Analysis of Comparison of Human Meibomian Lipid Films and Mixtures with Cholesteryl Esters In Vitro Films using High Resolution Color Microscopy

Thomas J. Millar¹ and P. Ewen King-Smith²

PURPOSE. The lipid layer of the tears has been studied in vivo using high resolution color microscopy (HRCM). The purpose of these experiments was to gain further insight into the structure of the lipid layer by applying HRCM to in vitro meibomian lipid films.

METHODS. Films of human meibomian lipids, cholesteryl nervonate, cholesteryl palmitate, or their mixtures, were spread on a Langmuir trough. Changes to the films were monitored using HRCM as the films were compressed to different surface pressures. The penetration of albumin into a meibomian lipid film also was studied.

RESULTS. Small amounts of meibomian lipids at low pressures formed very thin films estimated to be 5.2 nm thick. Compression caused spots to appear in the films. At higher concentrations, micro lenses were a feature of the film. Cholesteryl nervonate formed a multilayered oil slick that did not change with surface pressure. Cholesteryl palmitate formed a stiff film that collapsed at high compression. Mixtures of cholesteryl nervonate and meibomian lipids showed that they mixed to increase surface pressures above that of the individual components. HRCM also allowed albumin to be seen penetrating the meibomian lipid film.

CONCLUSIONS. HRCM combined with in vitro surface pressure measurements using a Langmuir trough is useful for modeling meibomian lipid films. The films often resemble the appearance of the lipid layer of in vivo films. The data indicate that the lipid layer might be modeled best as a duplex film containing an array of liquid crystals. (*Invest Ophthalmol Vis Sci.* 2012; 53:4710–4719) DOI:10.1167/iovs.12-10022

The seminal work of Holly and his co-workers^{1–6} signalled the beginnings of trying to understand the physical properties of the tear film in relationship to its chemical components. At that time, the lipid layer was perceived as just a blanket preventing evaporation,⁷ but Holly began to investigate the role of meibomian lipids in spreading of the tear film across the ocular surface and stabilising the film. This led to a model involving lipids and then a mucin lipid mixture

spread from the lid margins across the ocular surface upon eye opening.^{2,3}

Since this time, to understand further the structure of the lipid layer, a great deal of effort has been placed on determining the composition of meibomian lipids from humans and animals.^{8–19} The analysis from several groups now suggests a composition of mainly cholesteryl and wax esters (~80%), triglycerides (~3%), and omega hydroxy acyl fatty acids (~5%).^{8–19} Although the major portion being wax and cholesteryl esters is consistent across the literature, the ratios vary considerably from 13–68% for wax esters and 8–67% for cholesteryl esters. The latest analyses indicate that cholesteryl esters increase with age¹⁸ and wax esters in human meibum estimates are around 40%.¹⁹ From their own analysis of meibomian lipid composition, McCulley and Shine proposed a model that described the major lipid components as self assembling into a structured hydrophobic phase at the air interface interacting with a surfactant polar phase at the aqueous surface,⁹ which was consistent with a functional model proposed by Holly that meibomian lipids formed a duplex film.^{2,3} Current models have extended the original concept to include proteins, and describe the lipid layer as likely to be a mosaic of lipids, proteins and mixtures of these,²⁰ rather than a crystalline structure such as that indicated by the McCulley and Shine model.

Much of the evidence for and testing of such models comes from in vitro work. Our group and others have measured surface pressures, and correlated these data with composition and appearance of films of meibomian lipids.^{2,3,20–25} Recently, a technique has been developed to observe the lipid layer in vivo using a high resolution microscope; the original monochrome camera recently has been replaced with a color camera, so the method will be referred to as a high resolution color microscopy (HRCM).²⁶ Many of the micrographs of in vivo lipid films have an appearance similar to those seen from micrographs of meibomian lipid films spread in vitro on a Langmuir trough. Therefore, the purpose of our study was to combine these two methodologies to investigate the appearance of meibomian lipid films in vitro, determine if these were similar in appearance to meibomian lipid films in vivo or to films of other lipids, and from these data suggest models for a meibomian lipid film. In this initial study, the meibomian lipid films were compared with two cholesteryl esters, cholesteryl nervonate (cholesteryl-C24:1) and cholesteryl palmitate (cholesteryl-C16). Analysis of meibomian lipids has shown that cholesteryl nervonate forms a relatively large proportion of cholesteryl esters found in meibomian lipids, and cholesteryl palmitate has been studied in physical chemistry previously,^{27,28} and models of saturated cholesteryl esters, such as cholesteryl arachidate, which have been detected in meibomian lipids.^{12,13} In another experiment, the film was seeded with albumin as a model protein to observe the adsorption of the protein to the lipid layer.

From the ¹University of Western Sydney, School of Science and Health, New South Wales, Australia; and ²College of Optometry, Ohio State University, Columbus, Ohio.

Supported by the Australian Government VisionCRC grant and by the National Institutes of Health Grant R01EY017951.

Submitted for publication April 12, 2012; revised May 28, 2012; accepted June 4, 2012.

Disclosure: T.J. Millar, Allergan (F), Alcon (F); P.E. King-Smith, None

Corresponding author: Thomas J. Millar, School of Natural Sciences, University of Western Sydney, Parramatta Campus LZ118, Locked Bag 1797, Penrith NSW, Australia 2751; t.millar@uws.edu.au.

FIGURE 1. Pressure-area curves of isocycles of different amounts of meibomian lipid films spread at 20°C and 35°C. The first and equilibrium isocycles are shown. Note that for (B), sequential isocycles were the same as the first, so for clarity are not shown.

METHODS

Two cholesteryl esters were chosen: cholesteryl nervonate, which has the longest acyl chain with a double bond (C24:1, ω9) that is available commercially (mp 49°C),²⁹ and cholesteryl palmitate, which has a C16 acyl chain and no double bond (mp 75–77°C).³⁰ These were purchased from Nu-Chek-Prep Inc. (Elysian, MN). Bovine serum albumin was purchased from Sigma-Aldridge (Sydney, Australia) and meibomian lipids were expressed from a single 56-year-old man and collected using a stainless steel spatula. Local ethics permission was obtained and complied with the tenets of the Declaration of Helsinki. The lipids were dissolved at a concentration of 1 mg/mL in high grade chloroform (high performance liquid chromatography [HPLC] grade; Labscan, Seaclyff, SA, Australia).

Lipids were spread onto the surface of an artificial tear buffer, as described by Mirejovsky et al.³¹ but not containing lipids or proteins, in an 80 cm³ double barrier Langmuir trough with a surface area of 80 cm² as described previously.²⁴ In one case, a mixture of meibomian lipids and cholesteryl nervonate was used. This was prepared by mixing 1 mg/mL solutions of cholesteryl nervonate: meibomian lipids in a ratio of 1:4 vol/vol. The temperature of the subphase could be maintained through a water jacket.

Bovine serum albumin was used as a model for observing protein penetration of a meibomian lipid film. In this case, the meibomian lipid film was spread between the barriers in the Langmuir trough and several isocycles were carried out to relax the film. The barriers then were closed to an area that gave a surface pressure of 10 mN/m and the area was maintained. An albumin solution made up in the subphase buffer (1 mg/mL) then was injected (100 µL) into the subphase close to but outside the

barriers, and the surface pressure monitored. Once penetration had reached equilibrium, pressure-area isocycles were performed.

A high resolution color microscope²⁶ was mounted above the trough; the original monochrome camera was replaced by a Basler Scout sCA640-74fc color camera. The microscope used a stroboscope to illuminate the surface and micrographs were taken at selected pressures using 75 Hz frame rates. All micrographs presented are in color. The color indicates the thickness of the film based on constructive interference of different wavelengths of light. Most micrographs are gray, which indicates films less than about 40 nm. The images also were enhanced digitally using a complex curve using Photoshop curves mode in the RGB channel. The anchor points in the curve are 77,82; 112,210; and 202,31.

RESULTS

Different amounts of meibomian lipids were spread on the trough at 20°C and 35°C to determine the effect of increasing the concentration and the effect of temperature on pressure area curves. Spread films using 20 µL of meibomian lipids at 20°C gave typical complex curves, and the film slowly expanded with the number of isocycles (increase in maximum surface pressure between starting isocycle and last isocycle) and showed a distinct hysteresis (Fig. 1A). HRCM of these films at low pressure initially showed uniform dark gray lakes within a uniform lighter gray surrounding area (Fig. 2, upper left). If the lakes correspond to bare saline, then the thickness of the

FIGURE 2. Micrographs showing the appearance at progressively higher surface pressures of a spread film at 20°C and 35°C of 20 μ L 1 mg/mL meibomian lipids during different isocycles. In this and subsequent figures, the legend at the top of each panel represents the surface pressure and the isocycle number when the micrograph was taken (e.g., 27.5 iso12 means the micrograph was taken when the surface pressure was 27.5 mN/m in isocycle 12). Inserts are 175% enlarged and digitally enhanced areas from the same micrographs. *First row:* the inserts highlight the initial patchiness of the film, which then forms spots. The right insert in the upper left image is from an average of 20 images, at increased contrast showing uniform dark gray lakes within a uniform lighter gray surrounding. It is similar in the 12th isocycles, but at higher pressures the spots appear to be smeared. At 35°C, spots and microlenses are apparent when the film was first spread. Scale = 100 μ m.

light surround can be estimated by the method in the Supplementary Material (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10022/-/DCSupplemental>) to be about 5.2 nm. As the pressure increased, the patches disappeared and spots appeared (Fig. 2). At higher pressures, when the isocycle profiles had reached equilibrium (isocycle 12), the spots appeared smeared as indicated by a light gray patchiness in the sea between the spots in the enhanced views (Fig. 2; 27.5–33 iso12). At 35°C, the isocycles were very smooth with little hysteresis and the maximum surface pressure was less than at 20°C (Fig. 1B). Sequential isocycles at 35°C had the same pressure area curves as the first isocycle. HRCM of the film when first spread showed that it contained microlenses that were larger than the spots seen at higher pressures in films spread at 20°C (Fig. 2; 0 iso0). These were separated by an even sea, and as the pressure increased the patches of sea became smaller and the lenses appeared more prominent (Fig. 2). At the highest pressure, there was some smearing of the film. These films emulate closely that shown *in vivo* for a normal (Figure 6 in the study of King-Smith et al.²⁶).

Films made with very large amounts of meibomian lipids also were examined. When 100 μ L of 1 mg/mL (1.25 μ g/cm²) of

meibomian lipids were used, it caused a starting surface pressure of 12.1 mN/m, and during the compression part of the first isocycle there were notable inflections at $\sim$ 16 and $\sim$ 23 mN/m where the film became stiffer (gradient increases, Fig. 1C). During subsequent isocycles, the pressure area curve became smooth with a marked increase in stiffness (curve becomes steeper) at $\sim$ 24 mN/m. Notable was that the films did not collapse, despite the high amounts of lipids on the surface. This is indicated by the consistent maximum surface pressure and take-off pressure between different isocycles. HRCM of these films showed that when spread initially, a number of small spots and larger composite islands with a compact center and amorphous surround formed on a thin background sea (Fig. 3). At $\sim$ 15–28 mN/m the composite islands enlarged, often with multiple compact centers, while the background still contained the tiny spots, best appreciated as small pale dots in the enhanced insert. At $\sim$ 36 mN/m, the amorphous surrounds of the composite islands appear to have coalesced fully with the compact centers still visible within it. Even at the highest pressure, there are small gray areas of sea, but detailed analysis indicates that these are lighter, indicating that they are thicker than the background layer at the lowest pressures. In subsequent isocycles (Fig. 3; iso5), at

FIGURE 3. Micrographs showing the appearance of a 100 µL, 1 mg/mL meibomian lipid film after initial spreading (12.2 iso0) during the first and fifth isocycles at 20°C, and a 80 µL 1 mg/mL meibomian lipid film during the first isocycle at 35°C (*bottom row*). The inserts are 175% enlarged and digitally enhanced areas from the same micrographs, and in particular the background sea appears even in grayness. Scale = 100 µm.

low pressures, composite islands did not appear, but instead, broken-up thick conglomerates were present and these were pushed together gradually as the pressure increased. The background layer did show some variance in thickness and some spots at the edges of different thickness layers could be seen. It is notable that the consolidation of the film at around 24 mN/m (not shown) corresponded to where the pressure area curve begins to become steeper (Fig. 1C). Spreading 80 µL of 1 mg/mL meibomian lipids on the surface at 35°C resulted in a similar equilibrium isocycle pattern to 100 µL of 1 mg/mL spread at 20°C except the maximum pressure was greater (Figs. 1C, 1D). HRCM showed that when the lipids first were applied, small lenses formed over a uniform sea (Fig. 3). This was similar to the appearance of the film at 20°C, except that the lenses had replaced the composite islands. Spots in the background became evident at ~10 mN/m. As the pressure increased, the lenses became closer together, but not substantially larger in size. The background sea still was obvious as an even gray with increasing numbers of spots. Further isocycles lead to a similar appearance, except that at higher pressures, the lenses became more smeared as indicated by the light gray auras around the lenses (not shown). Some of these figures have features similar to those shown in Figures 8 and 10 in the report of King-Smith et al.²⁶

Since cholesteryl esters are a significant component of meibomian lipids, pure films of the unsaturated cholesteryl

nervonate were compared to films of the saturated cholesteryl palmitate at 20°C. The amounts chosen to spread on the Langmuir trough were such that the films had a take-off at or close to maximum surface area. The initial isocycle of cholesteryl nervonate (10 µL, 1 mg/mL) resulted in a pressure area curve that had similarities to a meibomian lipid film in that it showed a complex curve on compression and had a large hysteresis (Fig. 4A). Further isocycles lead to the curve gradually having take-off moving to larger areas, becoming smoother, increasing maximum pressure, and decreasing hysteresis. HRCM of cholesteryl nervonate films showed a multilayer structure with the boundaries of each layer tending to be circular. As described in the Supplementary Material (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10022/-DCSupplemental>), the thickness of a single layer is estimated to be about 4.4 nm. The film was very fluid and the array of interference colors varied across the film independent of pressure or the isocycle (Fig. 5). This oil-slick appearance is typical of a duplex film³ and gray represents very thin areas of the film (see Table). This contrasted sharply with a pure film of cholesteryl palmitate. The rapid rise in surface pressure upon compression of the cholesteryl palmitate film (Fig. 4B) indicated that it was very stiff, and there was extremely strong hysteresis, which normally is an indication that a stiff film is fracturing into islands during expansion. Take-off in subse-

FIGURE 4. A comparison of pressure-area isocycles of cholesteryl nervonate applied to the surface at 20°C (A) with cholesteryl palmitate (B), and a mixture of cholesteryl nervonate and meibomian lipids (C).

quent isocycles moved to smaller surface areas and the maximum surface pressure decreased. This indicated that the film had collapsed. HRCM supported these observations. The initial film was a relatively evenly spread thin film (Fig. 5). There were regions thicker and thinner than the main uniform gray area (Fig. 5; 0 iso1). As the pressure increased, the film developed folds in the surface, indicated by black streaks, and these regions collapsed to form lines across the film that remained even at low pressures (Fig. 5; 0 iso2). This repeated in subsequent isocycles.

When cholesteryl nervonate was premixed with meibomian lipids (1:4 vol/vol solution) and spread (20 µL of 1 mg/mL), the first isocycle take-off was at about 1 mN/m, but the maximum surface pressure was reduced greatly, indicating that the film was forming multiple layers. There was some hysteresis (Fig. 4C). These features differ markedly from the pressure area isocycle curves of a pure meibomian lipid film and a pure cholesteryl nervonate film, suggesting that there was good mixing of the different components. Further isocycles showed a dramatic increase in maximum surface pressure, but no change in take-off. The appearance of the film in the first isocycle as shown by HRCM was similar to cholesteryl nervonate alone (Fig. 5). There appeared to be slightly more light gray areas, but this may have been an anomaly of sampling. In subsequent isocycles, the areas of color became smaller and more light gray areas predominated, indicating that the cholesteryl nervonate and meibomian lipids were mixing

(Fig. 5). At higher pressures, or after more isocycles, the film became stringy on a light gray sea.

Since there is evidence that proteins from the aqueous also absorb to meibomian lipid films,^{2,20,21} penetration of albumin from the subphase into a spread film of meibomian lipids was used as a model. Albumin (100 µL; 1 mg/mL), applied into the subphase, slowly penetrated a spread meibomian lipid film initially set at a surface pressure of 10 mN/m (Fig. 6A). Isocycles carried out after the penetration showed a marked increase in take-off, and maximum surface pressures and increased hysteresis, indicating that the albumin had penetrated the film and was very surface active (Fig. 6B). With further isocycles, there was a further increase in take-off and maximum surface pressures, indicating that there was reorganization of the film and possibly further denaturation of albumin (Fig. 6B). HRCM showed that, after 43 minutes, pale irregular gray-shaped elements started to appear between the spots of the meibomian lipids (Fig. 7). This continued to happen with time and, in some cases, dark patches also could be seen, and there were brighter gray patches as well. When isocycles were commenced, the film initially showed a patchy sea, similar to that seen at low pressures with meibomian lipids alone, but the spots seen at moderate pressures were much smaller and the film appeared to have a stringy appearance at high pressures, indicating that albumin had penetrated the film and was mixing with the meibomian lipids. At later isocycles, a network of thickened cords appeared over the surface of a

FIGURE 5. Micrographs showing the appearance of a spread film of 10 μ L 1 mg/mL cholesteryl nervonate (*top block*) compared to cholesteryl palmitate (*middle block*), and a mixture of meibomian lipids and cholesteryl nervonate at 20°C, and then at progressively higher surface pressures and different isocycles. The inserts are 175% digitally enhanced areas of the same films to show subtle differences (thicknesses) in the films. Scale = 100 μ m.

TABLE. Guide to Layer Thickness Based on Interference Colors

Thickness (nm)	Color
360	Dark purple
330	Red
310	Orange
290	Yellow
270	White (very pale green)
240	Pale blue
210	Deep blue
180	Black or dark purple
150	Brown
120	Light orange
90	White
45	Gray (very pale blue)
0	Black

relatively even background sea (Fig. 7). The thickened cords did not correspond with surface pressure; their appearance or lack of appearance depended upon sampling, which meant that they were distributed randomly across the film.

DISCUSSION

In our study, the equipment (HRCM in Columbus) did not allow simultaneous comparison between fluorescent micrographs and HRCM. Therefore, fluorescent micrographs from similar studies performed in Sydney were compared. The data compared well to fluorescence micrographs of meibomian lipid films seeded with a fluorescently tagged dipalmityl phosphatidylcholine (NBDPC, Fig. 8) spread on a Langmuir trough and show almost identical features. HRCM has allowed better interpretation of the fluorescent micrographs with variation of intensity in the background, the black spots (Fig. 8) being similar to the bright spots (Fig. 2). Analysis of data not shown indicates that spots are seen at lower pressures with HRCM than with fluorescence microscopy, which could be because scattering can occur from point sources below the resolution of the fluorescence microscope, and only after these nuclei have grown to a sufficient size can they be resolved in the fluorescent microscope. Fluorescence microscopy and light scattering microscopy have been compared in studies of lung surfactant extract (LSE).³² Similar spots forming in LSE at high surface pressures, as we have described, were reported. Schief et al. hypothesized that it represented nucleation of micelles, which then formed above the surface.³² This possibility was tested cleverly by these investigators in an experiment where, if the micelles formed below the surface, then the fluorescence would be quenched and they would not be seen, whereas if they formed above the surface, then they would be seen. The micelles formed above the surface with LSE films and below the surface with phospholipid films. On the basis of these data, it is likely that the spots seen in meibomian lipid films are excluding the phospholipid marker and forming above the surface (dark spots).

Overall, the implication is that spread lipids in the Langmuir trough can be used to model what is occurring on the ocular

surface. In particular, combining and comparing data from the high power interference microscopy with the fluorescence microscopy provides some overlapping and some complementary information about the behavior of the meibomian lipids. The fluorescence techniques seem particularly useful for examining the background sea in the micrographs (what is happening at the aqueous lipid interface), which is much more difficult to identify in HRCM. HRCM makes clear what is occurring above this layer. Therefore, a combination of these techniques provides the potential for an elegant in vitro platform for testing artificial tear supplements that are directed at altering the lipid layer, and then directly transferring the evaluation to in vivo testing. Some indication that it could be used to model the in vivo situation comes from the experiments of mixing cholesteryl nervonate with the meibomian lipids.

However, the purpose of studying a mixture of cholesteryl nervonate with meibomian lipids was to gather some insight on the possible structure of the meibomian lipid films. In physical chemistry, the behavior of cholesteryl esters in bulk and at surfaces has been a foundation for the field of liquid crystals. For further information in this area the appropriate chapters in the studies reported by Fisch,³³ Ginsburg et al.,³⁴ and Alonso et al.³⁵ are recommended. Many studies show that saturated cholesteryl esters interdigitate to form semicrystalline monolayers or multilayers at an aqueous interface, and these resemble their bulk characteristics.^{34,35} Here, such a crystalline film appears to have been formed with cholesteryl palmitate. The stable structure was very rigid and folded under pressure as illustrated by the collapsed lines in the film. This is consistent with previous reports.^{27,28} When a cis double bond occurs in the acyl group, the interdigitation is more difficult but has been shown to occur in bulk crystals formed upon cooling. Crystallography of bulk crystals illustrates that they have a complex arrangement where the cholesterol rings of reversed pairs of molecules interdigitate along the sides of the sterol rings, and then these form layers with other pairs of molecules.²⁹ The oil-slick formed by cholesteryl nervonate is typical of a duplex film that has been allowed to reach equilibrium. This is characterized by a monolayer at the aqueous surface and lenses forming above. In the Supplementary Material (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10022/-DCSupplemental>), the thickness of layers of cholesteryl nervonate was estimated to be 4.38 nm, which is comparable to the layer thickness of 4.25 nm in the bulk phase at 295°K,²⁹ suggesting that the structure in the film is similar to the bulk structure.

All these data taken together support the suggestion by Holly that meibomian lipids form a duplex film.^{2,3} However, the greater resolution of the film provided by HRCM suggests an interesting functional model for lipid layer that would give the lipid layer an extraordinary tolerance to outside parameters. This model rests on the concept that the duplex film formed by meibomian lipids contains a surfactant region at the aqueous surface and a superficial “collecting” layer. When spread across a surface, duplex films are unstable, and eventually reach equilibrium by transforming into a monolayer and lenses (e.g., Fig. 2). In the micrographs shown here, the background gray sea would represent the monolayer. HRCM suggest that this layer is about 5 nm. Although the major component of this layer is likely to be surfactant lipids, such as the omega hydroxyacyl fatty acids, it is hypothesized that this monolayer is more likely to be a liquid crystal layer rather than a single layer of surfactant molecules. This is because some differences in grayness can be seen in this layer (Fig. 8) indicating different thicknesses and cholesteryl esters are significant components of meibomian lipids and have a strong tendency to form liquid crystals.³⁵ Interestingly, this means that the structure of this layer would be similar to that modelled by McCulley and Shine,⁹ with all of the molecules roughly oriented in the same direction out from

FIGURE 6. Penetration of 100 μ L of 1 mg/mL albumin into a meibomian lipid film set with an initial surface pressure of 10 mN/m (A) and the pressure area curve of isocycles after albumin penetration (B).

the surface, but the horizontal spacings between the molecules would vary. If this layer were in the form of a liquid crystal, the large amount of cholesteryl esters suggests that it might be thermotropic and have a chiral nematic structure.³⁵ Although there might be considerable variance in the crystalline structure, because the types of crystals formed by pure cholesteryl esters depend upon the nature of the alkyl chain³⁴ and also depend upon whether there are other molecules, such as hydrocarbon chains, capable of spacing between them,³⁵ in meibomian lipids this could be waxes. This means that it would not be one layer of molecules as one typically might imagine, but there may be molecules interdigitating and tilting causing a complex spiral layering up from the surface. Technically, this still would be a monolayer. Higher resolution techniques, such as neutron scattering, would be needed to test this idea. On this matter, it has been shown that the melting profile in bulk (not of spread films) of cholesteryl esters is not sensitive to the addition of wax esters,³⁶ which would tend to indicate that these components remain separate. This could mean that in a spread

film, the cholesteryl esters are floating on a sea of wax esters if it behaved in the same manner as in the bulk. However, some evidence that spread films behave differently from bulk components comes from the observation in our studies that cholesteryl nervonate is solid at room temperature, but behaves as a liquid as a spread film.

In addition to lipids, proteins, particularly surfactant proteins, also could be a part of this layer and, hence, it may be a mosaic rather than have a single structure. This possibility is supported by the studies using albumin penetration into the film. In future studies, it would be of interest to examine mucins because the ability of mucin to decrease surface tension of meibomian lipid films caused Holly to suggest that it was a critical component of meibomian lipid films.^{2,3} More recently, other surfactant proteins, such as surfactant proteins B and C, have been identified and, therefore, it is likely that this layer is a mixture of surfactant proteins and lipids. The function of this layer would be to provide the physical chemistry necessary for the spreading of the tear film and lowering the surface tension.

FIGURE 7. Micrograph showing the appearance of a meibomian lipid film after penetration of albumin into the film and the appearance at different pressures during isocycles. Time (*min*) after application of albumin to subphase is indicated (*row 1*) and insets are enhanced graphically 1.5× samples from the micrographs. The appearance of the film before albumin penetration is shown (7.2 mN/m). Albumin penetration is indicated by the gray polygonal regions (*arrows*). These condense into brighter zones (*white arrow*) and darker zones also appear at longer times (*broken arrows*). After several isocycles (*iso14*) strands appear on the surface of the film. Scale = 100 μm .

The uniformity would be maintained by pushing molecules into and out of this layer as required. This is best illustrated by the formation of spots in meibomian lipid films (e.g., Fig. 2). From the *in vivo* studies using HRCM,²⁵ it appears that this also is the case in the tears as illustrated by Figure 6 in the study of King-Smith et al.²⁶ Superficial to the surfactant layer is a collecting layer that comprises mainly hydrophobic molecules, but can recruit surfactant molecules if required from the surfactant layer when they are squeezed out. Its role is to bundle up (denature and entrap) any exotic molecules entering the surfactant layer. This means that it can take on many forms depending upon the contaminants. To some degree, different forms of it were seen when excess meibomian lipids were applied to the trough (Fig. 4). Its scavenging role is illustrated here with albumin because the albumin eventually is denatured and bundled up to form stringy ropes (Fig. 7), and also when a meibomian lipid film was seeded with cholesteryl nervonate. In this case, after many isocycles, the cholesteryl nervonate appeared to be bundled into crystals to be removed (Fig. 5). This did not happen with films of cholesteryl nervonate alone. The appearance of this cleaning layer *in vivo* would depend upon which foreign molecules were being managed, and only the structure of the surfactant layer would indicate the physical condition of the lipid layer. Close examination of the *in vivo* micrograph from a dry-eye patient in

Figures 10b and 14 in the study of King-Smith et al.²⁶ illustrates a deformity in the surfactant layer despite the emphasis in the article on the superficial scavenging layer.

A limitation of these experiments, which has led to this hypothesis, is that expansion of the films (perhaps equivalent to *in vivo* break-up) was not examined. It could be that the nature of the outer layer influences break-up of the surfactant layer. Such studies of what occurs in the expansion phases of the isocycles may prove beneficial if compared to *in vivo* observations of the lipid layer following a blink. In particular, it may give insight into what factors lead to breakdown of the background sea exposing the aqueous layer. Such studies should reveal stepwise thinning leading to the abrupt formation of holes that grow from molecules moving from the superficial layers into the holes (sheeting), and as the holes grow they coalesce with other holes to form a new thinner layer. This process may repeat several times as the film becomes progressively thinner. The thinning of such films has been modelled mathematically and parameters in the model that affect thinning could be tested.³⁷

Acknowledgments

Heather Chandler and Kathy Reuter provided technical advice and help for the HRCM recordings.

FIGURE 8. Micrograph of meibomian lipid film at 12 mN/m seeded with 0.5% 1-acyl-2-12-([7-nitro-2-1,3-benzoxadiazol-4-yl]amino dodecanoyl)-sn-glycero-3-phosphocholine (NBDPC). The lighter areas are more strongly fluorescent.

References

- Holly FJ, Lemp MA. Wettability and wetting of the corneal epithelial. *Exp. Eye Res.* 1971;11:239-250.
- Holly FJ. Formation and rupture of the tear film. *Exp. Eye Res.* 1973;15:515-525.
- Holly FJ. Formation and stability of the tear film. *Int. Ophthalmology Clin.* 1973;13:73-96.
- Holly FJ. Surface chemistry of tear film component analogs. *J Colloid Interface Sci.* 1974;49:221-231.
- Holly FJ, Lemp MA. Tear Physiology and dry eyes. *Surv Ophthalmol.* 1977;22:69-87.
- Holly FJ, Hong B. Biochemical and surface characteristics of human tear proteins. *Am J Optom Phys Opt.* 1982;59:43-50.
- Mishima S, Maurice DM. The oily layer of the tear film and evaporation from the corneal surface. *Exp Cell Res.* 1961;1:39-45.
- Nicholaides N, Kaitaranto JK, Rawdah TN, Macy JI, Boswell FM III, Smith RE. Meibomian gland studies: comparison of steer and human lipids. *Invest Ophthalmol Vis Sci.* 1981;20:522-536.
- McCulley JP, Shine WE. A compositional based model for the tear film lipid layer. *Trans Am Ophthalmol Soc.* 1997;95:79-88.
- McCulley JP, Shine WE. Polar lipids in human Meibomian gland secretions. *Curr Eye Res.* 2003;26:89-94.
- Butovich IA, Millar TJ, Ham BM. Understanding and analyzing meibomian lipids—a review. *Curr Eye Res.* 2008;33:405-420.
- Butovich IA. Cholesteryl esters as a depot for very long chain fatty acids in human meibum. *J Lipid Res.* 2009;50:501-513.
- Cory CC, Hinks W, Burton JL, Shuster S. Meibomian gland secretion in the red eyes of rosacea. *Br J Dermatol.* 1973;89:25-27.
- Mathers WD, Lane JA. Meibomian gland lipids, evaporation, and tear film stability. *Adv Exp Med Biol.* 1998;438:349-360.
- Butovich IA. The meibomian puzzle: combining pieces together. *Prog Retin Eye Res.* 2009;28:483-498.
- Chen J, Green-Church KB, Nichols KK. Shotgun lipidomic analysis of human meibomian gland secretions with electrospray ionization tandem mass spectrometry. *Invest Ophthalmol Vis Sci.* 2010;51:6220-6231.
- Lam SM, Tong L, Yong SS, et al. Meibum lipid composition in Asians with dry eye disease. *PLoS One.* 2011;6:e24339.
- Shrestha RK, Borchman D, Foulks GN, Yappert MC, Milliner SE. Analysis of the composition of lipid in human meibum from normal infants, children, adolescents, adults, and adults with meibomian gland dysfunction using ^1H -NMR spectroscopy. *Invest Ophthalmol Vis Sci.* 2011;52:7350-7358.
- Butovich IA, Arciniega JC, Lu H, Molai M. Evaluation and quantitation of intact wax esters of human meibum by gas-liquid chromatography-ion trap mass spectrometry [published online ahead of print April 24, 2012]. *Invest Ophthalmol Vis Sci.* 2012.
- Millar TJ, Tragoulias ST, Anderton PJ, et al. The surface activity of purified ocular mucin at the air-liquid interface and interactions with meibomian lipids. *Cornea.* 2006;25:91-100.
- Gouveia SM, Tiffany JM. Human tear viscosity: an interactive role for proteins and lipids. *Biochim Biophys Acta.* 2005;1753:155-163.
- Mudgil P, Torres M, Millar TJ. Adsorption of lysozyme to phospholipids and meibomian lipid monolayer films. *Colloid Surface B.* 2006;48:128-137.
- Mudgil P, Millar TJ. Surfactant properties of human meibomian lipids. *Invest Ophthalmol Vis Sci.* 2011;52:1661-1670.
- Millar TJ, Mudgil P, Butovich IA, Palaniappan CK. Adsorption of human tear lipocalin to human meibomian lipid films. *Invest Ophthalmol Vis Sci.* 2009;50:140-151.
- Leiske DL, Raju SR, Ketelson HA, Millar TJ, Fuller GG. The interfacial viscoelastic properties and structures of human and animal meibomian lipids. *Exp Eye Res.* 2010;90:598-604.
- King-Smith PE, Nichols JJ, Braun RJ, Nichols KK. High resolution microscopy of the lipid layer of the tear film. *Ocular Surf.* 2011;9:197-211.
- Kwong CN, Heikkilä RE, Cornwell DG. Properties of cholesteryl esters in pure and mixed monolayers. *J Lipid Res.* 1971;12:31-35.
- Viswanath P, Suresh KA. Monolayers and 3D films of cholesteryl derivatives at the air-water interface. *J Phys Chem B.* 2004;108:9198-9207.
- Sawzik P, Craven BM. Conformation and packing of unsaturated chains in crystals of cholesteryl nervonate at 123K. *J Lipid Res.* 1984;25:851-856.
- Ginsburg GS, Small DM, Hamilton JA. Temperature-dependent molecular motions of cholesteryl esters: a carbon-13 nuclear magnetic resonance study. *Biochemistry.* 1982;21:6857-6867.
- Mirejovsky D, Patel AS, Rodriguez DD, Hunt TJ. Lipid adsorption onto hydrogel contact lens materials. Advantages of Nile red over oil red O in visualization of lipids. *Optom Vis Sci.* 1991;68:858-864.
- Schief WR, Antia M, Discher BM, Hall SB, Vogel V. Liquid-crystalline collapse of pulmonary surfactant monolayers. *Biophys J.* 2003;84:3792-3806.
- Fisch MR. Liquid crystals. In: *Liquid crystals, Laptops and Life*. Singapore, : World Scientific Publishing Co.; 2004:163-182.
- Ginsburg GS, Atkinson D, Small DM. Physical properties of cholesteryl esters. *Prog Lipid Res.* 1985;23:135-167.
- Alonso C, Kuzmenko I, Jensen TR, Kjaer K, Lahav M, Leiserowitz L. Self-assembly of crystalline films of interdigitated long-chain cholesteryl esters at the air-water interface. *J Phys Chem B.* 2001;105:8563-8568.
- Borchman D, Foulks GN, Yappert MC, et al. Human meibum lipid conformation and thermodynamic changes with meibomian-gland dysfunction. *Invest Ophthalmol Vis Sci.* 2011;52:3805-3817.
- Bergeron V, Jimenez-Laguna AI, Radke CJ. Hole formation and sheeting in the drainage of thin liquid films. *Langmuir.* 1992;8:3027-3032.