically significant; both individually (within) and between their interactions ($P \leq 0.045$).

Table 5-3. Repeated measures ANOVA statistical results for cholesterol penetration comparing various incubation methods and contact lens materials

Repeated Measures ANOVA statistical results for cholesterol penetration					
Variable	**Sum of Squares**	**Degrees of Freedom**	**Mean Square**	***F***	***P***
Incubation Methods	1094750.82	1	1094750.82	615.43	< 0.001
Contact Lens Materials	2287925.12	6	381320.85	90.33	< 0.001
Incubation Times	200928.78	1	200928.78	47.60	< 0.001
Incubation Methods * Contact Lens Materials	475027.12	6	79171.19	44.507	< 0.001
Contact Lens Materials * Incubation Times	193438.91	6	32239.82	7.64	< 0.001
Incubation Methods * Incubation Times	7148.49	1	7148.49	4.02	0.045
Incubation Methods * Contact Lens Materials * Incubation Times	295658.03	6	49276.34	27.701	< 0.001
Error (Incubation Methods)	2844359.78	1599	1778.84		

5.4.1 Impact of Contact Lens Material

Overall, SiHy lens materials accumulated significantly more ($P \leq 0.001$) of the fluorescently-labeled lipid than CHs, with the exception of nesofilcon A CLs ($P \geq 0.209$), which showed similar amounts of accumulated NBD-cholesterol as the somofilcon A and narafilcon A lens materials. The general pattern found, after pooling all data points for each CL material and statistically comparing between them, was: delefilcon A > somofilcon A ≥ nesofilcon A ≥ narafilcon A > etafilcon A > ocufilcon B > nelfilcon A, with no statistically significant differences between the SiHy lenses ($P \geq 0.117$). Within the CH materials, however, the differences of accumulated cholesterol were statistically significant ($P \leq 0.003$), except between etafilcon A and ocufilcon B ($P = 0.992$).

After the 4 h vial incubation, the deposition sequence was the same as the previously listed general pattern, however, these differences were not statistically significant between all SiHy lenses ($P \geq 0.582$), the SiHy lenses and nesofilcon A ($P \geq 0.721$), as well as between etafilcon A and narafilcon A ($P = 0.130$), nesofilcon A ($P = 0.141$), and ocufilcon B ($P = 1.000$). The pattern after the 12 h vial incubation varied slightly and was: delefilcon A > narafilcon A > somofilcon A > nesofilcon A > etafilcon A > ocufilcon B > nelfilcon A. All those differences were statistically significant ($P \leq 0.012$), except between delefilcon A and narafilcon A ($P = 1.000$), somofilcon A and nesofilcon A ($P = 0.989$), and ocufilcon B and nelfilcon A ($P = 0.092$).

For the OcuFlow incubation method and after 4 h, the pattern was: delefilcon A > narafilcon A > somofilcon A > nesofilcon A > ocufilcon B > etafilcon A > nelfilcon A. All of the differences were significant ($P \leq 0.001$), except between somofilcon A and narafilcon A ($P = 0.342$) and etafilcon A ($P = 1.000$), narafilcon A and nesofilcon A ($P = 0.451$), etafilcon A and ocufilcon B ($P = 1.000$) and nelfilcon A ($P = 0.978$), and between ocufilcon B and nelfilcon A ($P = 0.855$). After 12 h of incubation, the pattern varied: nesofilcon A > somofilcon A > delefilcon A > etafilcon A > narafilcon A > ocufilcon B > nelfilcon A. All differences were statistically significant, except between nesofilcon A, somofilcon A ($P = 0.973$) and delefilcon A ($P = 0.475$), between delefilcon A, somofilcon ($P = 0.827$) and etafilcon A ($P = 0.064$), between narafilcon A and etafilcon A ($P = 1.000$), and between ocufilcon B, narafilcon A ($P = 1.000$), etafilcon A ($P = 0.957$), and nelfilcon A ($P = 0.328$).

Interestingly, the NBD-labeled cholesterol permeated through the entire thickness of the SiHy lens materials; the sole exception was in somofilcon A lenses, where the lipid only accumulated on the anterior and posterior margins (Figure 5-3A and Figure 5-3B). Unexpectedly, the CH lens material nesofilcon A revealed the same pattern of cholesterol penetration using both of the *in vitro* methods. Also, etafilcon A and ocufilcon A showed noteworthy penetration patterns after 4 h incubation with the vial method, where the NBD-cholesterol was found to deposit in the front section of those lens materials. These penetration patterns however subsided after 12 h and lipid penetrated through the entire lens material thickness (Figure 5-3B) for both incubation methods.

5.4.2 Impact of Incubation Method

Based on the RIF and subsequent to both incubation times (4h and 12h), the uptake of the lipid was elevated considerably after 12h compared to the 4h exposure time for most lens materials. Figure 5-3 and Figure 5-4 depict substantial differences of NBD-cholesterol between our OcuFlow model and the common vial incubation method, with a superior amount of uptake and penetration using the latter *in vitro* procedure ($P < 0.001$). In particular, the general pattern of NBD-cholesterol accumulation for the incubation methods was ‘vial 12h’ > ‘vial 4h’ > ‘OcuFlow 12h’ > ‘OcuFlow 4h’. All differences were statistically significant ($P \leq 0.007$), except between the incubation times of the vial method ($P = 0.109$).

Comparing the differences between the incubation methods within each CL material, the order of the general pattern changed slightly, nevertheless, the traditional vial method always showed greater amounts of accumulated NBD-cholesterol over the OcuFlow platform.

Interestingly, somofilcon A, delefilcon A, nesofilcon A, and ocufilcon B showed higher rates of accumulated NBD-cholesterol after 4h of vial incubation compared to 12h. This, however, was only statistically significant for delefilcon A and ocufilcon B CLs ($P \leq 0.001$). Most of the CLs accumulated the lowest amount of lipid after the 4h incubation using the *in vitro* platform, except for narafilcon A, that showed the least overall amount of NBD-cholesterol after 12h incubation with the OcuFlow, which however was not statistically significant ($P = 0.833$) compared to the 4h time-point.

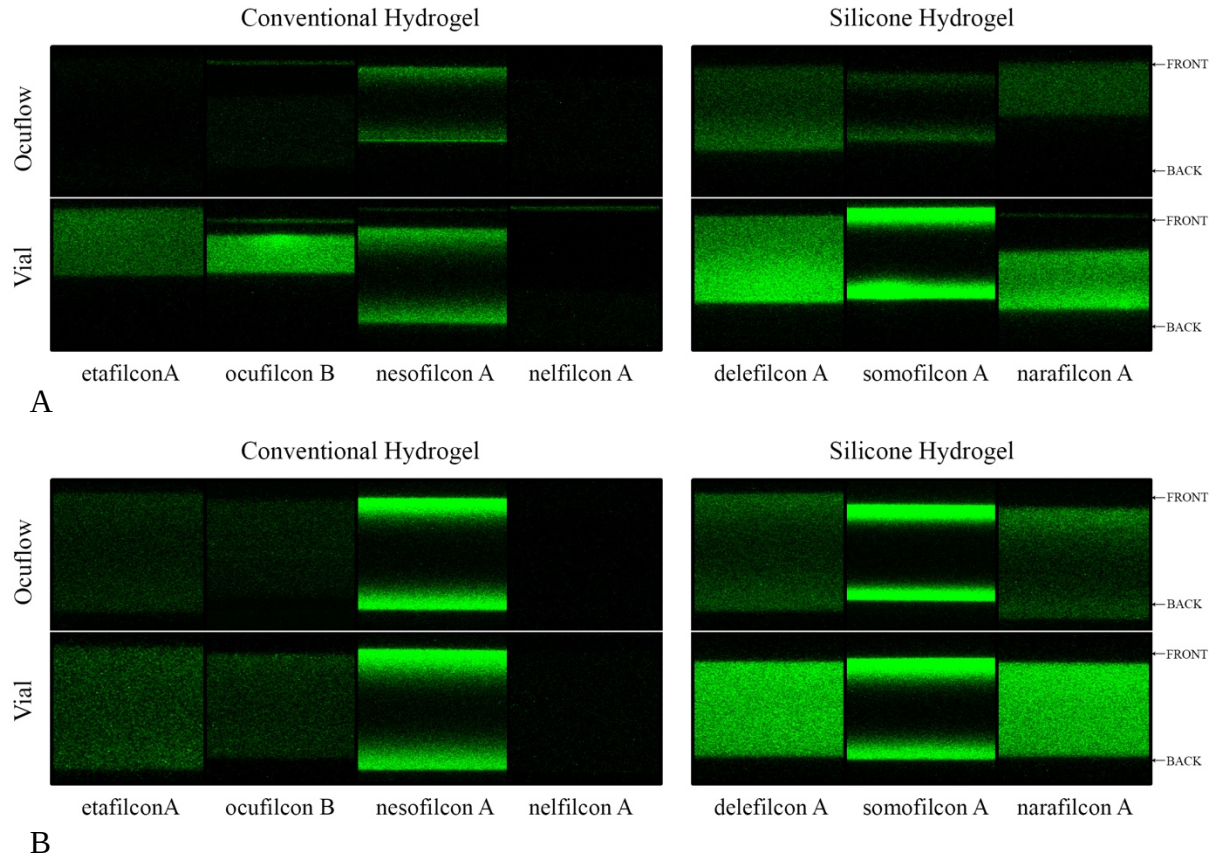


Figure 5-3. Confocal images showing a cross-section of etafilcon A, nelfilcon A, nesofilcon A, ocufilcon, delefilcon A, somofilcon A, narafilcon A after incubation with NBD-cholesterol in the vial and OcuFlow model after 4h (A) and 12h (B)

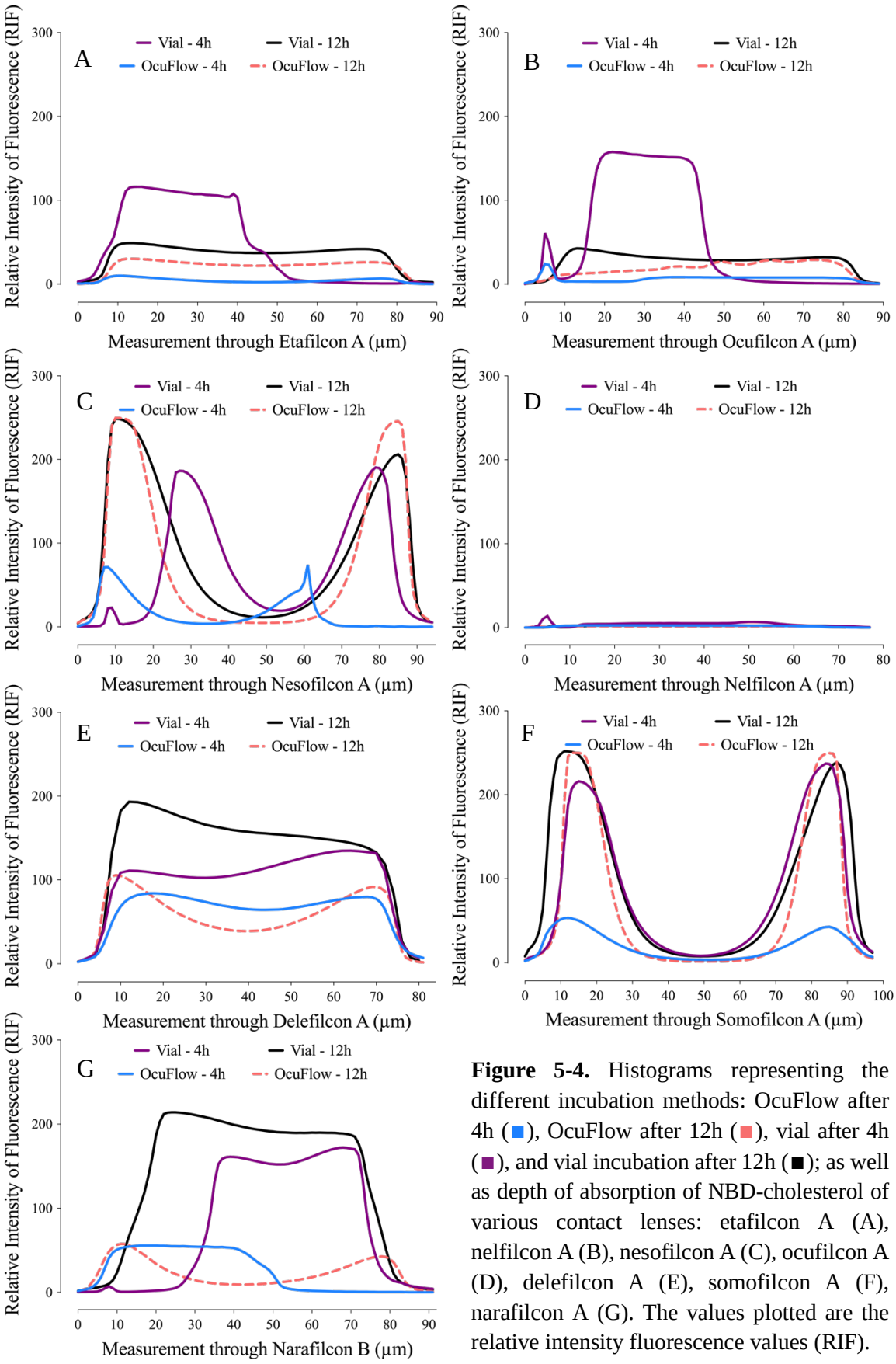


Figure 5-4. Histograms representing the different incubation methods: OcuFlow after 4h (■), OcuFlow after 12h (■), vial after 4h (■), and vial incubation after 12h (■); as well as depth of absorption of NBD-cholesterol of various contact lenses: etafilcon A (A), nelfilcon A (B), nesofilcon A (C), ocufilcon A (D), delefilcon A (E), somofilcon A (F), narafilcon A (G). The values plotted are the relative intensity fluorescence values (RIF).

5.5 Discussion

In recent years, researchers have recognized the various limitations [375-379, 461] of using a vial as an *in vitro* method for evaluating their interactions with tear-film components (Figure 5-5). Subsequently, to better simulate the ocular environment, several unique *in vitro* eye models have been developed such as the inclusion of a microfluidic tear replenishment component,[375-379, 461] intermittent air exposure,[266] and/or a mechanism of *in vivo* fouling of soft contact lenses.[270] Not surprisingly, the results generated from these experiments are very different than those obtained with the conventional vial model and might more closely resemble *in vivo* data.[266, 270, 375-379, 461] Our unique eye model incorporates multiple key elements of the ocular environment, including tear flow, tear volume, air exposure, and mechanical rubbing, to provide the best simulated environment possible for *in vitro* CL evaluation.[459]

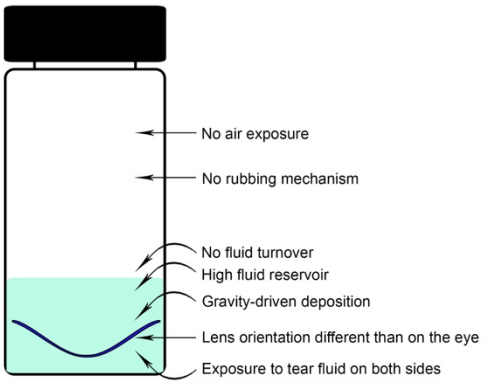


Figure 5-5. Drawbacks of using a simple vial model to evaluate contact lenses.

Being able to correctly visualize the localization of lipid deposits on CLs provides a better understanding on how the deposition of certain tear elements could progressively lead to discomfort. Previous research has been limited to quantifying lipid deposits on CLs, which provided useful data for comparing the relative performance of different CLs.[58, 61, 62, 257, 260, 266,

[325] However, to fully understand the mechanism leading to discomfort, it is also important to characterize how lipids penetrate the lens material over time.

Conventional methods of evaluating tear fluid (TF) deposition on CL have been performed in vials. As shown in Figure 5-5, there are several drawbacks when CLs are evaluated in this manner, which may significantly skew the results of lipid deposition on CLs. In particular, the high incubation volume and the incorrect horizontal orientation of the CL will facilitate lipid deposition on the CL, leading to an overestimation of lipid deposits. Not surprisingly, the penetration and fluorescence intensity of NBD-cholesterol for all materials were considerably higher in the vial than the eye model, especially after 4h of incubation time. Furthermore, we also observed uncharacteristic deposition patterns for two CH, etafilcon A and ocufilcon B in the vial, but not in the eye model. Typically, CH hydrogels are hydrophilic and therefore do not absorb a high quantity of lipids.[58, 61, 62, 257, 260, 266] However, as seen in Figure 5-3, more cholesterol penetration was seen in etafilcon A and ocufilcon B within their lens matrix when incubated using a vial at 4h. After 12h of incubation time, the uncharacteristic deposition pattern for these two CHs subsided and the fluorescence intensity of NBD-cholesterol decreased. We speculate that this effect could be due to the diffusion of the lipid through the lens materials over time. Most likely, there was no further accumulation of cholesterol within the 4h and 12h time-points but instead more of a “spreading” (i.e. balancing out) of the lipid within the CL materials. Therefore, the total amount of lipid deposited may be similar between the time-points; however, the fluorescence intensity appears vastly different because the NBD-cholesterol is distributed more uniformly after 12h than 4h. This inconsistency also highlights

the drawback of using a vial in which the CL is positioned horizontally. In this orientation, the deposition of TF on the CL is partially facilitated by gravity, rather than the material properties of the CL. In contrast, these artifacts were not observed in the OcuFlow, which can be attributed to the unique vertical orientation of the CL on the model.

For most part, there was still a strong agreement between the vial and the model in regards to the overall pattern of lipid penetration and deposition on the materials. For instance, both conditions showed that there was no lipid penetration in the matrix of the lens for nesofilcon A (CH) and somofilcon A (SiHy) at either the 4h or 12h time-point. With the exception of nelfilcon A, cholesterol deposition and penetration increased between 4h and 12h for all lens types in both the vial and the OcuFlow model. Similar and consistent patterns in lipid penetration was also observed for nelfilcon A, delefilcon A, and narafilcon A. The penetration and fluorescence intensity was higher in SiHy than CH CLs in both models. Therefore, results from the vial experiments could still be considered relevant for evaluating the relative performance of CLs in regards to lipid deposition.[325]

For commercially available CH and SiHy lenses, the effective pore sizes are approximately 150 nm.[205] The molecular size of cholesterol is estimated to be 1.6 nm across its length.[462] Since none of the DD CLs used in this study were surface coated, we expected that NBD-cholesterol would be able to penetrate throughout the lens material.[462] Interestingly, for some lens materials, such as nesofilcon A (CH) and somofilcon A (SiHy), cholesterol was localized mostly on the surface of the lens but not within the polymer matrix, even after 12h. The reasons for this lipid localization is unclear. We propose that it could be due to a structural arrangement

of the polymers within these lenses, which favors binding to NBD-cholesterol at the surface. Furthermore, this interaction is likely not solely due to hydrophobic interactions, but rather the result of side chains between the polymer and the lipid. Other lenses, such as delefilcon A and narafilcon A, also exhibited a similar deposition pattern after 12h for the OcuFlow system. Interestingly, nelfilcon A showed almost no sign of cholesterol penetration even after 12h.

An important property that cannot be simulated in a vial incubation is the formation of the pre- and post-lens TF, created when the CL sits on the cornea *in vivo*. While the pre-lens TF is continually replenished, there is very little tear exchange occurring behind the post-lens TF.[463-465] Consequently, we initially expected that there would be minimal lipid deposition occurring on the posterior side of the lens. To date, there are few studies that have investigated the differential deposition of TF on CLs, but this observation has been noted previously with the deposition of vitronectin.[466] However, even using the OcuFlow model, we were unable to observe a significant difference between the front and back surface deposition for NBD-cholesterol. This suggests that there is enough CL distortion and movement to allow for sufficient tear fluid to deposit on the back surface of the lens.[467] One of the problems with our current model is that the eyepiece is synthesized from polydimethylsiloxane (PDMS), a highly hydrophobic material. As such, the post-lens seal created between the eyepiece and the contact lens in our model may be less tight and, thus, may not yet achieve the perfect fit that an *in vivo* lens does on the eye. As a result, as the eyelid piece presses on the corneal piece, there is enough pressure to force fluid beneath the lens. We predict that using a more hydrophilic

material for the eye-piece will generate a different penetration profile for the back surface of the CL and this warrants further experimentation.

LSCM can provide useful insights on how tear components are absorbed within a lens material.[233, 357] However, one of the main drawbacks in using fluorescently-labelled probes, such as NBD-cholesterol, is the assumption that the labelled compound will behave similarly as its native counterpart. In the case of NBD-cholesterol, the fluorophore contains functional groups not found on the native lipid, which may interact differently with the lens materials. In addition, the fluorescently tagged lipid mass (494.63 g/mol) is significantly higher than the mass of the native cholesterol (386.65 g/mol). To our knowledge, there are no studies that compare the sorption of cholesterol and NBD-cholesterol on CLs. However, a study evaluating sorption of fluorescently-labeled proteins on hydrogels has shown that CLs adsorb labelled proteins much higher than native proteins, and the effect is significantly pronounced for silicone hydrogels.[468] Consequently, quantitative determinations of lipid deposition based on fluorescently-tagged lipids may be unreliable. However, for visualizing deposition patterns on CLs, we do not expect major significant differences between NBD-cholesterol and cholesterol, as both molecules are relatively small and hydrophobic.

In conclusion, the OcuFlow system presented in this study can be used as a model to evaluate lipid deposition on CLs. The platform mimics key ocular parameters and can replace conventional vial-based studies to provide better insights on the performance of CLs on the eye. The localization of lipid deposits and penetration on a CL, in tandem with the amount

deposited, may play a significant role in determining CL discomfort. The current system described here can also be extended to evaluate deposition of other tear components.

5.6 Acknowledgment

This study was funded by the Canadian Optometric Educational Trust Fund (COETF) and the NSERC 20/20 Network for the Development of Advanced Ophthalmic Materials.

In this next chapter, a novel *in vitro* method will be presented to determine tear break-up times over CLs and comparisons of NIBUTs over different commercially available DD lens materials using this method, will be made.

Chapter 6

Novel *In Vitro* Method to Determine *Pre-Lens* Tear Break-up Time of Hydrogel and Silicone Hydrogel Contact Lenses

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Author	Concept/Design	Data Acquisition	Analysis	Write Up/Publication
Walther	Y	Y	Y	Y
Subbaraman	Y			Y
Jones	Y			Y

6.1 Outline

6.1.1 Purpose

To develop a novel, *in vitro* model to determine pre-lens non-invasive break-up time (NIBUT) and to subsequently use this method to compare the NIBUT over five contemporary daily disposable (DD) contact lenses (CL).

6.1.2 Methods

Three silicone hydrogel (SiHy) and two conventional hydrogel (CH) DD lens materials were incubated in an artificial tear solution (ATS) containing a variety of proteins and lipids. A device designed to model the drying effect that occurs on lens surfaces during the inter-blink interval (a model blink cell; MBC) was utilized to mimic intermittent air exposure. CLs were incubated by repeatedly being submerged for 3 seconds (s) and exposed to air for 10 s over periods of 2, 6, 12, and 16 hours (h). NIBUTs over the CL materials (n=4) were determined out of the blister pack (T_0) and at the end of the incubation period. To measure NIBUTs, a corneal topographer (CA-100, Topcon Canada) was used to illuminate the lens surfaces and the reflected placido ring images on the lens surfaces were captured with a digital video camera (Canon-XA10).

6.1.3 Results

Overall, NIBUTs ranged from 26.19 ± 5.79 s to 1.23 ± 0.13 s, with nesofilcon A showing the longest NIBUT (P <0.001). At T_0, CH CLs revealed significantly longer NIBUT ($P \leq 0.001$) than SiHy CLs. After 2 h, nesofilcon A showed the longest NIBUT, however, this was only

statistically significant compared with delefilcon A ($P \leq 0.001$). After 6 h, nesofilcon A NIBUT was significantly longer than all other CLs ($P \leq 0.001$). Etafilcon A showed a significantly superior NIBUT ($P \leq 0.001$) over all other CLs after 12 h and delefilcon A had the longest break-up time ($P \leq 0.001$) after 16 h of incubation. Statistically significant ($P \leq 0.05$) changes of NIBUT within the lens materials varied between the examined time points. NIBUT decreased significantly ($P \leq 0.002$) between T_0 and 2 h for all CLs, except for somofilcon A ($P = 0.728$). After 16 h, all CLs showed significant reductions in NIBUTs ($P \leq 0.001$) in comparison to T_0.

6.1.4 Conclusion

NIBUT values reduced gradually over time and varying levels of deposition on different CLs impacted the measured pre-lens NIBUT of various lens materials. While NIBUT of CH materials are longer than that obtained with SiHy materials immediately out of the blister pack, it appears that after TF exposure, the NIBUTs obtained between CH and SiHy DD materials are very similar.

6.1.5 Keywords

tear film break-up time, lipid, contact lens, daily disposable, model blink cell, deposition, NIBUT

6.2 Introduction

Contact lenses (CL) are a very convenient and common device to correct vision and are available in a wide variety of materials.[186, 469] Most wearers use soft lenses, which are typically replaced after time periods between 1 day and 4 weeks.[396] Although CLs are effective at correcting vision, they remain plagued by issues associated with end of day dryness and discomfort,[175, 197, 198, 216] which may lead to cessation of CL wear or "contact lens dropout".[197, 198, 201, 470, 471]

One way to improve the performance of CLs (and potentially reduce dropouts) is to increase the replacement frequency of CLs.[275] The ultimate for this concept is to replace lenses every day, and daily disposable (DD) CLs were introduced in the mid-1990's [424, 425, 429, 430] and continue to increase in popularity.[396, 447, 472] Their use has been associated with a decrease in CL-related inflammation,[437] microbial keratitis,[434] improved overall comfort and visual acuity,[473] and reduced tear film (TF) deposits.[325, 429, 452, 473, 474]

The pre-corneal TF coats the ocular surface to prevent dehydration, nourish the cornea, and provide a smooth layer for clear vision.[98, 475, 476] To carry out all of these functions, the integrity and stability of the TF is important and if the stability of the TF over the lens surface is poor, then CL-related complications may arise.[79, 105, 215, 477] The TF is a complex structure composed of a wide variety of mucins, proteins, lipids, and salts that are organized in a certain order.[175] When CLs are placed onto the ocular surface they disrupt the TF by splitting it into a pre- and post-lens TF [175] and by accumulating TF proteins [237, 239] and lipids.[62, 63, 257, 268, 275] These

processes result in decreased TF stability compared with that seen without a lens in place.[98, 215, 477]

TF stability is commonly measured through the determination of the tear break-up time (TBUT), which measures the thinning/instability of the TF layer [98, 478] and can be tested invasively or non-invasively.[98, 385] To measure the stability invasively, a small amount of sodium fluorescein is added to the TF on the ocular surface to permit the visualization of the TF.[478] However, this method changes the physiological TF integrity and leads to a reduced TBUT compared with measures that do not disturb the TF.[479, 480] By projecting various grids or patterns onto the TF overlying the cornea, it is possible to measure TBUT non-invasively (NIBUT).[481, 482] Most methods to determine NIBUT utilize a topographer that projects uniform placido ring mires onto the patients' ocular surface and any changes in the structure of these mires depict a break-up of the TF.[98, 481, 482] Average BUTs in normal eyes are often >20 seconds (s), where <10 s is considered abnormal and ≤ 5 s is often associated with dry eye symptoms,[479, 483] whereas during CL wear BUTs generally lie between 3 to 10 s.[98, 175, 238, 453, 484-487]

To-date, very little data exists on the NIBUTs associated with DD lens materials. Given their increasing popularity this study sought to develop a novel *in vitro* model to determine *pre-lens* NIBUTs and to subsequently use this method to compare NIBUTs between various contemporary DD CLs.

6.3 Material & Methods

6.3.1 Contact lenses and pre-treatment

Three silicone hydrogel (SiHy) lenses [delefilcon A (Alcon), somofilcon A (CooperVision), narafilcon A (Johnson & Johnson)], and two commercially available conventional hydrogel (CH) DD CLs [etafilcon A (Johnson & Johnson), nesofilcon A (Bausch+Lomb)] were evaluated in this study. All lenses had a dioptric power of -3.00 and base curve of 8.5 or 8.6 mm and were obtained from the manufacturer in the original commercial packaging. Table 6-1 details the properties of the CLs examined.

Table 6-1. Properties of the contact lenses used in the study.

	DAILIES TOTAL1®	**clariti™ 1day**	**1-DAY ACUVUE® TruEye®**	**1-DAY ACUVUE MOIST**	**Biotrue 1Day**
United States adopted name (USAN)	delefilcon A	somofilcon A	narafilcon A	etafilcon A	nesolfilcon A
Manufacturer	Alcon	CooperVision	Johnson & Johnson	Johnson & Johnson	Bausch + Lomb
Water content (%)	33% (surface >80%)	56%	46%	58%	78%
FDA group	V	V	V	IV	II
Centre thickness (mm)	0.09	0.07	0.09	0.08	0.05
Oxygen permeability ($x10^{-11}$)	140	60	100	28	42
Oxygen transmissibility ($x10^{-9}$)	156	86	118	25.5	42
Principal monomers	Not disclosed	Not disclosed	MPMDSM, DMA, HEMA, siloxane macromer, TEGDMA, PVP	HEMA, MA	HEMA, NVP

USAN: United States Adopted Names FDA: Food and Drug Administration HEMA, hydroxyethyl methacrylate; MA, methacrylic acid; PVP, polyvinyl pyrrolidone; NVP, N-vinylpyrrolidone; DMA, N,N-dimethylacrylamide; MPDMS, monofuncional polydimethylsiloxane; TEGDMA, tetraethyleneglycol dimethacrylate;

6.3.2 Artificial Tear Solution

The composition of the artificial tear solution (ATS) used has been previously reported.[61] In short, the solution contains various lipids (oleic acid methyl ester, cholesterol, triolein, phosphatidylcholine, cholesteryl oleate, and oleic acid), various salts, urea, glucose, proteins (lysozyme and hen egg albumin), and mucin, the concentrations of which were based on those in normal human tears.[61]

6.3.3 Model Blink Cell

A model blink cell (MBC) was utilized to incubate the CLs in the ATS. The MBC used in this study is an updated version to that which has been previously described by our group,[266] which enables us to incubate up to 48 CLs at a time. Specifically, it is composed of a polytetrafluoroethylene (PTFE/Teflon™) trough that is divided into 4 chambers that can each be filled with 250 mL of the ATS test solution. Each CL is placed on a Teflon button (Figure 6-1A) that is positioned on a Teflon plate, which is attached to a motor that raises and lowers the plate in and out of the test solution. In order to further secure the CLs and prevent them from floating off the Teflon buttons, each lens is held in place with a clip. A gap of 100 µm between the button and clip-on allows the CLs to float freely and prevents the mounted CLs from getting damaged. Furthermore, the mechanics are set up within an environmental chamber (Figure 6-1B) that enables the regulation of humidity and temperature during lens incubation.

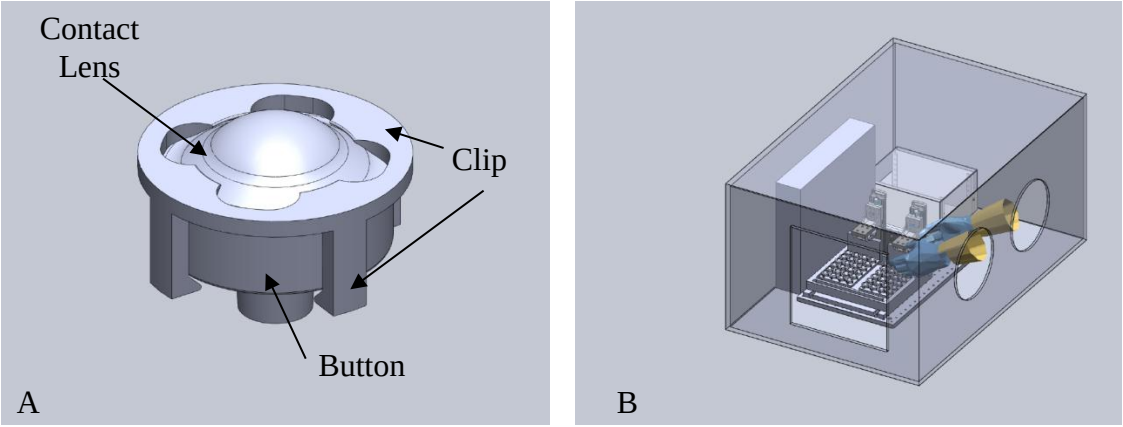

Figure 6-1. Teflon button for holding lenses (A) and Model Blink cell controlled environmental chamber (B).

6.3.4 Topographer

To measure the NIBUTs, we utilized a corneal topographer (CA-100, Topcon Canada) to illuminate the upper CL surfaces and project a uniform Placido ring structure onto them (Figure 6-2 & Figure 6-3). An additional video camera (Canon-XA10; Figure 6-2) was used to capture the changes of the Placido ring appearance by recording the LCD on the topographer.

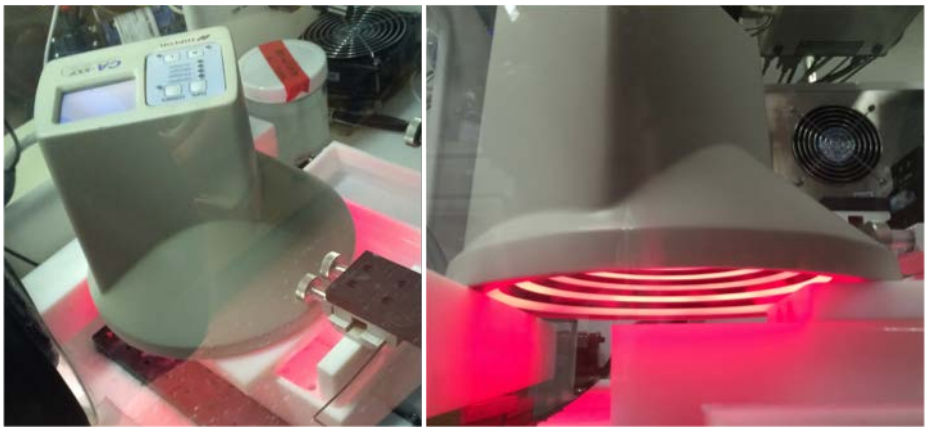

Figure 6-2. Experimental set up of the topographer over the MBC.

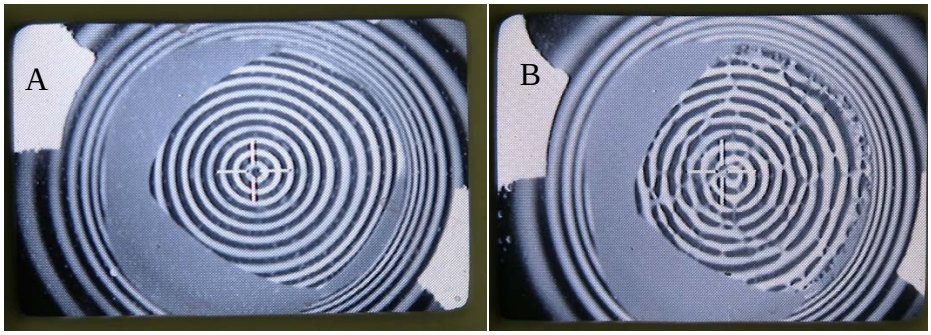

Figure 6-3. Placido ring image over a "fresh" contact lens (A) and a dried-up lens surface (B). The smooth rings in A compared with the irregular rings in B indicate the difference between a confluent and a broken tear layer.

6.3.5 Experimental Outline

At the start of the experiment, four lenses of each type were taken out of the blister pack, placed onto a Teflon button in the MBC, and the initial (T_0) NIBUT was measured. Thereafter, the CLs were submerged in the ATS for 3 s and exposed to air for 10 s, to mimic intermittent air exposure. The environment in the MBC chamber was set at a humidity of 50±5% and a temperature of 34±4°C. After 2, 6, 12, and 16 h of incubation each lens was raised into focus of the topographer and the BUTs were determined. After each measurement was taken, the lenses continued to cycle in and out of the ATS until the 16 h time-point.

6.3.6 Data Analysis

NIBUTs were determined by analysing the recorded videos manually by comparing the video sequences frame by frame until a significant change in the structure of the mires occurred. To achieve this, we focused on the innermost 6 to 8 concentric rings reflected from a CL, compared the initial Placido ring structure, once it came out of solution, and measured the time until the first distortion of the Placido mires occurred (n=4; Figure 6-4). The observer

who recorded the first disruption in the mires was masked as to the lens material and time of ATS exposure during this process.

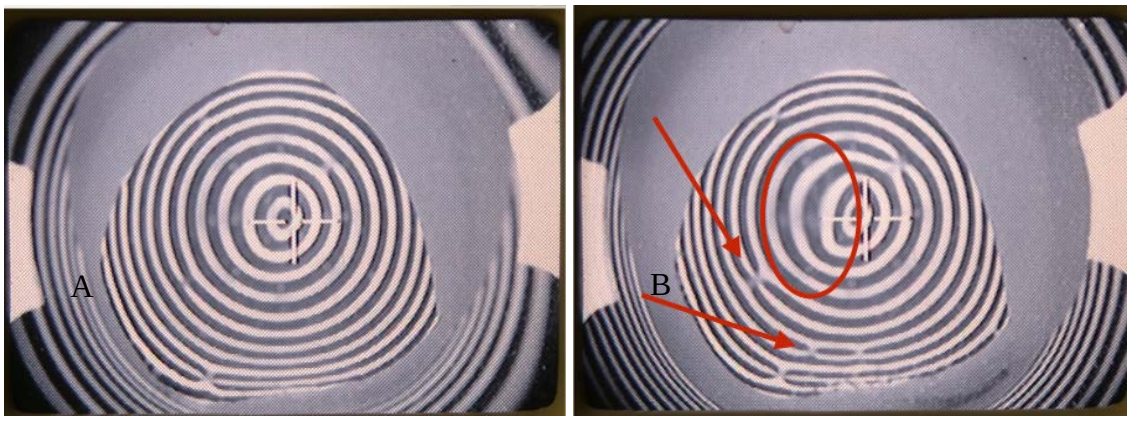

Figure 6-4. *In vitro* placido ring images retrieved from the experiment; T_0 (A) and first break-up (13 sec) over lens material (B)

After assessing the NIBUTs of every lens material, data analysis was conducted using repeated measures-analysis of variance (RM-ANOVA) and univariate analysis to test for any significance within each time-point and lens material, using SPSS Statistics 23. An alpha level of $P < 0.05$ was considered significant. Individual differences were analyzed using a Tukey post-hoc analysis.

6.4 Results

The results of the study are reported in Table 6-2, Table 6-3, and Figure 6-5. Table 6-2 illustrates that the NIBUT measurements depended significantly on the duration of incubation, the type of CL material, as well as the interaction between these factors ($P < 0.001$). When a general comparison was made between all tested CL types (all time-points pooled), CH lens materials showed significantly greater NIBUTs than SiHy lenses ($P \leq 0.001$), but no significant difference between the two CH CLs themselves ($P = 0.276$) or between the SiHy lens materials

($P \geq 0.912$). Nesofilcon A had the longest average NIBUT of all tested CLs, with this difference being significant ($P \leq 0.001$) over the SiHy materials only.

Table 6-2. Repeated-measures analysis of variance statistical results for NIBUTs over various DD contact lens materials

Repeated-measures analysis of variance statistical results for NIBUTs across time					
Variable	**Sum of squares**	**Degrees of freedom**	**Mean square**	***F***	***P***
Duration of incubation	2608.09	4	651.02	343.94	<0.001
CL material	351.11	4	87.78	34.44	<0.001
Incubation time * CL material	665.99	16	41.62	21.99	<0.001
Error	113.57	60	1.89		

Table 6-3. NIBUT over contact lenses in seconds (mean ± SD)

USAN	Delefilcon A	Somofilcon A	Narafilcon B	Etafilcon A	Nesofilcon A
T_0	11.44 ± 1.76	11.81 ± 0.61	10.79 ± 0.64	26.20 ± 5.79	22.00 ± 1.32
2 h	8.66 ± 0.22	11.30 ± 1.08	12.85 ± 1.03	13.81 ± 0.60	13.48 ± 2.00
6 h	7.86 ± 0.18	8.54 ± 0.52	7.33 ± 0.18	13.29 ± 1.78	8.60 ± 0.48
12 h	4.76 ± 0.20	3.64 ± 0.26	3.74 ± 0.15	3.03 ± 0.67	6.13 ± 0.36
16 h	3.60 ± 0.26	2.77 ± 0.31	1.23 ± 0.13	1.84 ± 0.37	2.70 ± 0.31

USAN: United States Adopted Names

As shown in Table 6-3, at T_0, the two CH lenses revealed significantly longer NIBUTs ($P \leq 0.001$) than all three SiHy materials, but no significant difference was found between the two CH lens materials ($P = 0.262$) or the SiHys ($P \geq 0.984$). After 2 h of lens incubation, delefilcon A showed the shortest NIBUT ($P \leq 0.037$) in comparison to all other CLs and etafilcon A presented the longest NIBUT; however, this was only statistically significant compared with delefilcon A ($P \leq 0.001$). After 6 h, the NIBUT of etafilcon A was significantly longer than all other examined CLs ($P \leq 0.001$). After 12 h of incubation, the CH nesofilcon A showed a

statistically superior NIBUT over all other tested CLs ($P \leq 0.001$). With a NIBUT of 3.6 ± 0.26 s, delefilcon A had the longest BUT after 16 h of incubation and this difference was significant ($P \leq 0.001$).

Statistically significant ($P \leq 0.05$) changes of NIBUT within the lens materials varied between time-points. Overall, NIBUT decreased significantly ($P \leq 0.002$) between T_0 and 2 h for all CLs, except for somofilcon A ($P = 0.728$) - which showed no relevant change - and narafilcon B, which marginally increased. NIBUT between T_0 and 6, 12, and 16 h was significantly lower for all CLs ($P \leq 0.001$). For delefilcon A, the reduction in NIBUTs was statistically significant ($P \leq 0.001$) among most of the time-points, except between 2h-6h and 12h-16h ($P = 0.638$ and $P = 0.299$, respectively). For somofilcon A lenses, we found statistically significant difference in NIBUT decrease amongst all time-points ($P \leq 0.002$), except between T_0-2h and 12h-16h ($P = 0.782$ and $P = 0.328$, respectively). As mentioned above, narafilcon B revealed an increase of NIBUT after 2h of incubation, whereas, all other time-points showed a continuous decrease in NIBUT. All differences between the time-points within narafilcon B were statistically significant ($P \leq 0.001$). For etafilcon A, statistically significant differences ($P \leq 0.045$) in NIBUT were found between all time-points. Nesofilcon A showed significant reduction differences ($P \leq 0.001$) amongst some time-points, except between 2h-6h and 12h-16h.

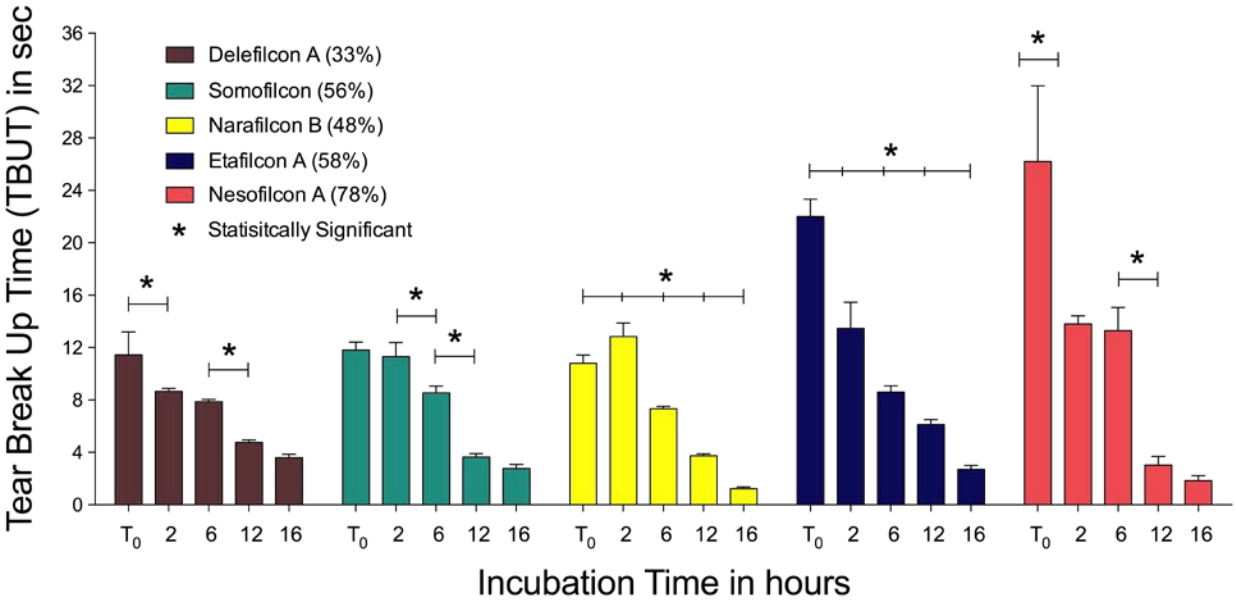

Figure 6-5. Histogram representing the NIBUTs for the five daily disposable materials for up to 16 hours after incubation in a model blink cell. Statistically significant differences (*) are indicated for $P \leq 0.05$.

6.5 Discussion

The purpose of this study was to develop an *in vitro* model which could be used to determine pre-lens NIBUT values and to subsequently use this concept to compare the NIBUT of contemporary CH and SiHy DD materials over a period of 16 hours. The *in vitro* model used a methodology in which lenses were incubated in an ATS that mimicked the composition of the TF and during their incubation were intermittently exposed to the air, in an attempt to better mimic *in vivo* CL wear. In general, we found that the NIBUTs for CH were longer than those for SiHy materials, particularly when immediately removed from the blister pack, and that the NIBUTs for all materials reduced gradually over time.

Close inspection of Figure 6-5 shows graphically how the NIBUTs reduced with increasing exposure to the ATS over time. Of interest, is that the NIBUT reduced for all materials, such that after 16 h the differences in NIBUT were relatively small, as compared with the T_0 times, in which the CH materials had clearly longer NIBUT. It would appear that over time, exposure to the ATS and the MBC doping procedure was a great "leveler", with this process reducing any major differences in NIBUT between all 5 materials.

The most likely reason for this reduction in NIBUT relates to deposition of the lenses with components of the ATS. Previous work has shown that, on average, CH materials tend to preferentially deposit proteins (particularly group IV materials such as etafilcon A, which has a strong attraction for lysozyme)[160, 225, 229, 235, 239, 332, 390, 488] and SiHy materials tend to preferentially deposit lipids.[58, 62, 160, 187, 257, 260, 268] This deposition holds true even for those materials replaced on a DD basis such as those examined in this study.[229, 254, 260, 325, 332, 489] It is likely that the progressive accumulation of deposition over the course of the day, regardless of its type, resulted in a gradual reduction in surface wettability, measured by a reduction in NIBUT. To-date, there are no studies that have attempted to directly link levels of increasing deposition with reducing NIBUT, and thus our suggestion that increasing deposition led to the reduction in NIBUT remains a hypothesis only. However, several studies have shown that deposition is cumulative,[63, 229, 234, 235, 237, 254, 325, 332, 388, 489-496] that pre-lens NIBUT is often lower after a period of wear [255, 486] (although not always)[497] and that comfort reduces both over the day and across the replacement period.[275]

It is of interest to compare these *in vitro* results with previously published *in vivo* values for T_0 NIBUTs. The results of this study for the SiHy materials are nearly two times longer than previously published results, with Varikooty *et al* [453] and Kojima *et al* [487] reporting in eye pre-lens NIBUT values of 5 to 8 s. For the CH CLs, the same relative difference is observed, with T_0 NIBUTs in this study being over 20 s, whereas *in vivo* data reported for CH materials are typically half this or lower.[406] These differences may exist for a variety of reasons. One difference between our *in vitro* setup and the in-eye situation relates to the large amount of ATS the lenses were exposed to (250mL), compared to the physiological levels of tears that are available at any given time (3mL/24h).[457, 458, 498] This may impact the data in two ways. Firstly, the volume of tears sorbed to the lenses in the *in vitro* test may result in reduced dehydration during the time that the lenses are exposed to the air, as excess fluid may accumulate on the lens surface. Previous work by our group has shown that deposition is influenced by the degree to which lenses dehydrate.[266] Thus, different levels of deposition between the lenses in this *in vitro* analysis may occur compared with that seen in eye, which could impact the subsequent NIBUT determined. Secondly, the excess amount of ATS would provide greater amounts of protein and lipid to be available to deposit on the lens materials than that seen in eye, again potentially impacting the NIBUT recorded. Previous work by us has shown that greater amounts of available lipid results in more lipid deposition[63] and that the amounts of proteins and lipids (and their relative concentrations) can impact lens deposition, particularly on SiHy materials.[62, 331] The deposition of lipid or denatured protein on lens materials may increase the hydrophobic nature of the lens surfaces, reducing the NIBUT.

Another aspect to consider that may help to explain these differences relate to the mounting of the lenses during the deposition process. The CLs in this experiment are mounted horizontally within the MBC rather than vertically, as they are when worn. This exerts a different force on the tear spreading over the lens surface, which may affect the observed NIBUTs and drying patterns. In addition, the MBC does not take the mechanical contribution of the blink of the eyelids into account. The eyelids, and the lid margins in particular, are thought to play a major role in the spread of the TF.[499, 500] The lids help to redistribute and re-establish the formation of the TF layers as the lids open. The meibum lipids that are expressed and secreted by the lids as we blink play a crucial role in stabilizing the TF and preventing TF evaporation, which affects the measured BUTs.[54, 70, 98, 477, 498] In our MBC model, the lipids are fully incorporated and solubilized in the ATS, creating a homogeneous mixture, and will therefore not form an outermost layer that could retard the dehydration of the TF, which may lead to faster NIBUTs than *in vivo* data shows. [238, 484, 485]

To our knowledge, this is the only study to present kinetic data over a day that investigates NIBUT over CH and SiHy materials so we are unable to determine how these values compare with previous data using other methods. One previous study examined pre-lens NIBUT over two CH materials after 5 hours [486] and found that the NIBUT only reduced for wearers who were symptomatic, with asymptomatic wearers showing no such reduction in wettability. Varikooty *et al* [453] tested tear breakup after 8h of SiHy CL wear and recorded times between 5.8 s (delefilcon A) and 4 s (narafilcon B) over the CLs. Our NIBUT measurements after 6-

12h are in line with these results, suggesting that our *in vitro* method for these time periods are comparable to *in vivo* studies.

One limitation of our methodology is that we determined the pre-lens NIBUTs manually, which could result in subjective bias when determining the first break in the projected placido pattern occurred. However, the observers were masked and thus any observations would be biased equally across all lenses equally. Ideally, all videos would be examined using automated software that would recognize such a distortion and this process is being undertaken.[501]

In conclusion, we have developed a system to measure pre-lens NIBUTs over CLs *in vitro* that exposes the lens materials to an ATS that mimics the composition of the TF and incorporates the lens surface drying that occurs during the inter-blink period. Using this system, we were able to obtain *in vitro* data that, after 6 hours of TF exposure, is comparable to *in* vivo data. While NIBUT of CH materials are longer than that obtained with SiHy materials immediately out of the blister pack, it appears that after TF exposure the NIBUTs obtained between CH and SiHy DD materials are very similar. Further work is warranted in which the pre-lens NIBUT is determined over the course of the day to determine if a progressive reduction does occur and, if so, whether such a difference is mitigated by the materials being worn.

In this final chapter, different commercially available assays for oxidative stress will be optimized and their capability to measure lipid oxidation by-products in tear samples will be assessed.

Chapter 7

Development of a technique to quantify oxidative stress in tear film lipids

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Author	Concept/Design	Data Acquisition	Analysis	Write Up /Publication
Walther	Y	Y	Y	Y
Subbaraman	Y			Y
Jones	Y			Y

7.1 Outline

7.1.1 Purpose

Tear film (TF) lipids create a protective layer to prevent tear evaporation. Oxidation degrades lipids and can lead to tissue damage, which can cause alterations in the tear lipid layer resulting in symptoms of dry eye (DE). The purpose of this project was to optimize and develop a method to determine and quantify lipid peroxidation by-products that will indicate oxidative stress in tears, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and oxidized low-density lipoproteins (OxLDL).

7.1.2 Methods

Tear samples were collected from 10 volunteers, using calibrated disposable capillary tubes (Drummond Scientific Company). A volume of 5 µL/eye was collected and transferred into a 0.1 mL sterilized plastic vial, capped, spun down with a centrifuge, and lyophilized to dryness for long-term storage (-80°C) until further analysis. Various assays that quantify lipid peroxidation by-products were compared against each other: thiobarbituric acid reactive substances (TBARS) assay, MDA-, 4-HNE-, and OxLDL-ELISA (enzyme-linked immunosorbent) assays. Pooled and individual tear samples were diluted in a wide range (10 µL to 0.5 µL and 2 µL to 0.5 µL, respectively) to determine the lowest volume of tears that could be used with the assays. Subsequently, the samples' fluorescence was measured with a fluorescence spectrophotometer at 530 nm (excitation) and 590 nm (emission), and absorbance at 450 nm.

7.1.3 Results

The concordance correlation coefficient of all assays showed high linearity ($R^2 \geq 0.989$) and thus were judged as reliable techniques to measure oxidized lipids. For TBARS, the detected MDA of individual tear samples were below the limit of detection (LOD) of the assay. However, after tear samples were pooled and concentrated, 0.056±0.004 µM MDA could be measured. For the OxLDL ELISA, in pooled and diluted tears between 71.39 ± 3.78 ng/mL to 28.81 ± 2.29 ng/mL were detected. When testing with individual tear samples (2.5µL), OxLDL ranged between 45.59 ± 2.95 ng/mL and 28.24 ± 4.66 ng/mL. All measurements using the MDA- and 4-HNE-Adduct ELISA assay were below the LOD.

7.1.4 Conclusion

Assays for oxidative stress were optimized for the use of tear samples and showed that oxidative stress is detectible in small quantities of tears (2.5 µL). Thus, these techniques could be employed to determine oxidative stress in TF lipids, which could potentially help to identify patients with dry eye and contact lens discomfort.

7.1.5 Keywords

tear film, lipid, contact lens, oxidative stress, oxidation

7.2 Introduction

The pre-corneal tear film (TF) is a protective and functional layer that serves to shield the cornea from the air and also provides some nutritional benefits.[75] It is composed of a wide array of elements but can be simplified into a mucin, an aqueous, and a lipid layer.[2, 68] The interactions between the outermost lipid layer and the underlying aqueous phase are of especially high value to prevent TF evaporation and dry eyes (DE).[442] The lipid layer provides a protective film to avert TF evaporation [442] and consists of two main lipid groups and layers: an outermost layer that mainly consists of non-polar lipids that help stabilize the TF layer, and polar lipids that link the hydrophobic non-polar lipid phase with the hydrophilic aqueous phase.[48, 54, 502] The innermost polar phase consists of amphiphilic lipids (phospholipids, sphingomyelin, ceramides, and long chain (O-acyl)-ω-hydroxy fatty acid (OAHFA)) [2, 33, 37, 42, 68, 503, 504] that contain both hydrophilic and hydrophobic functional groups, allowing them to interact with water and lipids at the same time and, thus, function as mediators.[48, 54, 502]

Lipids are thus major TF components that stabilize the tear film, protect the cornea, and prevent the evaporation of the aqueous layer.[75] Among normal individuals, the lipid composition is fairly similar but the quality of the lipids change in people with DE symptoms and/or meibomian gland dysfunction (MGD).[45, 349, 356] Structurally, most lipids are typically composed of long carbon chains (or cyclic groups in the case of sterols), which are defined by their degree of saturation. Unsaturated lipids contain one or more double carbon bonds, with each additional double bond increasing the degree of unsaturation of the molecule.[56] The amphiphilic lipids of the polar phase may contain unsaturated fatty acids that are affected by

oxidation or phospholipids may undergo enzymatic lipolysis (through secretory phospholipase A_2), which degrades lipids, and might cause deterioration of the native lipid structure, loss of functionality, and, may subsequently cause tissue or cell damage due to the formation of toxic species.[56, 97, 125, 126, 128, 129, 132-134] Some of the external stress factors that cause lipid oxidation are wind, extreme temperatures, UV radiation, pollutants, irritants, and smoke.[108, 132, 135-137] Lipid peroxidation is known to be influential in the development of Alzheimer's disease, Parkinson's,[138, 139] and cancer,[135] and in the eye specifically, lipid oxidation is associated with age-related macular degeneration,[140, 141] DE,[137, 142, 143] uveitis,[144] cataract,[145-148] and keratitis.[135, 149]

To determine oxidative stress in blood and serum samples of humans, studies have shown that a thiobarbituric acid reactive substances (TBARS) assay or MDA/4-HNE ELISA kits are efficient procedures to obtain the amount of oxidative stress.[128, 133, 142, 152, 153, 361, 362] These colorimetric methods measure the amount of the by-products malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and oxidized low-density lipoproteins (Ox-LDL) that occur during the lipid peroxidation process.[128, 133, 142, 150, 152, 153, 161, 361-364] Because these methods are primarily used to measure the oxidative stress in blood or serum, which are available in higher volumes than that possible in the collection of tears, the commercially available kits need to be optimized in order to examine human tears. To our knowledge, to-date only a few studies have investigated the amount of lipid peroxidation by-products in tears of DE patients and contact lens (CL) wearers. [106, 125, 142, 156, 159, 162, 163, 365] These studies found increased quantities of oxidative stress inhibitors in intolerant CL wearers,[125] CLs,[156, 365] the elderly,[106] and DE

patients.[142, 159, 162] Because a high rate of oxidative stress in human cells has been shown to be associated with diseases that are caused by cell death due to lipid oxidation,[133] oxidative stress in the TF might be a possible cause for DE.

To be able to assess lipid oxidation in tears, the objective of this project was to establish a sensitive method to detect natural by-products of lipid peroxidation and determine the amount of oxidative stress in the TF. The results from this project might provide previously unavailable information about the pathophysiology of DE by providing an insight into the cause of DE disease.

7.3 Materials and Methods

7.3.1 Tear Samples & Collection

Tear samples were collected from volunteer participants using calibrated disposable capillary tubes (Drummond Scientific Company, Broomall). A maximum amount of 5 µL/eye was collected and transferred into a 0.1 mL sterilized plastic vial, capped, spun down with a centrifuge, and lyophilized to dryness for long-term storage (-80°C) until further analysis.

7.3.2 Test Assays

Various colorimetric methods were used to detect and quantify lipid peroxidation in human tears.

7.3.2.1 Thiobarbituric Acid Reactive Substances Assay

The Thiobarbituric Acid Reactive Substances (TBARS; Cayman Chemicals Company, Ann Arbot, MI)[505] kit is a standardized assay to measure lipid peroxidation in cells by measuring the amount of MDA present in a sample (Figure 7-1). After TF collections, samples were diluted (ranging from 6 µL to 0.75 µL) to determine the lowest volume of tears that could be used with the TBARS assay. In addition, to assess the sensitivity of the assay kit, 200 µL of tears and artificial tear solution (ATS)[61] were pooled, dried using a centrifugal evaporator, and re-suspended with 15 µL of de-ionized water to prepare a 13x concentrated "tear pool". Subsequently, samples were measured fluorometrically using a SpectraMax M5 UV-Vis Spectrophotometer (Molecular Devices, Sunnyvale, CA) at an excitation of 530nm and an emission of 590nm.

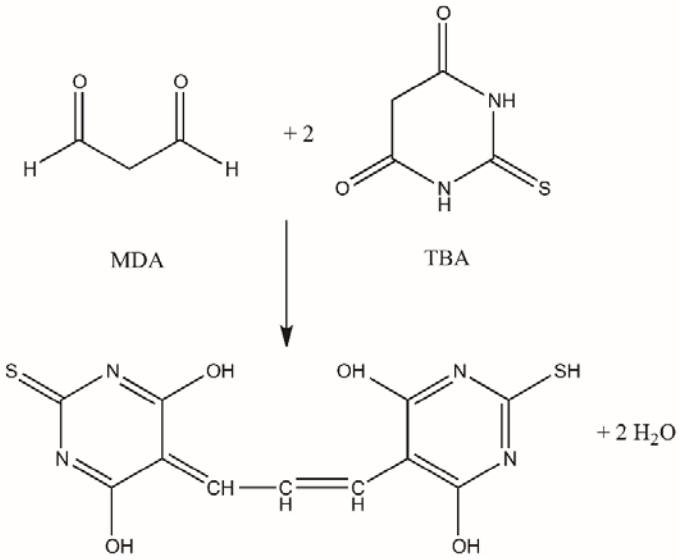

Figure 7-1. MDA-TBA bond principle in the TBARS assay.

7.3.2.2 ELISA Kits

TF samples were also assessed using various enzyme-linked immunosorbent assays (ELISA) that detect the oxidization by-products MDA (Cell Biolabs Inc., San Diego, CA)[506] and 4-HNE (Cell Biolabs Inc., San Diego, CA)[507] bound to TF proteins and low-density lipoproteins (OxLDL; Cell Biolabs Inc., San Diego, CA).[508]

7.3.2.3 OxLDL ELISA

This ELISA assay[508] detects OxLDL, which is a carrier of cholesterol and other fatty acids such as phospholipids that are essential for TF stability. To determine the amount of oxidative stress, individual tears and pooled tear samples were diluted in a wide range (2 µL to 0.5 µL and 10 µL to 0.5 µL, respectively) to determine the lowest volume of tears that could be used with this ELISA assay. Furthermore, a spike-and-recovery (SAR) test was performed to determine the accuracy of the ELISA when using it with tear samples. Therefore, tear and blood plasma samples were spiked with known amounts of MDA (7.81 ng/mL, 31.25 ng/mL, and 125 ng/mL) and compared to an un-spiked sample to determine the rate of recovery (RR%). A RR between 80-120% is considered an acceptable range and everything outside this range means that something in the analyte interferes with the assay.[509]

7.3.2.4 MDA- and 4-HNE-Adduct Elisa

In preparation for the MDA- and 4-HNE-Adduct ELISA assays,[506, 507] two different collection methods were examined: regular tear collection using capillary tubes and a flush method.[329, 330, 510] To perform the flush method, 50 µL of 0.9% sodium chloride was instilled

onto the superior conjunctiva. Thereafter, participants were asked to roll their eyes to ensure a complete wash of the anterior surface before the diluted (~10x) tears were collected with calibrated glass capillary tubes within 1 minute. This tear collection method was applied to rinse out all possible oxidation markers from the surface of the eye. For comparison, the tear samples from the traditional collection method were processed without further dilution. After sample preparations were completed, the absorbance was measured using the SpectraMax M5 UV-Vis Spectrophotometer at 450 nm for all ELISA assay kits.

Furthermore, we used surplus samples of tears from symptomatic and asymptomatic lens wearers from a previously conducted study in the CCLR to determine the amount of lipid peroxidation by-products. The tear samples were pooled to reach a quantity of 50 µL each, diluted, prepared, and tested according to the assay protocols.

In a further attempt to test if the MDA and 4-HNE-ELISA kits could be used to determine lipid oxidation, four volunteers were asked to wear four different CLs: lotrafilcon B (Air Optix Aqua, Alcon Inc.); balafilcon A (PureVision2, Bausch+Lomb); narafilcon B (TrueEye 1day, Johnson & Johnson); and etafilcon A (Acuvue2, Johnson & Johnson)] for 4 hours a day. Thereafter, a 6 mm disc was punched out of the lens material, placed into a well of both ELISA test plates, and assays were prepared and performed according to the test protocols.

7.3.3 Analysis

Calibration curves were generated for each assay to determine the coefficient of determination (COD), limit of detection (LOD), and quantification (LOQ).[511] The linearity equations of the calibration curves were used to interpolate the amount of detected oxidation

by-product. The LOD and LOQ were calculated based on the slope and the standard error of the predicted y-value for each x in the regression of the calibration curves and determined on a 3:1 and 10:1 ratio, respectively. All values are tabulated in Table 7-1.

Table 7-1. Linearity equations of calibration curves, LOD, LOQ, and coefficient of determination values of tested lipid oxidation assays.

Oxidation Assay	Biomarker	COD Equations	LOD	LOQ	R^2
TBARS	MDA	y = 3.617x – 0.2605	0.31µM	1.04 µM	0.989
ELISA	OxLDL	y = 0.011x – 0.0306	36.49 ng/mL	121.63 ng/mL	0.994
	MDA	y = 0.064x + 0.0075	6.76pmol/mg	22.53pmol/mg	0.991
	4-HNE	y = 0.765x + 0.0029	0.51µg/mL	1.69µg/mL	0.992

7.4 Results and Discussion

7.4.1 Thiobarbituric Acid Reactive Substances Assay

Figure 7-2 and Table 7-1 show the generated calibration curve and the linearity equation of the TBARS assay. The amount of detected MDA for tear dilution were similar, below the assays LOD, and depicted graphically in Figure 7-2: 0.039 ± 0.187 µM in 6 µL, 0.162 ± 0.063 µM in 3 µL, 0.113 ± 0.034 µM in 1.5 µL, and 0.089 ± 0.077 µM in 0.75 µL. When tears were pooled and concentrated (13x; Figure 7-3), the amount of MDA found in tears was 0.721 ± 0.058 µM, which would equate to 0.056 ± 0.004 µM in a normal concentration (1x) in tears. In the 13x concentrated ATS, we found 0.922 ± 0.047 µM of MDA, which suggests that the artificial solution could function as a control when testing lipid peroxidation using the TBARS assay.

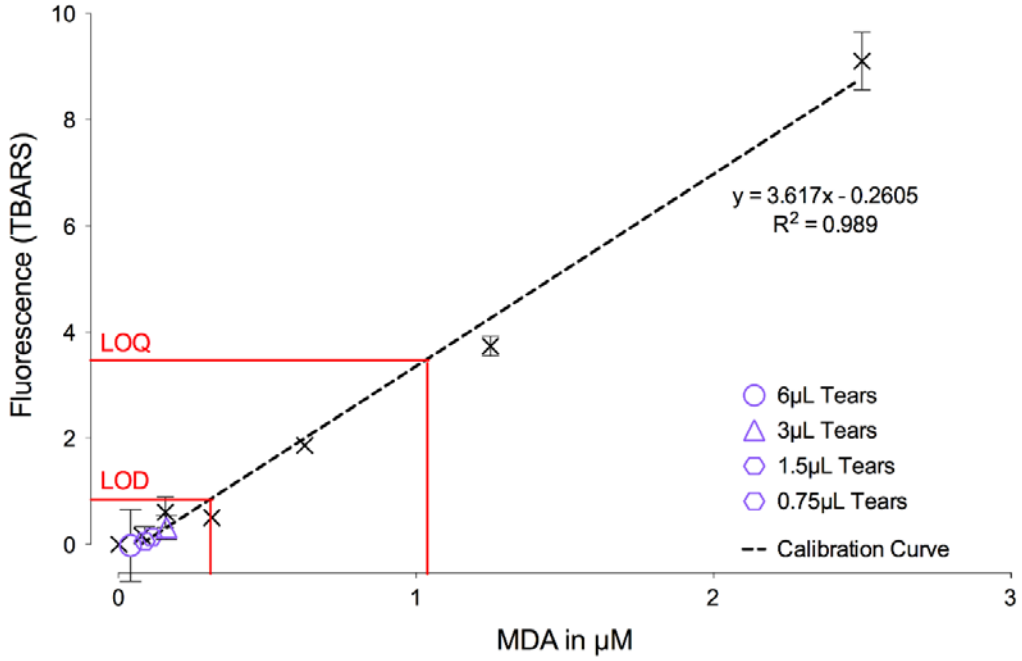

Figure 7-2. Generated calibration curve for TBARS assay and detected amount of MDA in diluted tear samples. Fluorescence was read at 530 nm (excitation) and 590 nm (emission).

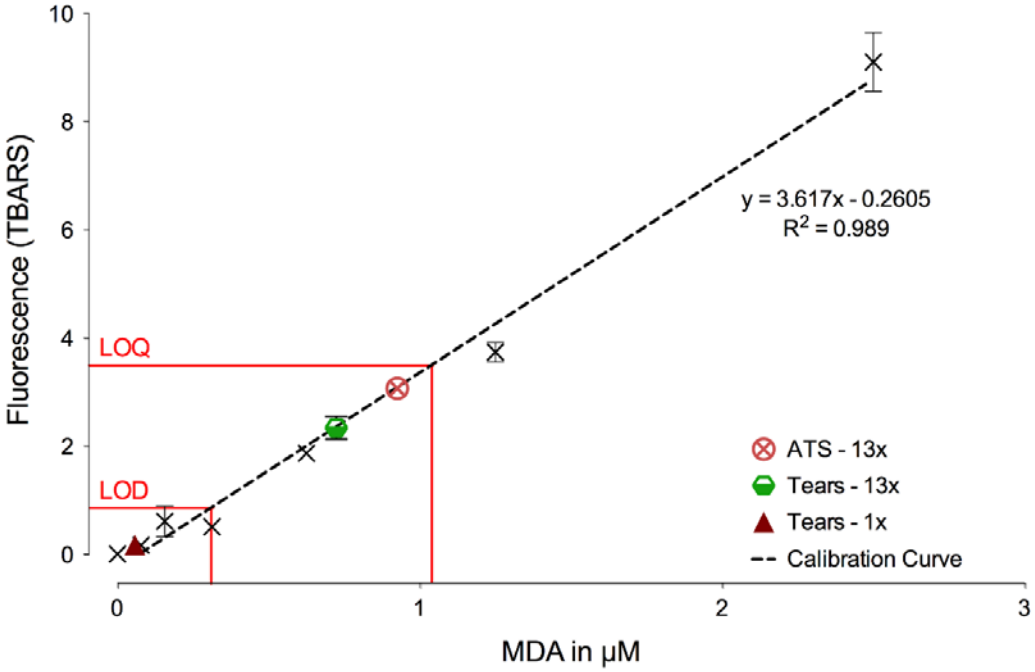

Figure 7-3. Generated calibration curve for TBARS assay and detected amount of MDA in concentrated tear pool and ATS. Fluorescence was read at 530 nm (excitation) and 590 nm (emission).

Using the TBARS assay, this study was not able to detect MDA within individual tear samples. To detect oxidative stress in tears, a pooling step of multiple tear samples (200 µL) from several subjects and creating a high concentrated solution (13x) of those samples was required. Similar to this study, other published work that used the TBARS assay to determine oxidative stress in tears reported that a pooling step was required. [142, 365] Following that, Augustin et al.[142] found an increase of MDA in tears of patients with DE symptoms, whereas for Mahomed and colleagues,[365] the detected quantity of the oxidative stress biomarker was below the assays LOD, similar to the results of this study. A more recent publication by Schuett et al. [163] oxidized various PUFAs *in vitro* to study the effects of varying degrees of oxidation on CL depositions. To test their oxidized lipid mixture for presence of MDA, 25 µL of the mixture were added to the TBARS assay. All of those studies have been able to detect MDA in artificial and tear samples, but the reported amounts of samples necessary to achieve that exceed the amount of tears that would be available on a patient's eye.

A different tear collection method employed by Benlloch-Navarro et al. [106] was the Schirmer's strip, presumably to maximize the amount of tear components within a sample. However, this method is more invasive than capillary tubes, which leads to reflex tearing, and in turn, may result in further tear dilution, oxidation, and decreased amounts of detected MDA. Also, extra steps are required to extract the tear samples from Schirmer's strips which potentially dilutes the samples further and may increase the amount of oxidation, depending on the methods that are applied.

It would appear that it is possible to measure MDA in tears of patient groups/categories through the TBARS method; however, the specificity of it is still in debate as thiobarbituric acid (TBA) has shown to react with other chemical species that might be present in the TF and may lead to misinterpretations of the results.[133, 366, 367] Further examination and optimization of the TBARS assay is therefore required to increase its sensitivity for testing individual tear samples at low volumes. A promising approach to further enhance the accuracy and account for any other TBA reactions in samples is to include a separation step of the test solution before measuring the MDA absorbance by including a high-pressure-liquid-chromatography (HPLC) [106, 133] step or by using liquid chromatography mass-spectrometry (LCMS). Incorporating such methods into the measuring procedure has shown to lower the amount of required tear samples and was successfully applied to determine increased amounts of oxidative stress in elderly.[106] As mentioned previously, using small sample amounts is especially important to investigate oxidative stress in individual tears from patients with DE or contact lens discomfort (CLD). Those patients usually present with very small amounts of tear fluid readily available to collect, and in order to provide them with a tailored treatment plan, a more specific and sensitive method to detect MDA in tears will be needed.

7.4.2 ELISA Kits

7.4.2.1 OxLDL ELISA

Due to the small amount of collectable tears from the subjects and to determine the smallest amount of tears necessary, we first created a tear pool of all subject samples and tested for the presence of OxLDL in 10 µL, 5 µL, 2.5 µL, 1.25 µL, and 0.63 µL. The results are shown in Figure 7-4 and OxLDL in pooled tears was: 71.39 ± 3.78 ng/mL, 50.01 ± 8.06 ng/mL, 41.86.86 ± 3.50 ng/mL, 33.86 ± 1.27 ng/mL, and 28.81 ± 2.29 ng/mL, respectively. Based on those results, it appears that the ELISA could be used with tear samples a small as 2.5 µL, which showed OxLDL above the assays LOD (36.49 ng/mL; see Table 7-1). Subsequently, we measured OxLDL in 2.5 µL of four individual tear samples and detected between 45.59 ± 2.95 ng/mL and 28.24 ± 4.66 ng/mL of OxLDL (Figure 7-5), where two tear samples presented OxLDL above the LOD and two others below it. Therefore, this ELISA assay might be feasible to be used to measure OxLDL in tears, but to determine if we can accurately measure oxidative stress in tears, we performed a SAR.

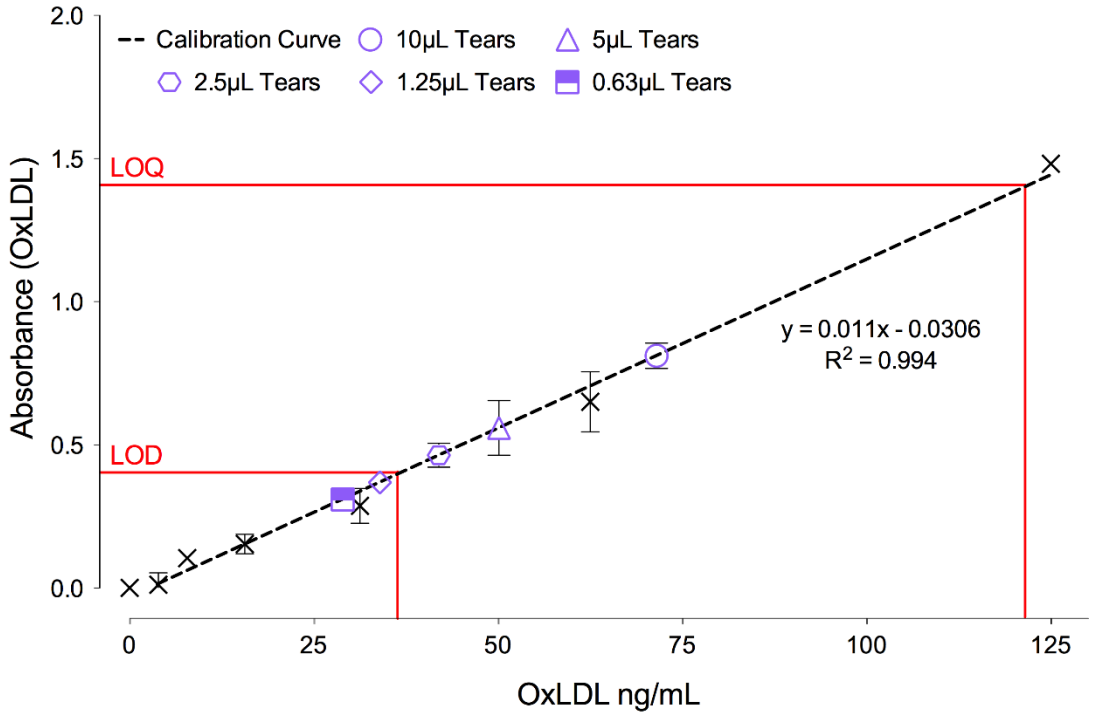

Figure 7-4. Generated calibration curve for OxLDL ELISA assay and detected amount of oxidation in pooled tear samples. Absorbance was read at 450 nm.

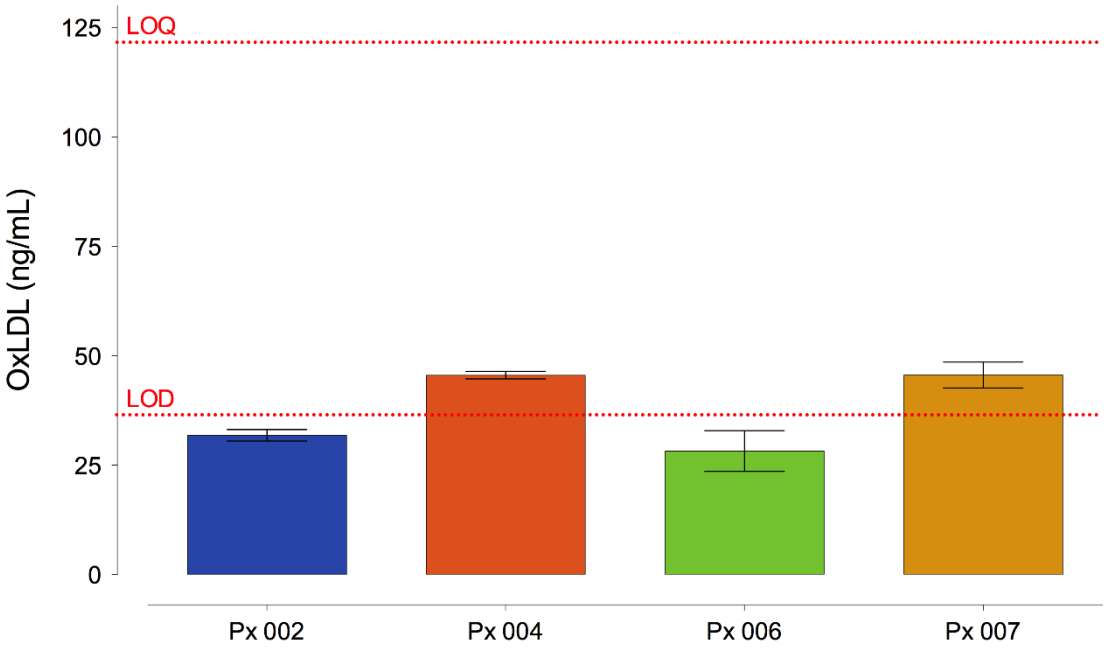

Figure 7-5. Amount of OxLDL detected in 2.5 µL of individual tear samples**.**

One of the first hurdles we had to overcome using this ELISA assay was a required chemical precipitation step to separate out proteins from test samples. When first using the assay with tear samples, no precipitation occurred. The ELISA kit is usually used for blood and serum samples that consist of significantly larger amounts of protein (65-85 mg/mL)[512] within a sample compared to tears (6.5-9 mg/mL),[513] which might be a reason why the separation step was not successful. To support the precipitation of proteins, 52 µg/µL lysozyme was added to the tear samples as a carrier protein; however, still no precipitation occurred. An additional approach of pooling and concentrating the tear samples also did not work in our favour to precipitate out enough protein to be measured with the assay. In a further step, an ammonium sulfate $(NH_4)_2SO_4$ solution of 75% concentration was created and 2 µL solution were added per 1 µL of the previously created lysozyme-tear mixture. After allowing the mixture to equilibrate for 15 minutes and centrifuging it for an additional 15 min at 2500g, the protein precipitation was successful and a pellet formed (Figure 7-6). After the precipitate and supernatant were separated, samples were re-dissolved in assay diluent and prepared for further use.

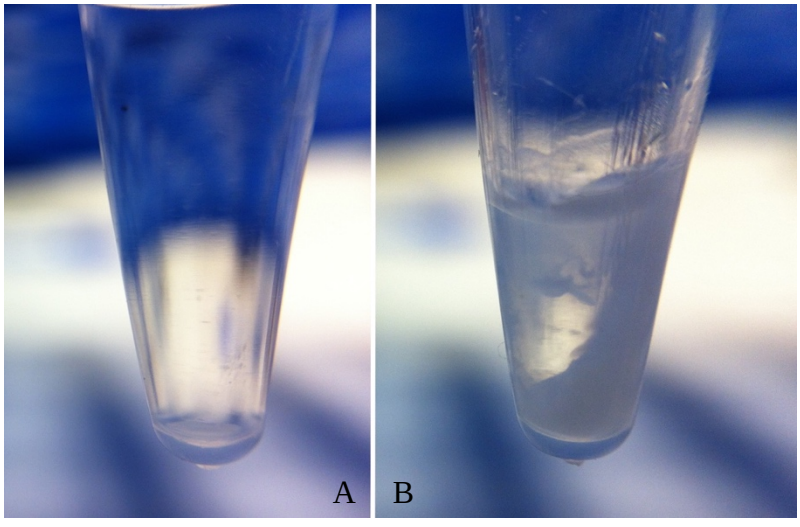

Figure 7-6. Precipitation of tear samples after addition of lysozyme and ammonium sulfate: before (A) and after (B).

The results of the SAR test revealed a recovery rate (RR) for the spiked plasma samples of 81%, 84%, and 111% and are therefore within the normal range 80-120% compared to the un-spiked plasma sample, which confirms that this OxLDL assay is an accurate technique to use with such sample types. For the tear samples, however, the recovery values were ≥156% and, therefore, out of the normal range. Furthermore, the high RR values show that the samples are interfering with the assay. A probable cause for the out of range RR could be the additionally added lysozyme and $(NH_4)_2SO_4$, but further analysis is necessary to determine the actual cause before this ELISA assay can be used confidently for the examination of tears.

7.4.2.2 MDA-, and 4-HNE-Adduct ELISA Kits

Figure 7-7 and Figure 7-8 show the calibration curves and Table 7-1 the linearity equations of the MDA- and 4-HNE Adduct ELISAs. No differences were found between the two tested collection methods (flushed vs. normal capillary collection). In addition, the received tear samples of symptomatic and asymptomatic DE patients showed similar MDA (Figure 7-7) and 4-HNE (Figure 7-8) amounts. All of the measured values are below the assays LOD and are tabulated in Table 7-2 and Table 7-3.

A recent study by Choi et al. [162] examined tears of non-Sjögren's Syndrome (SS) DE and non-DE patients and found increased amounts of both MDA and 4-HNE in non-SS DEs, using similar ELISA assays. However, comparing the results of non-DE patients,[162] the amount of detected MDA in our study was significantly smaller (3.80 ± 1.05 pmol/mg), whereas, the amount of 4-HNE (0.02 ± 0.03 µg/mL) was similar. These differences in oxidative stress biomarkers to the results presented by Choi et al. [162] might be a consequence of the amount of

tear samples (30 µL vs. 5 µL) that were used in both studies. Furthermore, we did not find significant amounts of oxidation biomarkers when examining samples from symptomatic and asymptomatic DE patients, which might be related to the age and long term storage of the samples used in this study.

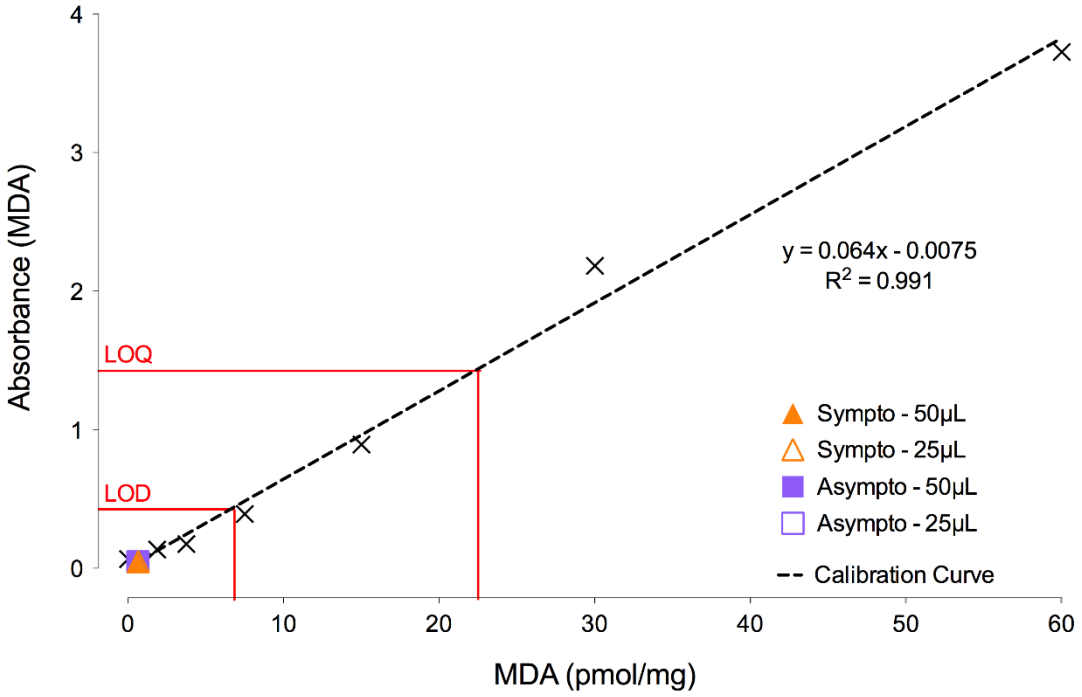

Figure 7-7. Generated calibration curve for the MDA ELISA assay and detected amount of oxidation in symptomatic and asymptomatic tear samples**.** Absorbance was read at 450 nm.

Table 7-2. Amount of MDA (pmol/mg) measured in tears and on various contact lens materials.

	Px 002	Px 004	Px 006	Px 007
Flushed Tear Collection	0.778 ± 0.039	0.586 ± 0.063	0.601 ± 0.051	0.595 ± 0.036
Traditional Tear Collection	0.563 ± 0.074	0.573 ± 0.056	0.604 ± 0.013	0.712 ± 0.019
Lotrafilcon B	1.336 ± 0.084	2.290 ± 2.019	3.676 ± 1.686	1.569 ± 0.396
Balafilcon A	2.009 ± 0.627	1.225 ± 0.094	1.644 ± 0.143	4.633 ± 3.867
Narafilcon B	1.205 ± 0.145	1.038 ± 0.196	0.846 ± 0.031	1.769 ± 0.968
Etafilcon A	0.901 ± 0.113	2.914 ± 2.171	2.588 ± 0.953	1.600 ± 1.054

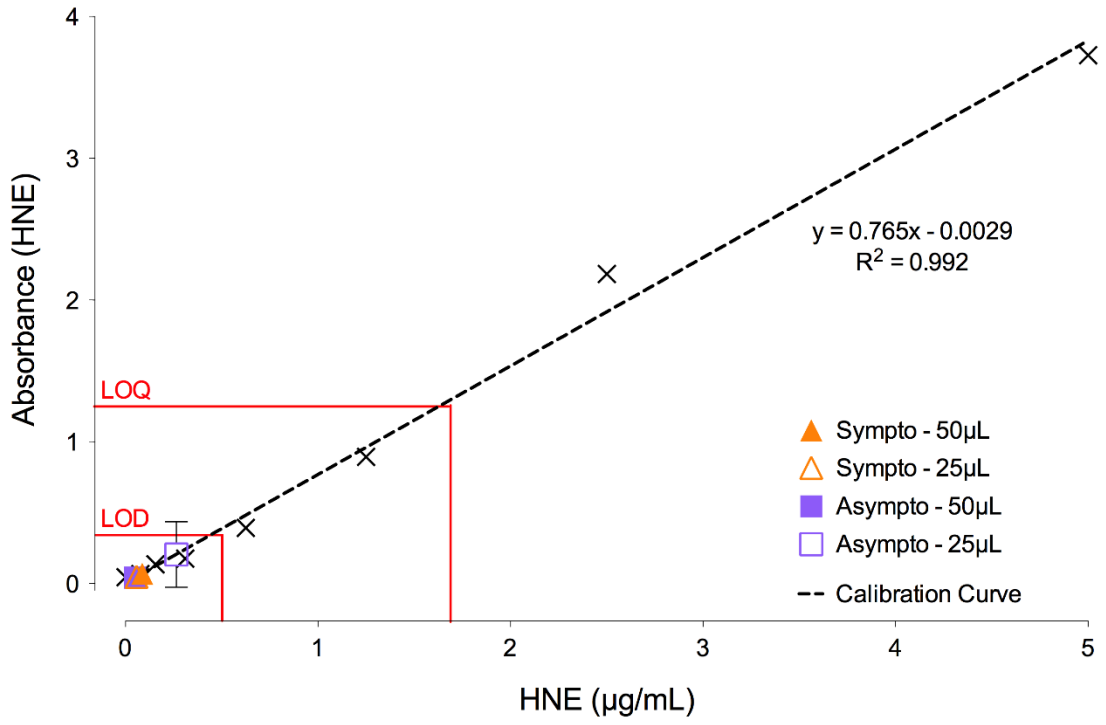

Figure 7-8. Generated calibration curve for the 4-HNE ELISA assay and detected amount of oxidation in symptomatic and asymptomatic tear samples.

Table 7-3. Amount of 4-HNE (µg/mL) measured in tears and on various contact lens materials.

	Px 002	Px 004	Px 006	Px 007
Flushed Tear Collection	0.206 ± 0.212	0.080 ± 0.031	0.056 ± 0.003	0.056 ± 0.001
Traditional Tear Collection	0.063 ± 0.008	0.082 ± 0.016	0.063 ± 0.002	0.052 ± 0.011
Lotrafilcon B	0.075 ± 0.120	0.195 ± 0.096	0.000 ± 0.207	0.412 ± 0.219
Balafilcon A	0.503 ± 0.059	0.428 ± 0.053	0.291 ± 0.023	0.372 ± 0.028
Narafilcon B	0.050 ± 0.051	0.510 ± 0.376	0.000 ± 0.021	0.231 ± 0.009
Etafilcon A	0.000 ± 0.018	0.074 ± 0.035	0.000 ± 0.099	0.301 ± 0.158

As a final test and preliminary study for future projects, we tested the amount of MDA and 4-HNE on contact lenses that were worn for 4 hours. The results are listed in Table 7-2 and Table 7-3 depict amounts below the assays LOD with ≤ 4.63 ± 3.87 pmol/mg of MDA and ≤ 0.50 ± 0.06 µg/mL of 4-HNE. However, the results show that amounts of measured MDA and 4-HNE on CLs varied greatly between patients and lens materials. Despite the low detected quantities, CLs seem to be a carrier for oxidative stress markers. Once lipids deposit on CLs, they become immobilized and are exposed to UV radiation and oxygen for far longer and are therefore more susceptible to oxidation,[132] which may explain why the amount of detected biomarkers on CLs in this study was greater than the detection in tears. Nevertheless, the degree of oxidation has been shown to affect the amount of total lipids depositing on CLs,[163] and once deposited, oxidation by-products can be sequestered from CLs materials.[163, 365] This further supports that CLs would be a good tool to collect oxidation by-products on the eye.

7.5 Conclusion

Oxidative stress may very well occur in the TF, but the impact on DE and contact lens discomfort (CLD) is still in debate.[132, 135, 137] The amount of occurring lipid oxidation by-products, however, depends on individual lipid composition, the turnover rates of the TF,[132] or the presence of lipid degrading enzymes in tears of DE patients or CL wearers.[97, 125, 126, 134] We believe that occurring lipid peroxidation may have a destructive effect on TF lipids and their stability, however, it is more difficult for oxidative stress by-products to accumulate over time because of the frequent TF renewal every time we blink. In general, researchers have been able to show that increased lipid oxidation causes health issues by testing blood or serum samples, but in contrast to tears, those samples are extracted from an enclosed system where oxidative stress can build up and cause more damage over time. Although, this study only found biomarkers of oxidative stress below the assay's LOD, some of the tested CLs showed greater amounts of detected biomarkers compared to tears, which supports the idea to use CLs as a medium to collect markers of oxidative stress that occur on the eye and build up over time in future studies.

Unfortunately, no current data exists that determined the amount of oxidative stress by-products in normal tears, which makes it difficult to compare the measured values of this or other studies. Therefore, future studies should focus on developing a more sensitive and specific method to determine lipid peroxidation in tears, to help create a database for oxidative stress in tears of normal patients, DE patients, and CL wearers. The development of a sensitive

test assay and such a database will directly benefit DE patients and practitioners to determine the most perfect and efficient treatment of DE or CLD.

The objective of this study was to develop a sensitive method to determine oxidative stress of individual tear samples. The results show that markers of oxidative stress can be detected using the examined assays, however, only in very small quantities. Specifically, the TBARS kit was optimized for the use of tear samples and showed that small quantities of MDA are detectible, when samples were pooled and concentrated. The OxLDL ELISA kit was optimized for the use of tear samples as low as 2.5µL. Both techniques could be employed to determine the oxidative stress in TF lipids collected from patients with DE and CL-related DE. However, further work is needed to increase the sensitivity of all presented assays to be used to determine the lipid peroxidation in small tear sample volumes from patients with DE or CLD. To further lower the amount of required tear volume needed to test for oxidative stress markers, the presented assays could be used in combination with an HPLC or LCMS.

7.6 Acknowledgment

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Chapter 8
Conclusions and Future Outlook

Despite the constant growth in the popularity of CL [396, 472] and the many improvements in CL materials over the past few decades, the CL market still reports high numbers of patients ceasing CL wear, mainly due to CLD.[197, 198, 454] The introduction of SiHy lens materials contributed immensely to the recent success of CLs,[396] which can mainly be attributed to the increased oxygen transmissibility that these lens materials provide.[177, 439, 440] However, SiHy materials are relatively more hydrophobic than conventional hydrogels, which may affect wettability,[404, 405, 441] and may lead to increased lipid deposition.[58, 62, 63, 227, 257, 258, 260, 262, 263, 267, 268, 398, 449] It is widely believed that lipid deposition on CLs has a detrimental effect on material properties and lens performance on the eye, and may contribute to CLD and dryness.[261, 271, 272] However, an extensive literature search was unable to show a strong correlation between reduced CL comfort and increased lipid deposition on CLs.[275]

A CL immediately interacts with the ocular surface after lens insertion and research has shown that TF deposition occurs instantaneously and continuously throughout its use.[62, 63, 160, 227, 238, 239, 255, 257, 267, 268, 332, 371, 514] Despite the modification of surfaces [188, 189, 441, 515] to reduce the affinity of lipids for SiHy lenses, they are still prone to deposit in greater quantities on these lens materials.[58, 62, 63, 227, 258, 263, 268, 449] Common strategies among practitioners to minimize lipid deposition and to improve end of day comfort with CLs includes the use of cleaning solutions to remove deposits or to reduce the replacement frequency and switch patients to a

DD lens wear modality. However, valuable information is still missing on whether such strategies might be helpful to improve CL comfort.

To first answer the question of whether existing cleaning procedures and solutions are capable of efficiently reducing or fully removing accumulated TF lipids from CLs, Chapter 3 examined various contemporary lens materials exposed to different MPSs. Through the use of radiolabelled cholesterol, this chapter assessed the lipid uptake onto five SiHy lenses after an incubation period of 16 h a day over a time period of 7 days. Additionally, CLs were either stored in a saline solution or cleaned (using a rub & rinse procedure) with one of five MPSs between the incubation times. After extracting the accumulated TF lipids from the CLs, it was found that balafilcon A and senofilcon A lens materials were the highest depositors of cholesterol, which was consistent with other studies.[58, 60-63, 263, 266, 268] Only one of the contemporary MPSs showed a significant effect in the reduction of deposited cholesterol, and only on the two highest-depositing CL materials. Overall, this study showed that the efficiency of current MPSs to reduce and/or remove accumulated TF lipids from CLs is highly dependent on the material/solution combination. In cases where lipid deposition remains an issue, changing the lens material or increasing the replacement frequency of CLs would be appropriate.

The majority of patients wearing SiHy DD lens materials report excellent comfort.[451, 452] Information about the affinity of TF lipids depositing onto DD CLs, however, is lacking. In Chapter 4, the *in vitro* quantities and hierarchy of cholesterol deposition onto various DD lens materials are described. In this chapter, three contemporary SiHy and four CH DD CLs were

incubated in a lipid-containing ATS over a time period of 16 h and the degree of lipid uptake was assessed over time, using a radiochemical methodology. It was found that SiHy accumulate significantly more lipid than CH lens materials even after only one day of exposure. Furthermore, the quantities of extracted lipids from the DDs were far less than reported values on DW CLs that also evaluated lipid deposition after one day.[58, 63, 449] This *in vitro* study was the first to investigate lipid uptake onto various DD lens materials and provides valuable information for manufacturers and practitioners alike.

An important question that remained unanswered to-date concerns the location of lipid deposition on SiHy CLs and whether it was largely surface-located or was within the bulk of the lens material. The differences in lipid uptake and penetration in DD lens materials was, therefore, assessed in Chapter 5. Furthermore, this chapter investigated a novel *in vitro* approach to evaluate deposition and penetration of TF lipids. Three SiHy and four CH DD CLs were incubated for 4 and 12h in an ATS using two different incubation methods: the traditional "in-vial" method and a novel *in vitro* platform. In contrast to using the traditional "in-vial" method, where CLs were statically incubated in 3.5 mL of ATS, CLs were mounted on a novel *in vitro* eye-blink platform that simulated physiological tear flow, tear volume, air exposure and blinking. Through the use of NBD-cholesterol and LSCM, it was found that SiHy lenses depicted higher fluorescence intensities than CH lenses and that fluorescence intensities varied between incubation methods and CL materials. Specifically, when CLs were incubated using the "in-vial" method, the fluorescence intensities of NBD-cholesterol were relatively higher than using the novel *in vitro* platform. This experiment supported the theory that the traditional

"in-vial" incubation method is rudimentary and that more advanced *in vitro* models that simulate important ocular factors such as intermittent air exposure, physiological tear volumes, and tear flow may provide better insight to elucidate the interactions between CLs and TF components. Additionally, the localization of lipid deposits on CLs may play a significant role in determining CLD and provide valuable information to optimize the efficiency of MPSs in removing lipid deposits from CLs.

To elucidate the impact of TF deposits on the wettability of DD SiHy lens materials, Chapter 6 described the development of a novel *in vitro* model and used it to compare NIBUTs of five contemporary DD CLs. Three SiHy and two CH CLs were incubated for up to sixteen hours in a complex ATS by using a model blink cell that specifically mimics intermittent air exposure, which was previously shown to impact lipid deposition.[266] To model the drying effect that occurs between blink intervals, lenses were continuously submerged in the ATS for three seconds and exposed to air for ten seconds. By using a corneal topographer, it was found that the initial NIBUT of CHs was longer than over SiHy CLs. However, after twelve hours the NIBUTs of SiHy CLs was longer but very similar to CHs. Overall, this method showed varying levels of deposition may affect the measured NIBUTs of CLs and that NIBUT values gradually decrease over time, which were comparable to *in vivo* data.

The final chapter of this thesis, Chapter 7, assessed and optimized various assays that determine lipid peroxidation by-products in tears, which may play a role in DE and CLD.[125, 132] Tear samples were collected with micro-capillary tubes, diluted and processed to determine the smallest volume of tears that could be used to measure the presence of MDA, 4-HNE, and

OxLDL. Despite the small quantities of detected oxidative stress markers, the assays were optimized for the use of small tear volumes and could be employed to determine oxidative stress and potentially help to identify the cause for patients that suffer from DE or CLD.

The work presented in this thesis has provided deeper knowledge about the ability of various cleaning solutions to remove lipids from CLs and added valuable information about the interactions of TF lipids with CLs, particularly about DD lens materials. Furthermore, the use of two novel *in vitro* methods provided constructive information about the location of lipid deposits and the impact of TF lipids on tear break up times. Together, all of these results may help better predict the performance of a CL, and thus provide further information for manufacturers during the design process and clinicians when deciding the best possible product on the market for their patients.

Despite the tendency of SiHy CLs to accumulate significant amounts of TF lipids, there is an ongoing debate on whether lipid deposition is generally good or bad for the performance of a CL.[160, 272] Future projects could, therefore, focus on the development of cleaning solutions that are capable of removing lipid contamination efficiently, or selectively removing lipid types that may result in poor lens performance. Moreover, further studies are required to determine if the levels of lipid deposits found on DD CLs may prove deleterious during wear and such studies should determine if a progressive reduction of pre-lens NIBUT does occur during CL wear.

To develop “smart” lens materials and lens care products that will be capable of selectively depositing or removing lipid deposits, future studies are warranted to determine and

differentiate between “bad” and “good” TF lipids by evaluating their impact on CL performance and comfort. Such studies could employ loading or coating contemporary lens materials with a specific lipid or lipid combinations and subsequently compare comfort ratings and lens performance of those treated lenses to untreated CLs *in vivo*. However, these studies are not easy to conduct. They will be time consuming, costly, could potentially result in infections if the lens materials are not sterilized appropriately before wear and will thus require extensive testing and ethics clearance before they can be carried out. Nevertheless, lipids that might be beneficial to deposit onto CLs include cholesterol or amphiphilic lipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Although cholesterol has been shown to be one of the major TF lipids depositing on SiHy lens materials, small quantities of deposited cholesterol on CLs may provide an antibacterial treatment option.[516] However, the exact amount of cholesterol on lenses that can exhibit an anti-bacterial effect is currently unknown and requires further investigation. PC and PE are lipids that are believed to support the spreading of the lipid phase by mediating between the TF lipids and the underlying aqueous phase.[48, 54, 502] Incorporating or coating lens materials with these amphiphilic phospholipids might therefore be favourable, as they could occupy the hydrophobic chains within the SiHy lens materials to prevent excessive and continuous accumulation of other TF lipids and might simultaneously enhance the hydrophilicity and wettability of CL surfaces.[262]

Further research is also necessary to determine the role of oxidized TF lipids and their by-products in the development of DED and to assess in which way lipid peroxidation has an impact on CL deposition and discomfort. Even though phospholipids might be beneficial for

CL performance and comfort, [68, 517-519] these lipids consist of unsaturated and PUFAs that are prone to oxidation. To further our understanding of the role of oxidized lipids in relation to DED and CLD, future studies need to identify the exact fragmentation and conformational changes in TF lipids that oxidation may cause. Therefore, in addition to using traditional ELISA-based assays, upcoming projects should employ more sensitive analytical methods, such as mass-spectrometry, Raman-spectrometry, or HPLC technology to precisely determine structural changes and by-products of oxidative stress.

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