Special Issue

The International Workshop on Meibomian Gland Dysfunction: Report of the Subcommittee on Tear Film Lipids and Lipid–Protein Interactions in Health and Disease

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Understanding the molecular composition (e.g., proteins and lipids) of the tear film (TF) and the contribution of the meibomian gland to the TF is critical in gaining knowledge about TF instabilities, dry eye syndromes, contact lens (CL) incompatibilities, and other eye diseases. Among its functions, the lipid layer of the TF slows evaporation of the aqueous component, preserves a clear optical surface, and forms a barrier to protect the eye from microbial agents and organic matter, such as dust and pollen.[1] The TF contains a complex mixture of proteins, enzymes, lipids, mucins, and salts that allows the TF to perform its functions (Fig. 1). Researchers believe the outer lipid layer is 5 to 10 molecules thick and is composed primarily of wax and sterol esters, possibly intercalated with each other and with proteins rather than forming distinct repeating layers of molecules.[2,3] Evidence from interferometric studies indicate that the TF lipid layer thickness ranges from 20 to 160 nm.[4] If the size of a lipid molecule is approximately 2.2 nm (22 Å), then the calculated thickness for one layer would be 11 to 44 nm. The addition of polar and nonpolar layers would add to the lipid thickness, which indicates that the lipid component of the TF may be multiple layers thick or have other contributing sources to correspond with reported thickness measurements.[5]

While the signs and symptoms of TF instability are reasonably well characterized, we are only beginning to understand the specific molecular components of the TF and their relationship with disease and TF stability. The purpose of this review is to examine the meibomian gland's contribution to TF lipids and lipid–protein interactions in health and disease.

Review of the Tear Film Lipid Layer

The meibomian glands are the main source of lipids for the human TF. The meibomian gland secretions consist of an extremely complex mixture of various polar and nonpolar lipids containing cholesteryl esters (CEs), triacylglycerol, free cholesterol, free fatty acids (FFAs), phospholipids, wax esters (WEs), and diesters.[6–9] It was not until 1981 that the term meibum entered the lexicon, to describe these secretions.[10]

Current models of the TF originated in the 1950s[11] and include three major, well-defined layers: the glycocalyx layer, the intermediate aqueous layer, and the outermost tear film lipid layer (TFLL). The glycocalyx layer, which covers the corneal epithelium, is believed to be relatively viscous because of the large amount of membrane-bound and secreted mucins. The aqueous layer is enriched in water-soluble proteins, mucins, and salts, whereas the TFLL is formed almost exclusively from lipids and attached and/or intercalated proteins.[2] The TFLL is usually depicted as a two-layer structure: polar lipids form the lower sublayer and nonpolar lipids form the upper portion that is in contact with the air.[11] This concept was first proposed by Holly[12]; Shine and McCulley later elaborated.[13] Each sublayer is distinct in its responsibilities: The upper sublayer forms a thick blanket that seals the underlying aqueous portion of the TF. The outermost lipid component is believed to retard water evaporation, as lipid films have low water vapor transmissivity, depending on the lipid film thickness and composition.[13] Nonpolar lipids are thermodynamically unstable when they are spread over an aqueous subphase; this allows them to collapse easily and form lipid droplets. When that happens, the aqueous portion of the TF is left unprotected and prone to rapid evaporation.[13] Interestingly, the lower lipid sublayer is thought to create an interface that helps stabilize this upper portion. In this interface, the polar lipids are thought to be oriented perpendicularly, with their hydrophobic tails immersed in the nonpolar lipid sublayer, and their polar heads exposed to the aqueous layer. Shine and McCulley[13] suggested that this polar lipid sublayer was one to three molecules thick. They further suggested that it is formed from phospholipids and other polar lipids, including phosphatidylcholine, phosphatidylethanolamine, sphingomyelin (SM), ceramides, and cerebrosides.[13] More recently, another group of amphiphilic lipids—namely, very long chain (*O*-acyl)-ω-hydroxy fatty acids, have been described as meibum components and are theorized to contribute to the polar lipid sublayer.[14] Polar-to-nonpolar lipid layer thickness measurements have not been performed; however, it has been suggested that the polar lipid

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Supported by the Tear Film and Ocular Surface Society (TFOS; http://www.tearfilm.org); individual author support is listed in the Appendix of the Introduction.

Submitted for publication December 6, 2010; accepted March 23, 2011.

Disclosure: Each Workshop Participant's disclosure data can be found in the Appendix of the Introduction.

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DOI:10.1167/iovs.10-6997d
Investigative Ophthalmology & Visual Science, Special Issue 2011, Vol. 52, No. 4

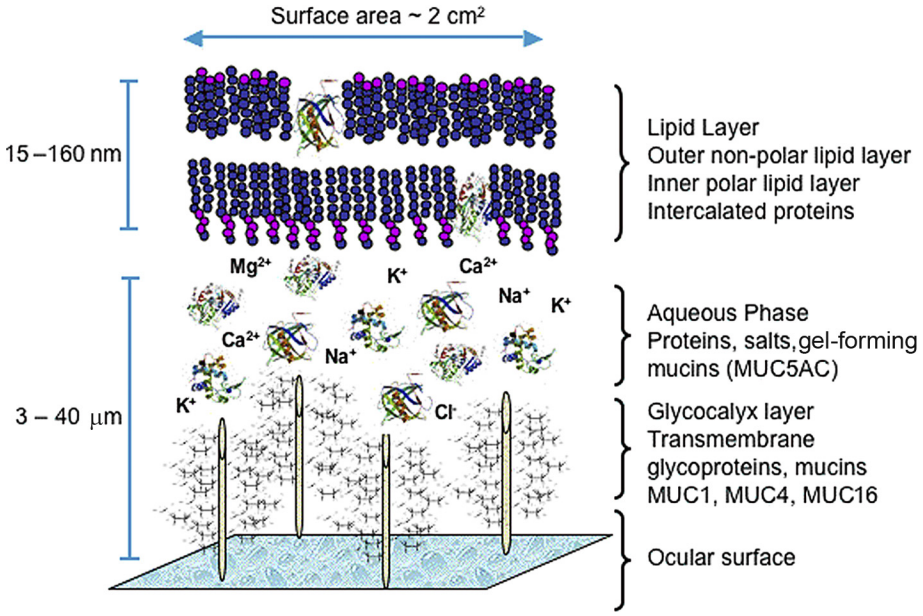

Figure 1. A proposed model of the precorneal tear film showing the relationship and interaction of lipid-binding proteins and the outer lipid layer.

is 5% to 15%[2,6] of the total lipid fraction. Therefore, this polar surfactant layer is hypothesized to be between 7 and 20 molecules thick and consists of more hydrophobic lipids above the polar lipid layer.[6,15] This estimate does not consider a (quite possible) redistribution of the lipids of different classes between the sublayers and is based on an assumption that the overall lipid composition of TFLL is identical with that of meibum.[2]

It is likely that lipids are not the only class of molecules from which the TFLL is assembled. For instance, proteins are now being considered an intrinsic part of TFLL.[2] Many proteins are surface active compounds, meaning they will spontaneously populate the air–liquid interface, lowering the surface tension of water and creating a surface protein layer. This translocation of proteins from the bulk aqueous phase to the air–water interface is typically accompanied by protein denaturing (i.e., irreversible conformational changes—typically, unfolding—that, in the end, prevent proteins from submerging back into the depth of the aqueous layer). This results in the formation of a protein layer.[2]

In the presence of meibomian lipids, proteins have to compete for the available surface space. This competition results in either protein penetration (intercalation) in the lipid layer or protein attachment to (or association with) the lipid layer. Both result in surface property alterations of the TF and TFLL. Indeed, Saaren-Seppälä et al.[16] demonstrated that TF lipocalin (Tlc) could actively interact with various artificial lipid membranes, regardless of overall charge and composition, and others have shown human Tlc binding to meibomian lipids organized in thin films. Similar experiments were conducted earlier to demonstrate that other tear proteins (such as lysozyme[17] and mucins[3]) could also penetrate the lipid layers.

Thus, an update to the classic three-layer model (Fig. 1) of the TF and two sublayers of TFLL is warranted. This new model should incorporate proteins (lipocalin, lysozyme, mucins, and others) intercalated in and/or adsorbed to the TFLL, and an addition of a novel class of lipids recently identified in human meibum, very long chain (*O*-acyl)-ω-hydroxy fatty acids, which may act in the formation of an intermediate surfactant lipid sublayer between the thick outermost nonpolar lipid sublayer and the aqueous layer of the TF.[8,15,18]

Analytical Methods for Lipid Evaluation

With the advancement in the lipid detection and identification technology, sample quality is critical. Modern instrumentation can detect a wide variety of compounds and provide accurate information on their structures. Thus, many of the analytes that previously had been impossible to detect and/or identify in meibomian lipids can now be discovered and categorized, even if present in minute quantities.

Methods of Sampling and Storing Meibum and Tear Film Lipids

Handling and storage of lipid samples generally follows the recommendations provided in lipid chemistry textbooks and protocols available online from lipid chemical companies (see, for example, http://www.avantilipids.com, technical support, lipid storage, and handling).[19] Care should be taken to minimize (or prevent) sample exposure or contact with any products made of plastic and silicone. Glass, stainless steel, noble metals, and Teflon (E.I. du Pont de Nemours and Co., Wilmington, DE) are the recommended collection and storage materials. The preferred conditions for storing lipid samples regardless of their origin are below temperature (−80°C), in an inert atmosphere (argon or nitrogen), and in a dark and dry state.

Meibum and TF samples can be collected using three main types of procedures: (1) soft or hard expression of meibum from the meibomian gland orifices[10,20–22]; (2) microcapillary collection of aqueous TF samples and meibum directly from the meibomian gland orifices[23] and (3) Schirmer test strips or similar tools to collect aqueous TF samples.[2,24,25] Another technique, the surgical removal of eyelids and/or meibomian glands, has been implemented in animal and human cadaver studies,[26–28] but is unrealistic with living human volunteers.

Soft expression, or expression/pressure from the outside only of the eyelid, is possibly less likely to contaminate the samples with surrounding tissues due to the gentler handling of the eyelids. The hard expression technique is often described as a squeeze technique in which a conformer or device is used behind the lid while pressure is applied to the front of the eyelid. This technique yields a greater sample volume, yet may be more uncomfortable to subjects. Both techniques can

yield samples contaminated by the tears as well as surrounding tissues (cells and debris). The contamination may vary by patient and/or examiner.

The use of powder- and latex-free gloves worn by the examiner is highly recommended to avoid contamination of the samples by examiner skin secretions. The use of a slit lamp during sampling allows a better visualization of the meibomian gland orifices and reduces the chances of contamination with lid margin tears and debris.

TF collection via a microcapillary technique is virtually painless and allows samples of aqueous tears to be collected directly from the tear menisci of the lower eyelids. The nonstimulated samples collected this way are typically between 2 and 5 μL, or a few micrograms, of wet sample.[18] The microcapillary technique has also been used for collecting meibum directly from the individual meibomian gland orifices.[22] The procedure is well tolerated by patients and is less invasive than hard expression, although the technique produces smaller sample volumes. The meibum sample solidifies at room temperature.[18,29] The solid meibum lipid is removed from the microcapillary by pushing out with a thin wire or simply by soaking in chloroform/methanol. If a spatula is used for meibum collection, the sample is immediately stored in a solvent. On average, approximately 15 mg of meibum is collected per eye by microcapillary collection, which is generally enough for downstream mass spectrometry analytical analysis, although protein extraction and/ or additional analyses may require pooling.

Patients accept the Schirmer test strip technique as a general component of an ocular surface examination. However, the Schirmer test may be less comfortable for patients than the microcapillary technique. This is more than offset by the test's high safety profile, aided by the lack of sharp or hard objects used in sample collection. The Schirmer test cannot be used for collecting a pure meibum sample, however, because it is virtually impossible to avoid wetting the strips with aqueous tears. Consequently, it seems that expression and microcapillary tube collection or spatula collection is well suited for collecting samples of pure meibum, whereas microcapillary tube and Schirmer test strips are better suited for collecting aqueous tears. Additional research evaluating alternate and/or optimal collection and storage of lipid samples is warranted.

Thin-Layer Chromatography

Thin-layer chromatography (TLC) is an established method of quantifying global classes of lipids; however, TLC requires large amounts of sample that can make it difficult to study tear and meibum samples. Detection is visual, via a stain (bromothymol blue) or charring.[30] To identify individual lipid species, the region of interest is removed from the TLC plate, and the lipids are extracted and either chemically derivatized to make volatile for gas chromatography or labeled with a chromophore or fluorophore for high-performance liquid chromatography (HPLC) detection. Bovine meibomian secretions were first analyzed by using this technique in 1976.[31] McCulley and Shine[32] used TLC followed by HPLC with ultraviolet detection and gas chromatography-mass spectrometry (GC-MS). While TLC still has many uses for quantifying lipids, newer techniques such as matrix assisted-laser desorption ionization (MALDI) and electrospray are gaining rapid acceptance and being used on their own or in conjunction with TLC. Lipid samples can be analyzed via MALDI and electrospray without derivitization or labeling.

Mass Spectrometry

Mass spectrometry is a very sensitive analytical method that allows for both detection and structural determination from very small sample amounts. Many mass spectrometry-based meibum studies have been published using analysis techniques including GC-MS,[10,33] liquid chromatography-mass spectrometry,[7,9,18,20,34] electrospray,[8,9,23,35,36] atmospheric pressure chemical ionization,[7,18,20] and MALDI.[37,38] Phosphorylated lipids from rabbit and human tears have been detected and analyzed using a unique extraction procedure and sample preparation for MALDI-TOF (time of flight) analysis.[38] No papers have been published on the detection of human meibum lipids using MALDI-TOF.

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) measures quantum mechanical magnetic properties of an atomic nucleus that possess a spin such as ^{1}H, ^{13}C, ^{31}P, ^{17}O, and ^{15}N.[39,40] As NMR spectroscopy does not destroy the sample, it could be a promising technique to quantify major lipid classes before using more complex HPLC or mass spectroscopic measurements. The NMR technique must be validated and the band assignments should be carefully confirmed. A disadvantage of NMR is that milligram quantities of sample are needed, and phospholipids are not soluble in deuterated cyclohexane, which is the solvent of choice for NMR analysis of waxes. Therefore, pooled meibum samples would be necessary for NMR analysis.

Infrared and Raman Spectroscopy

The basic principle of infrared spectroscopy is that when a bond with an electric dipole is changing at the same frequency as the incident radiation (infrared light), light is absorbed. From the intensity and frequency of the absorbed radiation, vibrational transitions of molecules are measured that provide compositional, environmental, and conformational information at the molecular level. Infrared spectroscopy and Raman spectroscopy are complimentary techniques; they both measure vibrational modes. However, symmetric bands are generally more intense in Raman spectra and asymmetric bands more intense in infrared absorption spectra. Fluorescence does not interfere with infrared spectra like it does with Raman spectra, but water bands, associated with most biological tissues, can overwhelm and interfere with infrared spectral features. Infrared bands are inherently broader and less resolved than Raman bands.[39,40]

Raman and infrared spectroscopy has been applied to characterize the conformation of human meibum and human tear lipids.[24,41–44] Meibum differs in composition from tear lipids in that it contains more C=C and CH_3 moieties than tear lipids. The conformation between meibum and tear lipids is also different.[24]

Chemical Properties of Lipids

Lipids are an extremely complex group of molecules, both structurally and functionally. One method of chemical property classification groups lipids into polar, amphiphilic and nonpolar lipids.[45] By definition, polar lipids are relatively water-soluble. They include short chain fatty acids, hydroxylated fatty acids, hydroxy-ceramides (OH-Cer), monoacyl glycerols (MAGs), glycosylated lipids, phospholipids, and others.[45] These lipids tend to have relatively high hydrophilic-to-lipophilic balance (HLB), which is an objective physicochemical parameter used to describe partitioning of solubilized molecules between polar (aqueous) and nonpolar (oil) subphases. Thus, in a water-in-oil emulsion, polar lipids would concentrate in the aqueous subphase. Nonpolar lipids, on the other hand, do not dissolve in water. Typical members of this family are hydrocarbons, very-long-chain acyl-ceramide, WE, CE, and triacyl glycerols.[45] Their hydrophilic-to-lipophilic balance is very

low; thus they easily partition into the oil phase of a water-in-oil emulsion. The borders between these three groups are blurry, as there are many factors that influence the solubility of lipids, such as the length of their carbon chains; the number and type of hydrophilic (hydroxy, amino, and carboxy) groups in their structures; the degree of unsaturation; *cis*, *trans*- and iso- and anteisomerism, for example.[45]

The formation of complex lipids happens through condensation reactions—mainly esterification and amidation. The reactions of esterification are involved in the biosynthesis of virtually every major complex lipid group (WE, CE, acylglycerols, and liposaccharides), while amidation is involved in the formation of fewer lipids such as fatty acid amides (FAs), Cer, SMs, cerebrosides.[45] These reactions are reversible, which means that in situ complex lipids may undergo enzymatic or nonenzymatic hydrolysis, in the course of which they will revert to a mixture of more simple lipids and other compounds such as glycerol (in the case of acylglycerols) and carbohydrates (in the case of liposaccharides).[45]

The second major type of lipid transformation relating to TF and TFLL is lipid oxidation. One of the major prerequisites for lipid oxidation is the presence of one or more double bonds in the lipid structure. The double bonds may undergo enzymatic or nonenzymatic oxidation. Many lipids can isomerize, either induced by enzymatic transformation or spontaneously. High temperature, UV light, and oxidation are typical causes of isomerization. These reactions inevitably change the physical and chemical properties of lipids and their mixtures because (1) hydrolysis products are typically more water soluble than the starting complex lipids; (2) lipid (per)oxidation products also become more hydrophilic because of the addition of oxygen and/or the formation of shorter, more hydrophilic scission products; and (3) isomerization influences lipid packing and the physical properties of lipids (e.g., melting points, boiling point, and density).[45]

For up-to-date, easily available information on lipids, the following web sites are recommended: lipidmaps.org, lipidlibrary.co.uk, cyberlipid.com, hplc-ms.byrdwell.com, and lipidbanks.jp, among others. An overview of lipidomics was also recently published and may elucidate the molecular composition of these biomolecules.[46]

Lipids of the Tear Film

Normal Meibum

Biochemical characterization of meibum began in 1897,[47] when Orlando Pes confirmed its lipid nature and suggested that it was rich in fats, FFAs, and cholesterol. Several decades later Linton et al.,[30] Andrews,[48] and Ehler[49] each demonstrated that meibum was rich in neutral fats, steryl, and WE. In subsequent studies, acyl glycerols, ceramides, phospholipids, and other polar lipids were reported to be present in meibum, and most meibum components are nonpolar lipids of different classes.[28,31,50–52] The polar lipids are a minority, though they have been implicated in playing a critical role in the TF stabilization and disintegration. Several reviews on the topic has been published,[53–56] including the most recent one by the International Dry Eye Workshop.[57] Table 1 summarizes all the lipids identified by a variety of techniques to date.[7–10,13–15,20,23,29,32–34,36,47–50,52,56,58–74]

The most often detected classes of meibomian lipids are probably ubiquitous WEs and CEs. Together, they are believed to represent up to 60% of meibum lipids. WEs and CEs are among the most hydrophobic members of meibum, whose lipophilicity is rivaled only by hydrocarbons.

WEs from normal human meibum have been recently characterized using HPLC-MS^n.[7,14,18,20] Numerous WE species were detected, and the most prominent compounds were $C_{18:1}$-fatty acid based esters of very-long-chain saturated fatty alcohols with C_{18} to C_{30} carbons.[7,18,20] In a later study,[17] additional types of WEs were described that included multiple structures of $C_{18:2}$-, $C_{18:3}$-, and $C_{18:4}$-fatty acid families. Their fatty acids were esterified to the same very-long-chain fatty alcohols, as were the $C_{18:1}$-fatty acids. Compared with the monounsaturated WEs, the polyunsaturated WEs were relatively minor, but still noticeable, components of meibum. A more detailed analysis of these polyunsaturated WEs revealed that many of the individual members of these families were present in several isomeric forms, which most likely differed in the *cis*, *trans* geometry of their double bonds.[17] For example, a compound with *m/z* 641 (an ester of a $C_{26:0}$ alcohol and a $C_{18:4}$ stearidonic acid, FAl:FA, $C_{26:0}$:$C_{18:4}$) was present as four isoforms, $C_{26:0}$:$C_{18:3}$ as three, and $C_{26:0}$:$C_{18:2}$ as two, whereas $C_{26:0}$:$C_{18:1}$ was represented by just one isomer. These observations suggest that the overall number of individual WE species in human meibum exceeds 100. Using direct-infusion ESI, WEs were found to be mainly $C_{18:1}$, but with a considerable amount of $C_{16:1}$.[8] Meibomian lipids from another group (namely, CEs) are equally complex.[8,15] Unlike WEs whose major fatty acids are of modestly long $C_{18:1}$ to $C_{18:4}$ variety, CEs detected in meibum can have very long saturated and unsaturated fatty acids, with their chains ranging between C_{18} and C_{34}. More than 20 individual CEs were observed in human meibum. The dominant species were CEs with C_{22} to C_{30} FAs. The molar ratio of the oleic acid–based $C_{18:1}$ CE—one of the common CEs in other tissues and organisms—was less than 5% of all meibomian CE. Free cholesterol appears to be less than 0.5% of meibomian sterols and steryl esters.[15]

The relative amounts of phospholipids in human meibum are very controversial and unresolved, but the amount of phospholipids appears to be far less than previously thought.[6,7,13,20,22,75,76] Discrepancies in phospholipid quantitation and identification may relate to (1) variations in sample collection techniques; (2) varying degrees of contamination of meibum samples with aqueous tears; and (3) differences in instrumentation and associated techniques. However, given that the meibomian gland secretes through a holocrine mechanism and that cell membranes are enriched in phospholipids, it would seem that phospholipids are, at least initially, secreted by the gland into meibum. The ability to detect such polar lipids, and to assess the nature and extent of possible variations between experimental groups, may depend on the methods of data analysis.[9,77–79] There are factors that can affect the sensitivity of an individual phospholipid molecular species, such as the unsaturation of fatty acyl substituent and chain length; however, the most significant factor is ionization efficiency, which is dependent on the polar head group of the individual phospholipid classes. Ion-suppression effects make it difficult to observe minor components when a major class is also present in the ion source. Finally, some phospholipids are more readily detected in the negative-ion mode, whereas the opposite is true for other classes of phospholipids that are more readily detected in the positive ion mode. Those limitations complicate any direct approach to accurate quantitation of phospholipids by mass spectrometry.[77]

The lipid patterns of human meibum samples show many similarities among individuals.[9,36,79] For example, Joffre et al.[73] and Souchier et al.[80] evaluated the changes in the FA composition of normal donors and subjects with meibomian gland dysfunction (MGD) or aqueous-deficient dry eye before and after minocycline treatment. The FA composition remained consistent in the normal subjects and did not differ much from those in subjects with aqueous dry eye. The MGD patients, however, produced a different, but a repeatable, pattern with a lower ratio of saturated to unsaturated FA, and a higher ratio

TABLE 1. Lipid Composition of Human Meibum*

Method of Analysis	Hydrocarbons	Monoacylglycerols	Diacylglycerols	Triacylglycerols	Wax Esters	Sterol Esters	Cholesteryl Esters	Free Sterols	Cholesterol	Free Fatty Alcohol	Long Chain Alpha Omegas	Free Fatty Acids	Polar Lipids	Phospholipids	Sphingolipids	Squalene
Chemical stains[46]				Pos					Pos			Pos				
PC[28]				Pos					Neg			Neg		Pos		
TLC[48]				Pos (trace)			Pos		Pos			Pos		Pos	Pos (trace)	
TLC[57]			Pos	Pos		Pos		Pos				Pos				Pos
TLC/GC[47]						33		Pos†				Pos (C18)‡		Pos		
TLC[49]			17.6	2.1	68.6		1.6		5		10.4					TLC
TLC/GLC/MS[51,55,58]	25–36	0.6–1.5		11–43	13–23		8–34	Pos	Pos	0–2		0–24	0–5	0.8–5		0.7–7
TLC/GLC/MS[10,59–62]	7.54			3.7	32.32	27.28		1.63		2	Pos	1.98	14.83			
TLC/GC/MS[63]				3.1	45.2	39.4			1.2			2.8				
GLC/MS[13,32,64–69]				Pos	Pos§	Pos‖	Pos		1.87–14.42	Pos (C14-18n)¶		0.21–1.3	Pos	Pos#	Pos**	
HPLC/ESI-MS[9,33,35,70]			Pos	Pos	Pos	Pos	Pos		Pos				Pos	Pos††		Pos
RP HPLC, MS, & TLC[7,15,18,20,71,72]		Neg	Neg	Pos	Pos	Pos	30–33		<0.5	Neg		Pos	Pos‡‡	<0.05	Neg	Pos
GC/MS[73,74] MS[22]				0.05	28	Pos	13	Small	Small			Pos (C16–18) 3	Pos‡‡	Neg		

* Numbers indicate percentages; PC, paper chromatography; TLC, thin later chromatography; GC, gas chromatography; MS, mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; RP, reverse phase chromatography; HPLC, high pressure liquid chromatography; Pos, positive; Neg, negative.

† 35.7% of alcohol.

‡ C18 25% of fatty acids.

§ 81.84% of all esters.

‖ 18% of all esters.

¶ Normal chain accounts for 48–68% of fatty alcohols.

PC and PE 38% and 16% of phospholipids, respectively.

** Ceramide and cerebroside 30 and 70% of sphingolipids, respectively.

†† PC > PE.

‡‡ (*O*-acyl)-ω-hydroxy fatty acids (OAHFAs).

of branched FA to straight-chain ones. Butovich et al.[20,22] found that the composition of meibomian lipids collected from normal donors was reproducible intersubject from sample to sample when compared visually.

Normal Tears

Recently, researchers have reported that human meibum and aqueous tears differ somewhat in lipid compositions and the relative amounts of individual lipids. The most noticeable difference was an increase in the molar ratio of lower molecular weight WE-type compounds.[7,9,20,79] Another difference is in the hydrocarbon chain ordering between lipids of meibum and aqueous tears: The former were less ordered at any tested temperature and had a lower phase transition temperature. This was explained by suggesting that both types of the samples had different lipid compositions.[44] Of particular interest, aqueous tear samples show the spectrometric signals of organic phosphate esters, similar to that found in phosphatidylcholines and SMs.[24,42,81]

ANIMAL MEIBUM AND TEAR FILM LIPIDS

Animal studies on meibum lipids have predominantly focused on bovines (castrated bulls) and rabbits, although other species such as the mouse, hamster, rat, and gerbil have also been studied. The major constituents of animal meibum have been identified as sterol and WEs,[10,28,31,82,83] although the mouse may contain predominantly CEs.[84] Cholesterol appears to be the major sterol in all animals tested,[28,31,59,61,82,85,86] apart from the rabbit, in which it has been reported that 24,25-dihydro-Δ8-lanosterol is the major sterol.[83] Other sterols identified have been 5-α-cholest-7-en-3/β-ol in the meibum of cows,[28] cholestanol in that of rabbits,[82] 3β-hydroxy-5α-cholestane and 3β-hydroxy-A7-cholestene in that of hamsters,[85] lathosterol and perhaps methyl sterol in that of rats,[58] and 3β-hydroxy-5α-cholestane in that of gerbils.[86] Wax and sterol esters combined make up between 63% and 70% of the percent-weight of lipids in cow meibum[30] and 78.5% of lipids in meibum of rabbit,[82] with CE being 32% to 41% of cow meibum.[10,31] Free cholesterol or other sterols make up only approximately 3% of cow or rabbit meibum.[10,82]

The fatty acids and fatty alcohols have also been examined in detail. Using an isolated meibomian gland model, Kalattukudy et al.,[28] found that the glands of steers synthesize a high proportion of anteiso-branched chains of both the acids and alcohols. Some acids with very long carbon chains, as long as C_{36}, have been found in the ω-hydroxy fraction. Anteiso-C_{25}, -C_{27}, and -C_{23} were the most highly labeled alcohols, confirming the findings of Baron and Blough,[31] who detected that the fatty alcohol moieties of the WE in isolated meibum are branched chain $C_{23:0}$ to $C_{27:0}$.

Nicolaides et al.[10] reported that the fatty alcohols in total lipids and WEs of the steer range from C_{18} to C_{31}. The major synthesized fatty acids in the WEs are anteiso-C_{15}, *n*-C_{16}, anteiso-C_{17}, and *n*-$C_{18:1}$, whereas anteiso-C_{25} and -C_{27} are the major labeled acids in the sterol esters. Steer fatty acids appear to be mostly composed of anteiso-branched and -saturated types.[10] The triglyceride fraction which contained 8% of the total lipids is composed of labeled fatty acids similar to those found in both the sterol and the wax ester fractions.[28] A group of ω-hydroxy fatty acids have been identified from the meibomian glands of steers and humans. These acids comprise approximately 10% of all the acids of the steer lipid, are primarily monoenoic, and constitute three homologous series with members ranging from C_{30} to C_{36} or C_{38}.[60,61]

For the rabbit, all chains of the fatty acids and alcohols are saturated. Fatty acids C_{15} to C_{29} predominate which are anteiso-branched. Three fatty alcohols have been identified; anteiso-C_{25}, -C_{27}, and -C_{29}.[82] Only esters of dihydrolanosterol have been found that contain anteiso-C_{15} to -C_{19} fatty acids. The principal WE contain anteiso-branched C_{25} to C_{31} fatty alcohols and anteiso-branched C_{15} to C_{19} fatty acids in combination, making esters in the C_{40} to C_{46} range.[83] FFAs makes up only 4.4% ± 0.2% of the total lipids.[82]

Harvey and Tiffany[84] reported that the fatty alcohols in mouse meibum are predominantly iso-C_{26} and anteiso-C_{27}. Monounsaturated fatty acids belong mainly to the ω-9 series, and saturated acids belong to the iso-, anteiso-, and *n*-series. Several 1,2-diols were also identified, with the most abundant of these being iso-C_{16} and iso-C_{20}. GC-MS studies on the intact WEs showed them to be composed of the branched-chain alcohols and both branched-chain and unsaturated acids.[84] Studies on the meibum of hamsters have shown fatty acids with chain lengths from 10 to 32 carbon atoms are found, the most common being C_{15} to C_{18} and C_{25} to C_{30}.[85] Chain types are predominantly iso- or anteiso-branched, monounsaturated (C_{16} and C_{18}), and straight. Fatty alcohols are mainly from the iso or anteiso series and tend to have longer chain lengths; the major alcohols have anteiso-C_{25} and -C_{27} and iso-C_{26} chains.[85] The tears of Golden hamsters contain considerable amounts of the unusual lipid, 1-alkyl-2,3-diacylglycerol.[87] The rat has fatty acids in its meibum with chain lengths of between C_{12} and C_{34} and a biphasic distribution of maxima around C_{16} to C_{18} and C_{25} to C_{27}. The chains are straight-chain iso, anteiso, and monounsaturated. The unsaturated acids have double bonds in the ω-7 and ω-9 positions. The alcohols have corresponding structures.[59] The gerbil has fatty acids in its meibum with chain lengths from C_{12} to C_{27} with, again, a biphasic distribution with maxima at C_{15} to C_{18} and C_{25} to C_{27}. Chains are predominantly iso- or anteiso-branched. Unsaturated fatty acids are mainly C_{16} and C_{18}. Fatty alcohols are mainly branched, with chain lengths between C_{25} and C_{27}, although there were also several fatty alcohols, both branched and unsaturated, with chains up to C_{33}.[86]

The phospholipids of rabbits have been examined in a unique NMR study on animal tears and meibum, Greiner et al.[27] reported that the phospholipid in meibum of rabbits is composed of 40% phosphatidyl choline, 18% phosphatidyl ethanolamine, 9% SM, 9% ethanolamine plasmalogen, 7% phosphatidyl serine, and 6% dihydrosphingomyelin. Ham et al.[37] reported that species related to platelet-activating factor and/or lysophosphatidylcholine, phosphatidylcholine, and SM were found in the tears of normal rabbits and rabbits made to have dry eye through surgical procedures. The varieties and the concentrations of SM were greater in tears of rabbits with dry eye than in those of normal rabbits.[37] In addition, the authors reported that they could not detect phosphatidylserine in the tears of normal rabbits, but this lipid was detectable in the tears of those with dry eye.[37] The amount of polar lipids in the meibum of bovine (steers) has been reported to be 13.3% of the total lipids.[10]

Meibum Lipid Changes in Disease

McCulley et al.[88] demonstrated that various forms of blepharitis are associated with changes to the lipid composition of the meibomian gland secretions. Also, meibomian secretions from patients with meibomian keratoconjunctivitis (MKC) have shown lower levels of unsaturated fatty acids and alcohols of the wax and cholesterol esters and occasionally differences in triglyceride profiles.[33,68,69]

There are generally low levels of phosphatidyl ethanolamine (PE) and SM in meibum of patients with blepharitis who also have dry eye symptoms.[89] In vitro, SM can inhibit peroxidation of unsaturated fatty acids in phosphatidyl choline monolayers,[90] and it has been shown that lipid peroxides can be significantly higher in tears of patients with severe-to-moderate dry eye ($P < 0.05$) or in those with good TF production but increased symptoms than healthy controls.[91] Alternatively, the increase in oleic acid in the meibum of those with meibomian seborrhea may help explain the clinically significant burning symptoms that this group of people reports.[92]

Some of the differences seen in lipid types associated with different forms of blepharitis may be due to the presence of certain types of commensal lid bacteria that can hydrolyze lipids. People with meibomian seborrhea with a clinical appearance of *Staphylococcus* infection appear to harbor significantly more coagulase-negative *Staphylococcus* (CNS) strains capable of hydrolyzing cholesteryl oleate than do normal individuals.[65]

In addition, differences in subgroups have been demonstrated in people with androgen hormone deficiency, including males taking antiandrogen therapy, females with complete androgen insensitivity syndrome, and persons with Sjögren's syndrome, all of whom show changes in the polar and nonpolar lipid components.[34,38,64,79]

Obstructive MGD is characterized by an abnormal structure of the gland with characteristic changes in viscosity of the lipid expressed.[64] An analysis of the lipid components in patients affected by MGD showed a significant decrease in triglycerides and cholesterol[93] and a decrease in the amount of monounsaturated fatty acid, specifically in oleic acid.[92] Decreased unsaturation of the nonpolar fatty acids tends to increase their melting point, leading to thickening of the meibum within the central duct. Infrared studies have shown that lipid order and phase transition temperatures are higher in meibum of donors with meibomian gland disease.[44]

Recently, Joffre et al.[74] demonstrated that the fatty acid profile of the excreta collected by Schirmer test strips in people with blepharitis is significantly different from controls. Total saturated fatty acids were 9.3% in those with blepharitis versus 24.6% in controls, with lower quantities of palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids. Branched-chain fatty acids were present in greater proportion in MGD patients. Interestingly, small differences were observed in fatty acid composition between those with blepharitis and those with dry eye, with 50% more linoleic acid in the dry eye group.[74]

The TF lipid layer changes secondary to MGD have a negative effect on the quality of vision measured as contrast sensitivity[94] and on evaporation of tears from the ocular surface. Gilbard et al.[95] demonstrated in rabbits that meibomian occlusion resulted in a rise of tear osmolarity, possibly as a consequence of an increased evaporation rate of water from the TF. Goto et al.[96] showed an alteration of the lipid layer determines an increased tear evaporation rate in people with MGD, which

TABLE 2. Summary of Soft Contact Lens Groups, as Classified by the FDA

Group	Water Content	Polymer Type	Lens Material
I	<50% H_2O	Nonionic polymer	Tetrafilcon A, Polymacon
II	>50% H_2O	Nonionic polymer	Lidofilcon A or B, Alfafilcon A, Omafilcon A, Nelfilcon A, Vasurfilcon A, Hioxifilcon A
III	<50% H_2O	Anionic polymer	Bufilcon A, Phemfilcon A, Ocufilcon A
IV	>50% H_2O	Anionic polymer	Etafilcon A, Vifilcon A

Source: U.S. Food and Drug Administration.

could be responsible for the increase of hyperosmolarity, which, in turn, is able to produce an inflammatory response and damage of ocular surface epithelia. The biochemical changes in meibomian gland lipids may have a direct toxic effect on ocular tissues, since FFAs have been shown to be able to irritate the skin in acne vulgaris.[76]

The consistent finding of higher than normal levels of FFA in MGD offers a potential basis for symptoms associated with MGD. However, a study of the effect of branched-chain fatty acids on cultivated conjunctival human cells treated with iso-C_{16} and iso-C_{20} has shown no effects on the parameters of cytotoxicity. Only the mitochondrial dehydrogenase activity was significantly decreased in relation to the isoC_{20} concentration increase.[74]

Ocular rosacea has been associated with MGD.[97] The concentration of triglycerides and FFA, especially the mono- and polyunsaturated forms, is increased and may be responsible for the activation of neutrophils and inflammatory mediators.[69] Furthermore, the analysis of the ocular microbiota of patients with rosacea demonstrated bacterial growth in all patients,[98] suggesting that rosacea may induce the production of lipases which in turn could disrupt the lipid layer of the TF and its protective role.

LIPIDS ON CONTACT LENSES

Types of CLs

There are two major classes of CLs: the rigid lenses and the soft lenses. Rigid lenses were initially made from polymethyl methacrylate (PMMA). The newer generations of rigid CLs, (rigid gas-permeable [RGP] lenses), add fluorine and/or silicone to the base material to facilitate oxygen transport. The rigid CLs are classified into four groups: group I materials contain no silicone or fluorine; group II materials contain silicone but not fluorine; group III contain silicone and fluorine; and group IV contain fluorine but not silicone.[99–101]

Soft CLs have been traditionally made from hydroxyethyl methacrylate (HEMA) and may add vinyl pyrrolidone or methacrylic acid. The U.S. Food and Drug Administration classifies soft CLs into several groups (I to IV) based on their water content and overall ionic nature of the lens material. Table 2 summarizes the properties of the soft CLs.

Since 1999, so-called silicone hydrogel CLs have been commercially available. These lenses were designed to achieve the oxygen permeability given by silicone and fluorine but in a soft lens form (>10% water content material). These lenses are classified in the FDA soft lens classification system outlined above. However, due to their very different chemical natures compared to classic HEMA-based soft lenses, there are proposals to separately classify these lenses into their own group (group V) within the soft lens system (Table 3).[101]

Lipid Deposition

While lipids from the meibomian gland appear to be essential for ease of lens wear,[102] investigators have analyzed the deposition of lipids onto the surface of CLs due to the possible clinical consequences. In in vitro experiments, RGP lenses lipid deposition has been shown to be dependent on the lens matrix hydrophobicity.[103] For the polymers siloxanyl alkyl acrylate and fluorosiloxanyl alkyl acrylate (silafocon A and paflufocon B, respectively), lipid in an artificial tear solution enhanced protein deposition but that protein in the artificial tear solution decreased lipid deposition on only the siloxanyl alkyl acrylate lens.[103]

In addition, differential lipid deposition can be seen by group. Group IV hydrogel lenses bind more phosphatidylcholine (although at <1 μg/lens) than other lens groups, possibly reflecting an interaction between the positively charged choline residue and the negative surface of the lens.[104]

Hydrogel lenses made of poly(2-hydroxyethyl methacrylate), poly(methy1 methacrylate)-poly(vinyl alcohol) or poly(2hydroxyethyl methacrylate)- poly(viny1 pyrrolidone)-poly(methacrylic acid) can all adsorb lipids from solution in vitro, and lenses made from poly(methy1 methacrylate)-poly(vinyl alcohol) tend to adsorb slightly more lipid.[105] Adsorption of cholesterol to poly(HEMA) lenses may collapse/condense the hydrogel lens material and expel water, whereas lipid binding to PMMA lenses was simply an adsorptive process.[106]

Cholesterol adsorbs in greater quantities than phosphatidylethanolamine for silicone hydrogel lenses or group IV lenses (Table 4).[107]

In vitro, the polyvinylpyrrolidone in both galyfilcon A and senofilcon A may be responsible for the increased binding to cholesterol or phosphatidyl ethanolamine. Silicone hydrogels bind cholesterol in relatively high levels and also bind squalene, CE, and WE.[108] Similarly, the level of lipid binding was greater for galyfilcon A (group Vd) and balafilcon A (group

TABLE 3. Proposed Classification of Silicone Hydrogel Lenses

Group	Basis of Categorization	Examples of Polymer Types
Va	Nonlinear relationship between Dk (oxygen permeability) and water content	Comfilcon A
Vb	Contain an ionic (anionic) component	Balafilcon A
Vc	Plasma or bonded surface modification	Lotrafilcon A and B; Asmofilcon A
Vd	"Released" wetting agent	Galyfilcon A; Senofilcon A

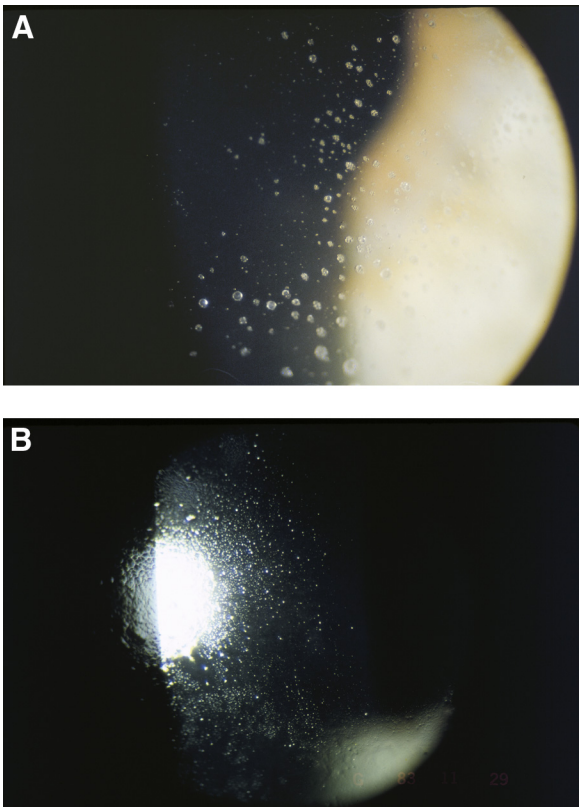

FIGURE 2. Soft contact lens lipid deposits seen on slit lamp examination (photos courtesy of the Brien Holden Vision Institute).

Vb) than for lenses from group Vc (again no group Va lens was tested).[107] Indeed, levels of cholesterol, squalene, cholesterol esters or wax esters on the group Vb lenses (lotrafilcon A and lotrafilcon B) were similar to levels adsorbed to a group IV HEMA-based hydrogel lenses.[108]

Initial in vivo studies demonstrated that lipid was present in deposits on CLs, with the principal lipid type being CE.[109] A particular form of deposit on lenses, often called jelly bump deposits, was shown to be composed of long and intermediate sized CE, triglycerides, and WE.[110] White spots, a similar particular type of deposits found on non-regularly replaced hydrogel lenses, are predominantly comprised of lipid.[111,112] The lipid white spot deposits have a distinct structural stratification with a lipid layer providing the interface between the CL surface and the deposit superstructure. This initial interfacial layer has been shown to be made from cholesterol, cholesterol ester and unsaturated lipids.[111,112] Of note, diet plays a part in formation of these white spots, and individuals who consumed larger amounts of alcohol, protein, and fat exhibited increased lipid deposition on their lenses.[113] As hydrogel CLs tend to be replaced much more frequently today compared to 15 years ago (typically on a weekly, bi-weekly, or monthly basis today versus annually in the past), the incidence of these white spot deposits (Fig. 2) has reduced and they are now rarely seen.

In vivo, RGP lenses deposit more lipid than many soft lens materials, probably due to the hydrophobicity of the lens.[114] Silicone-based RGP lenses also deposit more lipid than fluorine-containing RGP lenses, probably because silicone increases the hydrophobicity of the CL, whereas fluorine decreases hydrophobicity and thus decreases lipid deposition.[114] The level of lipid deposition on group 1 (polymacon and tetrafilcon A) and group III (Balafilcon A) appears to be related to characteristics of the wearer rather than lens material per se.[115] FDA group II lenses deposit the most lipid, and FDA group III lenses deposit the least.[103,114,116] On group II lenses (containing polyvinyl pyrrolidone) lipid deposition appears to increase over time (from 1–28 days of wear; $P < 0.0001$), whereas lipid deposition on the group IV lens reaches a maximum after 1 day and increases no further.[117] Lipid levels on group II lenses containing polyvinyl pyrrolidone are approximately twice that on group IV lenses,[118] and again, there was a significant intersubject variation in lipid deposition levels. Lipid deposition on lenses ex vivo is shown in Tables 4 and 5.

Overall lipid deposition increases with longer replacement schedules (3 months vs. 1 month).[121] These studies did not characterize the lipid types, but measured lipid adsorption using spectrophotometric methods, the sulfo-phospho-vanillin reaction or estimation of total phosphate (for phospholipids). The lipid deposits on worn hydrogel lenses were chemically analyzed, and detected WEs, fatty sterols, fatty alcohols, FFAs, and monoglycerides, whereas cholesterol, CEs, and triglycerides were not detectable.[122] However, cholesteryl oleate, cholesterol, oleic acid, oleic acid methyl ester, and triolein were detected in extracts from worn hydrogel and RGP lenses.[114] These discrepancies may be due to the types of lenses being investigated, with polar lipids depositing preferentially onto the more hydrophilic lenses compared to nonpolar lipids. Oleic acid methyl ester appears to adsorb less to group III and IV hydrogels and RGP than other lens types.[114]

For the silicone hydrogel lenses (groups Vb and Vc), the degree of lipid deposition in vivo appears to be substantially higher than that seen with conventional hydrogels.[123] Cholesterol was the most commonly deposited lipid, although oleic acid and oleic acid methyl ester was also detected.[123] Another study using balafilcon A, lotrafilcon A, and galyfilcon A lenses (groups Vb, Vd, and Vc, respectively) was also able to detect cholesterol in deposits,[123] but found very low levels of oleic acid or its methyl ester (summarized in Table 6).

Overall the levels of lipid deposition on the silicone hydrogels lenses were lotrafilcon A < galyfilcon A < balafilcon A,

TABLE 4. Deposition of Lipids onto Contact Lenses[107]

		Amount of Lipid Adsorbed In Vitro (μg/lens)			
Soft Lens Type	**Polymer Name**	**Cholesterol**	**Cholesterol Oleate**	**Phosphatidyl Ethanolamine**	**Dioleoyl Phosphatidylcholine**
Group I	Polymacon		0.5		0.1
Group II	Lidofilcon A		0.6		0.1
Group III	Phemphilcon A		0.7		0.3
Group IV	Phemphilcon A		0.9		0.3
Group IV	Etafilcon A	7.0		0.1	
Group Va	Balafilcon A	24.1		3.2	
Group Vc	Lotrafilcon B	3.0		1.5	
Group Vd	Senofilcon A	23.2		4.9	

TABLE 5. Amounts of Lipid Deposited on Lenses during Wear

Soft Lens Polymer Type	Polymer Name	Amount of Lipid Adsorbed In Vivo (μg/lens, unless otherwise stated)		
		Total Lipid	Phospholipids	Cholesterol
Group I	Polymacon	66.3,[103] 62[113]	2.1, 1180.01–0.05 (micromoles/lens)[117]	
Group II	Alphafilcon	427 (fluorescent units)[111]		
Group IV	Etafilcon A	44.1,[116] 29 (fluorescent units)[111]	1.8[118]	
Group Va	Balafilcon A		19 (ng/lens SM), 19 (ng/lens PC)[120]	4.1–8.2,[119] 3.9[120]
Group Vb	Lotrafilcon B			0.1–0.5[119]
Group Vd	Senofilcon A		59 (ng/lens SM), 195 (ng/lens PC)[120]	0.3–2.7,[119] 9.9[120]

SM, sphingomyelin; PC, phosphatidylcholine.

similar to the ranking and amounts seen during in vitro experiments.[107,108] The differences in the cholesterol deposition between the two studies (300–600 μg/lens), are not easily explained.[123,124] Another study examining the deposition of cholesterol onto various silicone hydrogel lenses had a ranking of lens types in their deposition of cholesterol; lotrafilcon B (group Vc) < senofilcon A (group Vd) < galyfilcon A (group Vd) < balafilcon A (group Vb), and the study identified the use of various cleaning/disinfecting solutions as a significant modulator of cholesterol deposition.[120] Saville et al.[114] found during silicone hydrogel lens wear (senofilcon A or balafilcon A) adsorption included a range of molecular type of both SM and PC, with SM $C_{16:0}$ and PC $C_{34:2}$.

Clinical Changes of Lipids and CL

The literature is unclear whether the deposition of lipid on CLs affects comfort, or whether clinical testing can be used to detect changes in lipid profiles on lenses. Clinically, galyfilcon A lenses (group Vd) tend to have more grade 3 to 4 lipid deposits than group IV lenses.[120] Soaking silicone hydrogel lenses (group Vc) in lanolin reduces the drying time of tears over the lens surface and TF appears thinner over lens surface.[125]

There is an apparent decrease in cholesterol levels in tears for around 10 hours after lens insertion occurs.[126] In addition, the phospholipid concentrations in tears of patients wearing polymacon (group I) or etafilcon A (group IV) lenses were 186 ± 39 μg/mL and 162 ± 33 μg/mL, respectively, with the latter concentration being significantly lower than that observed in the same subjects when not wearing CLs (220 ± 35 μg/mL; $P = 0.0023$).[127] This may be of significance as concentrations of these phospholipids in the tears of patients with marginal and moderate dry eye have been reported to be significantly lower than those in subjects without dry eye[128] and CL wear is well known to cause dryness and discomfort sensations in significant proportion of wearers.[129,130] Furthermore, two studies[131,132] reported that hydrogel lens wear altered the TF lipid composition by decreasing the levels of polar lipids and increasing levels of nonpolar lipids. These studies also found low levels of tear polar lipids (phospholipids) were associated with increased levels of tear instability during soft CL wear. It is possible that phospholipids in tears are degraded by group IIa secretory phospholipase A2 (sPLA2) deposited on CLs; etafilcon A (group IV) lenses deposit statistically significantly more group sPLA2 than polymacon (group I) lenses.[133,134] These changes to the biochemistry of the TF may manifest as overt changes to the clinical picture of the lipid layer on the surface of the TF.

It has been established that wearing most CL types results in a disruption to the lipid layer appearance of the TF.[135–137] Lipid layer thickness assessed via interference fringes is generally classified into six different types on the basis of the fringe patterns seen via a slit lamp system. These patterns—none, meshwork, wave, amorphous, colors, and other—increase in thickness layers form none to colors. During lens wear, the lipid layer does not uniformly coat RGP or soft hydrogel lenses.[135–137] There is, however, an increase in lipid layer continuity over high-water-content soft lenses.[138] Another study comparing two high-water-content hydrogel lenses, filcon 4A (67% water content) and lidofilcon (70% water content), found no difference between the two materials in terms of lipid layer appearance, but did demonstrate that the lipid layer over a lens just after waking was thicker than during normal open-eye conditions and that this correlated with more stable TF after waking than during open-eye.[139]

When the well-formed TF, including a healthy lipid layer over the CL, is missing or abnormal, evaporation of the TF during CL wear can occur, which may then lead to ocular discomfort. Thai et al.[140] demonstrated that wearing either soft hydrogel lenses or silicone hydrogel lenses leads to increased evaporation of tears from the eye when compared to nonwear. While there were no statistically significant differences between evaporation rates between lens types, individuals did show significant differences with different lens types.[140] Omafilcon A lenses have been noted in other studies to create thicker lipid layers.[140,141] The ability of these lenses to sustain a thicker lipid layer may be due to the biomimetic nature of the phosphorylcholine in the lens. In vivo, the galyfilcon A lenses (silicone hydrogel) have a thicker lipid layer than the alphafilcon A (group II HEMA), which may give the former a more stable TF than the latter.[141]

In the absence of lens wear, no difference has been found in the lipid layer thickness (lipid layer pattern) or TF stability in asymptomatic or symptomatic lens wearers.[142] However, TF stability is decreased in those intolerant of lens wear when compared with those who are tolerant, even though the lipid layer appearance was not different between the two groups.[143] Further, people intolerant of CL wear (defined as being unable

TABLE 6. Lens Materials and Various Deposit Levels[124]

Lens Material	Cholesterol (mg/Lens)	Oleic Acid (mg/Lens)	Oleic Methyl Ester (mg/Lens)
Balafilcon A	15.6 ± 3.9	1.0 ± 0.4	0.2 ± 0.4
Lotrafilcon A	0.5 ± 0.3	0.7 ± 0.5	0.0 ± 0.1
Galyfilcon A	9.9 ± 5.7	0.7 ± 0.7	0.1 ± 0.2

to wear CLs for more than 6 hours throughout the day) have increased levels of malondialdehyde and 4-hydroxy-2(E)-nonenal (degradation products of polyunsaturated fatty acid and related esters) in their TF. In addition, intolerant subjects had significantly more sPLA2 in their tears compared with tolerant subjects. No differences in the number of blocked meibomian glands were found between the two groups.[144]

Tear Lipid–Protein Interactions

The seminal paper on tear lipid–protein interactions was published in 1973.[12] Holly showed that the spread of lipids is facilitated by mucins.[12] With high surface pressure and low surface tension, the meibum lipid coalesces into a droplet and does not spread across the surface of water. When mucin is dissolved in water, such as with the mucin–aqueous gel gradient of the TF (Fig. 1), the surface tension is lowered allowing meibum lipids to spread across the aqueous surface.

Lipocalin

Cholesterol, fatty acids, fatty alcohols, glycolipids and phospholipids in the TF are bound by lipocalin and the binding remains after several levels of chromatographic separation.[145] Additional binding studies of tear lipocalin revealed that apo tear lipocalin has a high affinity for phospholipids and stearic acid (K_i) of 1.2 and 1.3 μM, respectively, and much less affinity for cholesterol (K_i) of 15.9 μM. For fatty acids, binding affinity correlates with the length of the hydrocarbon chain. Tear lipocalin binds most strongly to the least soluble lipids permitting these lipids to exceed their maximum solubility in aqueous solution. These data implicate tear lipocalin in solubilizing and transporting lipids in the TF.[145,146] Lipocalin is a major tear protein comprising 33% of total protein in a tear sample.[147] It is secreted by the lacrimal gland and has also been detected in meibomian gland secretions.[148] The structure, function, and molecular mechanisms of action of tear lipocalin have recently been reviewed.[149–151]

Conformational changes in tear lipocalin are evident when lipid binds the protein.[152] It has been proposed that lipocalin scavenges lipid from the corneal surface and may enhance the transport and equilibration of lipid in the lipid surface layer.[153] Recently, the solution structure of tear lipocalin bound to a native ligand was elucidated and the entire binding energy landscape was clarified by using a modification of site-directed tryptophan fluorescence.[154] Gasymov et al.[154] describe the process in which ligands explore multiple binding sites in nanoseconds before exiting the cavity of the protein. In Figure 3, the more intense red indicates greater static quenching or static binding. Subsequently, the tear lipocalin was crystallized in space group $P2_1$ with four protein molecules with bound artificial ligand 1,4-butanediol and its x-ray structure was solved at 0.026-nm (2.6 Å) resolution.[155] Breustedt et al.[155] showed that the loop region and adjoining areas of the β-barrel allow considerable conformational flexibility, which allows tear lipocalin to adapt to ligands that differ vastly in size and shape. This observed promiscuity in ligand recognition may be important in understanding the function of tear lipocalin and lipid–protein interactions on the TF.

Tiffany and Gouveia[156] found an interactive role of lipids and proteins in their tear viscosity study. It has been suggested that lipocalin forms dimers when lipid is bound to the protein. However, more recent work demonstrates that it is likely that tear lipocalin exists mainly as a monomer in the TF and that the dimeric form is minimal.[153,157]

Tear lipocalin deficiency is associated with meibomian gland dysfunction[158] and the studies above show that lipocalin sequesters lipids. Whether the lipocalin/ lipid complex interacts with the lipid layer has been the focus of recent studies. When human meibum was used in a in vitro study by Millar et al.,[159] lipocalin bound slowly to a human meibomian lipid film compared with lysozyme or lactoferrin. The adsorption of lipocalin to a human meibomian lipid film was very different from its adsorption to a bovine meibomian lipid film, indicating the nature of the lipids in the film is critical to the adsorption process.

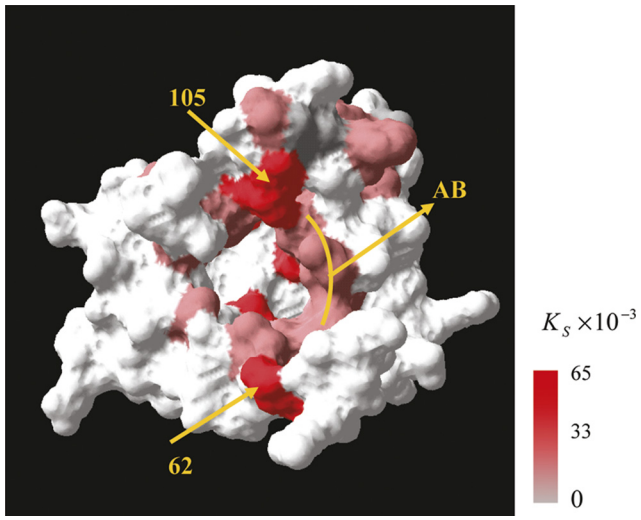

Figure 3. The solvent-accessible surface of TL. Residues are colored according to the static quenching constants observed with C12SL. Reprinted from Gasymov OK, Abduragimov AR, Glasgow BJ. Intracavitary ligand distribution in tear lipocalin by site-directed tryptophan fluorescence. *Biochemistry*. 2009;48:7219–7228 with permission from the American Chemical Society.

Based on these studies, it seems likely that tear lipid–protein interactions occur in vivo and that these interactions change the physical properties of tears. There are several gaps in knowledge that when filled could facilitate the development of therapies to reduce dry eye and MGD symptoms.

Lysozyme

Lysozyme, a major protein found in tears, acts as a bacteriolytic protein that depolymerizes mucopolysaccharides. Lysozyme does not sequester lipids as lipocalin does,[146] but it does interact with and binds in vitro to the phospholipids of membranes[160] and meibum films.[17,159,161,162] It is possible that lysozyme not only stabilizes the structure of the lipid layer but that loss of lysozyme in disease states disrupts this stability, causing an increase in the rate of evaporation.

Changes in the concentration of tear lysozyme by disease or drugs may disrupt the structure of the lipid layer of the TF. It would be useful to define interactions between lysozyme and tear lipids in vitro using spectroscopic approaches and to determine whether or not structural alterations in the lipid layer caused by a change in the concentration of lysozyme leads to increased rates of evaporation.

Apolipoprotein D

Apolipoprotein D (apoD) is a member of the lipocalin super family and has been shown to be produced in the lacrimal gland and has been found in the tears.[163] Although the physiological function of apoD is currently unknown, it has the ability to bind phospholipids, cholesterol, and other lipids. The function of this protein in tears may be to interact with the meibomian lipids present in human tear fluid and perhaps contribute to the surface spreading of these lipids. Another possible function could be as a

clearance factor, protecting the cornea from harmful lipophilic molecules.[163]

Phospholipid Transfer Protein

The presence of phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) in human tears was investigated using Western blot analysis and quantitated using ELISA.[164] PLTP was found to be present in tear fluid, whereas CETP was not. ELISA indicated that the PLTP concentration in tear fluid, 10.9 ± 2.4 μg/mL, is approximately two times higher than that in human plasma. PLTP-facilitated phospholipid transfer activity in tears, 15.1 ± 1.8 micromoles mL^{-1} h^{-1}, was also significantly higher than that measured in plasma. These results suggest that PLTP may be involved in the formation of the TF by mediating lipid transfer in tear fluid. However, the concentration of this protein is relatively small in tears compared to lipocalin and PLTP has not been specifically shown to bind with lipids in tears.

The studies by Millar et al.[3,162] show that mucin binds to meibum in vitro; however, mucin–lipid interactions have never been studied on a molecular level. Currently, there is no evidence that certain mucins may interact with the lipid layer; conversely, there is no evidence mucin–lipid interactions do not exist, either.[165]

Although the amount of fatty acid and cholesterol bound to lipocalin has been quantified, the amount and type of phospholipid and lysophospholipid bound to lipocalin has not. In addition, it can be hypothesized that most phospholipids in tears could be bound to lipocalin. Although the interactions between various lipids have been studied extensively, those directly relating to the mucin–lipid relationship, if any, remain a mystery. Research has yet to quantify whether binding of lipocalin, lysozyme or tear fluid components to meibum lipid cause molecular–structural changes to the proteins or lipids. Nor do we yet know whether compositional changes in meibum with age or MGD alter the binding of proteins to meibomian lipids.

Influence of Bacteria on Tear Film Lipids

The normal microbiota of the eye, including *S. aureus*, *Haemophilus influenza*, CNS, *Propionibacterium* sp., and *Corynebacterium* sp.,[166,167] produces enzymes that can degrade the lipids of the TF. CNS, *Propionibacterium acnes*, and coryneform bacteria from patients with chronic blepharitis, which along with *S. aureus* strains, produce lipolytic exoenzymes (cholesterol esterase, fatty wax esterase, and triglyceride lipase) that can hydrolyze cholesterol esters and WEs.[88,168] A higher number of CNS, able to produce these enzymes, has been found on lids of patients with seborrheic blepharitis or meibomian keratoconjunctivitis.[169] Changes in FFA of meibum secretions were also found in these patients. Of note, the ability of tetracycline to inhibit lipolytic enzymes before and during the inhibition of the growth of the bacteria[66] may explain the effectiveness of such antibiotics in the treatment of conditions such as blepharitis, with or without meibomitis. Treatment of patients with blepharitis with minocycline decreased the concentration of diglyceride, FFA, and free cholesterol in the meibomian secretions, suggesting that this antibiotic had inhibited lipolytic exoenzymatic activity on the parent triglyceride, cholesterol ester, and fatty wax molecules.[33] Clearly, the exact role of bacteria in changing TF lipids has yet to be fully resolved. There also should be further investigation into the molecular changes associated with bacterial colonization and use of antibiotics.

Surfactant Proteins of the Tear Film, Ocular Surface, and Lacrimal Apparatus

Superficially active substances, similar to the surfactant system of the lung, are of importance not only in TF but also in the auditory tube and on the skin.[170] The presence of surfactant-associated protein D (SP-D) has been described in TF and lacrimal glands.[171–173] Ni et al.[174] were able to show that SP-D is also present in the cornea of mice and shows protective effects against keratitis caused by *P. aeruginosa*. It was recently demonstrated that not only SP-D, but also the surfactant-associated protein A (SP-A), along with SP-B and SP-C, is present at the ocular surface (conjunctiva, cornea) in the lacrimal apparatus (lacrimal glands, nasolacrimal ducts) and in tears.[175,176] SP-A and SP-C in tear fluid, and SP-C in all examined tissues, show an expression pattern different from that of lung surfactant proteins, with the exception of lowering surface tension.[166] This difference is probably due to tissue-specific posttranslational or posttranscriptional modifications of the proteins and may lead to differences in the activity spectrum of the surfactant proteins. SP-A, -B, -C, and -D are found in the acinar cells of the lacrimal gland and the accessory lacrimal glands of the lids, in the conjunctival epithelial cells and in columnar epithelial cells (particularly apically) as well as in serous portions of seromucous glands in the efferent tear ducts. Goblet cells do not produce any of the four SPs, and in contrast to tear fluid, the aqueous humor does not show the presence of SPs under physiological conditions.[176] SP-B and SP-C are absent inside the cornea and on its surface.[175] Thus, besides SP-D, which has already been reported as present in TF,[172–174] SP-A, SP-B, and SP-C proteins are also present in the lacrimal fluid and on the ocular surface.[175,176]

Seeing that the hydrophobic surfactant proteins SP-B and SP-C exert an expanding influence on the surface tension of the air–liquid interface atop the alveolar lining layer, a similar effect could be discussed in relation to the TF and the tear fluid at the human ocular surface and efferent tear ducts. Within this context, a modified scheme of the TF could contain the surfactant proteins as evidenced and investigated with respect to their physicochemical properties and putative functions as discussed in tears, the lacrimal apparatus and at the ocular surface. This view demonstrates the possibility of small hydrophobic surfactant proteins B and C embedded into the lipid component of the TF, oriented according to their amphiphilic character. Furthermore, the water-soluble and polymerizeable collectin-like surfactant proteins A and D could be arranged within the aqueous component of the TF along with the various secreted and shed mucins that are already known. This hypothetical model supports possible functions of surfactant proteins in relation to severe diseases of the ocular surface (e.g., dry eye syndrome and as bacterial and viral infections). Hypothetically, absence of the small hydrophobic surfactant proteins B and C could result in alterations of TF stability and, as a consequence, interruption of the TF itself, leading to signs of dry eye syndrome (such as dry spots).

Further research is needed to establish a system of expression in which all four surfactant proteins can be manufactured by using recombinant methods, including open reading frames for mature forms and pre forms of the proteins. The recombinant surfactant proteins produced in this manner could be used in functional studies of the ocular surface and lacrimal system. Some recombinant surfactant proteins have already been manufactured.[177,178] With the software programs and methods available today, it is possible to use comparative protein modeling or threading to create a reliable model of the 3-D structure of proteins and to identify the active sites. Blocking or modification of assumed active sites could alter the

functionality of the proteins and lead to completely new proteins at the functional level.

Conclusions

Understanding the molecular composition (proteomics, lipidomics) of the TF and the contribution of the meibomian gland to the TF is critical to understand and describe TF instabilities, dry eye syndromes, CL incompatibilities and other eye diseases.

Elucidation of the compositional components of meibomian gland secretion and the TF has been challenging in the past because of limitations to analytical and biochemical techniques. Most analytical techniques had low sensitivity and low resolution, required large sample amounts (requiring pooling), and chemical derivitization for detection. This limitation caused possible degradation of the sample because of prolonged exposure time; low sample recovery due to derivation, isomerization and/or decomposition due to sustained high temperature analysis; long analysis time; lack of information on the actual molecular composition of the lipids; and contamination. Recently, new advances in analytical and biochemical techniques have allowed researchers better methods to examine the meibomian gland secretions and TF components with the ability to identify the actual specific molecular composition of lipids, proteins, posttranslational modifications and protein–lipid interactions. Although the signs and symptoms of TF instability are reasonably well characterized, we are only beginning to scratch the surface of understanding the specific molecular components of the TF and their relationship with MGD and TF stability.

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