Application of a Novel Interferometric Method to Investigate the Relation between Lipid Layer Thickness and Tear Film Thinning

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PURPOSE. The lipid layer of the tear film forms a barrier to evaporation. Evaporation is a major cause of tear thinning between blinks and tear breakup. The purpose of this study was to investigate the relation between tear film thinning and lipid layer thickness before and after instillation of an emulsion eye drop.

METHODS. Fifty non-contact lens wearers were studied. Spectral interferometry was used to measure the thinning rate of the precorneal tear film for up to 19 seconds after a blink. Simultaneously, lipid layer thickness was measured based on an absolute reflectance spectrum. After a 2-minute recovery, the measurement was repeated. A drop of the lipid emulsion was then instilled; 15 minutes later, two interferometry measurements were performed similarly.

RESULTS. A histogram of thinning rates was fitted by a bimodal distribution with narrow and broad peaks corresponding to slow and rapid thinning, respectively. The correlation between repeated thinning rate measurements was modest, but repeatability was considerably more significant when analyzed in terms of the slow/rapid dichotomy. Similarly, the correlation between thinning rate and lipid thickness was modest but was more evident when analyzed in terms of the slow/rapid dichotomy. Instillation of an emulsion eye drop significantly increased the thickness of the lipid layer but did not significantly alter the thinning rate.

CONCLUSIONS. The proposed slow/rapid dichotomy of thinning rates presumably relates to a good/poor barrier to evaporation of the lipid layer. The imperfect correlation between thinning rate and lipid thickness indicates that other factors, such as the composition and structure of the lipid layer, are important (e.g., sufficient polar lipids may be needed to form good interface between nonpolar lipids and the aqueous layer). (*Invest Ophthalmol Vis Sci.* 2010;51:2418–2423) DOI:10.1167/iovs.09-4387

According to the report of the Dry Eye Workshop (DEWS),¹ the two major classes of dry eye are aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE). The DEWS Report¹ also lists two core mechanisms of dry eye, tear hyperosmolarity and tear film instability. The latter was observed by reduced breakup time (BUT). The first core mechanism, hyperosmolar-

ity, is a sensitive indicator of dry eye disease.² It stimulates inflammation and ocular surface damage.¹ Tear osmolarity depends on the ratio evaporation rate divided by tear secretion rate³ and is an increasing function of this ratio. In EDE the ratio is increased because the evaporation rate is increased, whereas in ADDE the ratio is increased because the tear secretion rate is reduced.

There is evidence that the second core mechanism, tear film instability (reduced BUT), is often closely related to local evaporation from the tear surface.^{4,5} This would cause local hyperosmolarity of the ocular surface.⁶ In this case, the two core mechanisms would have a common origin—evaporation—causing both hyperosmolarity and tear film instability. Any ocular surface damage related to instability (breakup) could be caused by local hyperosmolarity induced by evaporation. We suggest that tear film instability—sudden, spontaneous breakup in models such as Holly's⁷—has less support from experimental evidence. Instability (measured by BUT) may therefore be an inappropriate term for a property that may depend mainly on evaporation.

These considerations indicate that better understanding of both ADDE and EDE requires improved analysis of the mechanisms of evaporation. Evaporation is controlled by both extrinsic and ocular factors. Extrinsic factors include the relative humidity, temperature, and flow velocity of the ambient air.⁸ Ocular factors include the surface temperature of the tear film and, more important, the quality of the outer lipid layer of the tear film, which acts as a barrier to evaporation from the aqueous tear film.⁹ The resistance of the lipid layer to evaporation may be expected to depend on its thickness and on its structure and composition. Thus our aims were to use interferometry to measure the thinning rate of the tear film between blinks as a measure of evaporation and to compare this with simultaneous measurements of lipid thickness.

METHODS

Interferometry

The use of spectral interferometry to measure the thickness of the precorneal tear film and its thinning between blinks has been described.^{10,11} For the current study, the method was modified to improve measurement of lipid layer thickness. Figure 1a shows reflectance of the lipid layer as a function of lipid thickness and wave number (1/wavelength) derived from the interference theory of thin films.^{12,13} Published values were used for the refractive indices of the lipid and aqueous layers,^{14,15} and the effect of dispersion (variation with wave number) was included. In these plots, which use an absolute scale of reflectance, it is seen that reflectance increases as a function of lipid thickness up to approximately 100 nm, accompanied by changes in the slope and shape of the spectral reflectance curve. In previous versions of our optical system, measurement of absolute reflectance was not possible because any misalignment of the eye reduced the intensity of light entering the entrance slit of the spectro-

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FIGURE 1. (a) Absolute value of calculated reflectance from the front of the tear film as a function of wave number and lipid thickness. (b) Relative reflectance (curves scaled to match at a central wave number).

photometer. Given this uncertainty in absolute reflectance, lipid layer thickness was estimated from the slope and shape information contained in the relative reflectance spectra in Figure 1b, where the spectra have been scaled to match at a central wave number. It is seen that the relative reflectance spectra of Figure 1b give more limited information about lipid thickness than the absolute reflectance spectra of Figure 1a; for example, lipid thicknesses of 40 and 60 nm give similar relative reflectance spectra (Fig. 1b), whereas the absolute reflectance spectra show a much larger difference (Fig. 1a).

A simplified diagram of the optical system for measuring absolute spectral reflectance is shown in Figure 2; further details, including the ocular alignment system and the calibration of spectral reflectance, have been described.^{10,11} The aim was that a constant fraction of the light reflected from the tear film should enter the spectrophotometer, despite any moderate misalignment of the eye, such as shown in Figure 2. A narrow beam of light (thick rays in Fig. 2) was focused down to a small spot (33- μm diameter) on the tear film. If the eye misalignment was not too great, the reflected beam (Fig. 2, thin rays) was not obstructed by the optical system (e.g., lenses L_2 and L_3); hence, the recorded intensity was directly proportional to the spectral reflectance of the tear layer. A relatively broad entrance slit of the spectrophotometer was used to avoid any obstruction of the reflected beam.

Lipid layer thickness was estimated in two different ways: it was assumed to be either uniform or variable within the measurement spot. In the latter, it was assumed that the probability distribution of lipid thickness was given by a Gaussian function of log thickness. The reason for using this probability distribution rather than simply a Gaussian function of thickness was that it avoids the logical impossibility that some of the lipid layer would have a negative thickness. Plotted on a linear thickness scale, the assumed probability distribution is skewed so that the mean thickness is greater than the median thickness. Thus, three estimates of lipid thickness were obtained, one on the uniform lipid thickness assumption and two—mean and median thickness—on the variable thickness assumption. Least square fits to the reflectance spectra for the variable thickness assumption were generally considerably better than for the uniform thickness assumption. Additionally, high-resolution imaging studies indicated that some variability of lipid thickness is to be expected within the measurement spot.¹⁶ However, the simplicity of the uniform thickness assumption made it worth considering. When the eye was badly misaligned so that some of the light did not pass through the collecting optics, the apparent absolute reflectance was not consistent with the slope and shape of the reflectance spectrum; in this case, the root-mean-square error of the least squares fit was increased, and data from that spectrum were rejected if this error was greater than 1% or 3% for the variable thickness or uniform thickness assumption, respectively. It is important to emphasize that lipid thickness is measured at the identical time and location as the measure of tear film thinning. Thus, the method is able to simultaneously measure the impact of lipid layer thickness on tear film thinning (e.g., evaporation).

Subjects and Experimental Studies

The study adhered to the principles of the Declaration of Helsinki and was approved by the Biomedical Institutional Review Board of the Ohio State University. Informed consent was obtained from all subjects after explanation of the procedure. Subjects were eligible for the study if they were older than 18 years and had not worn contact lenses for 3 months before the study. To provide a broad measure of the adult population, possible cases of dry eye were not excluded, but the study was not powered to show statistical differences between subjects with and without dry eye.

Spectral interferometry was used to measure tear film thinning of the right eye after a blink, as in previous studies,¹¹ with simultaneous measurement of lipid thickness. Subjects were asked to blink 1 second after the start of a 20-second recording and then to try to keep their eyes open for the remaining 19 seconds. Spectra were recorded at a rate of 10/s using a spectral range of 476 to 909 nm. After a 2-minute rest period, this procedure was repeated. One drop of lipid emulsion eye drop (Soothe; Bausch & Lomb, Rochester, NY) was then placed in the right eye. The subject completed a demographic form, and the Ocular Surface Disease Index (OSDI) was scored using previously published guidelines.¹⁷ Finally, two additional tear film interferometry measurements were taken 15 minutes after the original eye drop instillation, with a 2-minute rest interval again between the measurements. Mean ($\pm$ SD) temperature was $22.1^\circ\text{C} \pm 1.6^\circ\text{C}$, and mean relative humidity was $48\% \pm 16\%$. Because histograms of lipid thickness and thinning rates differed significantly from those of a normal distribution, nonparametric statistics (Wilcoxon signed ranks test, Spearman correlation, McNemar test, and Fisher exact test, all as two-sided tests) were used for data analysis.

RESULTS

Fifty subjects (22 women, 28 men) participated; mean $\pm$ SD age was 34 ± 12 years. The average OSDI score was 10.7 ± 12.1 . Seven subjects (included in the data analysis below) had scores of 21 or more, indicating possible dry eye.

Initial Measurements before Instillation of Lipid Emulsion

Mean lipid thickness was 42 nm for the uniform thickness assumption; for the variable thickness assumption, it was 35 nm and 42 nm based on use of the median and mean values of the fitted probability distribution (see Methods). Correlations

FIGURE 2. Simplified diagram of the optical systems with the eye slightly misaligned. L_1 , L_2 , L_3 , lenses; thick rays, illuminating beam; thin rays, reflected beam.

FIGURE 3. Histogram of lipid thickness (uniform thickness assumption, $n = 100$). Error bars indicate 1 SE, derived from Poisson statistics.¹⁸

between lipid thickness values for the uniform thickness and variable thickness assumptions were $r^2 = 0.989$ and $r^2 = 0.990$ using median and mean values of the fitted probability distribution, respectively. In view of these high correlations, further results will be presented only for the uniform thickness case.

A histogram of lipid thickness, based on 100 trials (from two measures from all subjects), is given in Figure 3.¹⁸ Thickness values ranged from 15 to 157 nm, with a mean of 42 nm. Distribution was skewed and differed significantly from normality ($P < 0.0005$, Anderson-Darling test).

A histogram of tear film thinning rates is given in Figure 4 ($n = 99$; one trial was excluded for insufficient data); the mean thinning rate was $3.4 \mu\text{m}/\text{min}$. The histogram was fit, using a maximum likelihood method, by the sum of two Gaussian functions labeled slow (solid curve) and rapid (dashed curve).¹¹ Mean thinning rates were 1.6 and $8.4 \mu\text{m}/\text{min}$ for the slow and rapid distributions, respectively. Corresponding standard deviations of the Gaussian functions were 0.9 and $6.3 \mu\text{m}/\text{min}$; slow thinning constituted 74% of the overall distribution. Slow and rapid distributions intersected at a thinning rate of $4.0 \mu\text{m}/\text{min}$ (Fig. 4, vertical line); therefore, thinning rates slower and faster than this value are considered slow and rapid, respectively. The idea that thinning rates may be considered as either slow or rapid is called the dichotomous classification of thinning rates.

Figure 5 is a plot of the second measurement of lipid thickness as a function of the first measurement. The Spearman correlation coefficient was $R = 0.835$ ($P < 0.0005$), indicating fairly good repeatability of lipid thickness measurements by this method. Filled circles indicate seven eyes with OSDI scores 21. Lipid thickness for these subjects did not differ significantly from that of the other 43 eyes ($P > 0.05$, Mann-Whitney Test); it should be noted again that the study was not

FIGURE 4. Histogram of thinning rates ($n = 99$). Error bars indicate 1 SE, derived from Poisson statistics.¹⁸ The histogram has been fit by a sum of two Gaussian distributions corresponding to slow (solid curve) and rapid (dashed curve) thinning. The intersection of the two Gaussian functions occurs at a rate of $4 \mu\text{m}/\text{min}$ and is marked by the vertical line. This rate is considered to be the boundary between slow and rapid thinning.

FIGURE 5. Second measurement of lipid thickness as a function of the first measurement ($n = 50$). Filled circles: seven eyes with OSDI scores 21 (possible dry eye). Dashed line: equality of the two measurements.

statistically powered to demonstrate differences between subjects with and without dry eye.

Figure 6 is a plot of the second measurement of thinning rate as a function of the first measurement. The Spearman correlation coefficient was $R = 0.325$ ($P = 0.022$), suggesting poorer repeatability than for lipid thickness. Horizontal and vertical lines indicate the boundary ($4 \mu\text{m}/\text{min}$) between slow and rapid thinning rates of the dichotomous classification. The numbers of observations in the four resultant regions is indi-

FIGURE 6. Second measurement of thinning rate as a function of the first measurement ($n = 49$). Filled circles: seven eyes with OSDI scores 21. Dashed line: equality of the two measurements. Horizontal and vertical lines: boundary between slow and rapid thinning. Numbers of subjects in the four resultant areas are indicated.

FIGURE 7. Thinning rate as a function of lipid thickness; data have been averaged for each subject ($n = 50$). *Filled circles*: seven eyes with OSDI scores ≥ 21 . *Horizontal line*: boundary between slow and rapid thinning. *Inset*: ROC curve indicating the ability of lipid thickness measurements to predict rapid thinning.

cated (e.g., four subjects had slow thinning on the first measurement and rapid thinning on the second). Of the eight subjects who had rapid thinning on the first trial, seven had rapid thinning again on the second trial (sensitivity, 87%). Of the 41 subjects who had slow thinning on the first trial, 37 had slow thinning again on the second trial (specificity, 90%). Analyzed in this way, repeatability was considerably more significant ($P = 0.00003$, Fisher exact test) than for the Spearman correlation. The observation that analysis in rapid and slow thinning gives a more significant result than Spearman correlation further supports the dichotomous model of thinning rates.

Averaging thinning rates for the two trials, 4 of 12 subjects with rapid thinning had OSDI scores ≥ 21 , whereas only 3 of 38 subjects with slow thinning had OSDI scores ≥ 21 . Based on these figures, subjects with high OSDI scores were significantly more likely to experience rapid thinning ($P = 0.048$, Fisher exact test).

Figure 7 is a plot of thinning rate as a function of lipid thickness; data from the first and second measurements have been averaged for each subject. The Spearman correlation coefficient is $R = -0.385$ ($P = 0.006$). The solid horizontal line is the boundary between slow and rapid rates of the dichotomous model. Some predictability of rapid versus slow thinning (dichotomous classification) is evident; for example, the five subjects with the thinnest lipid (<24 nm) experienced rapid thinning, whereas 31 of 33 subjects with the thickest lipid (>30.5 nm) experienced slow thinning. It is remarkable, however, that one subject, with a relatively thick lipid layer (85 nm) experienced rapid thinning; this subject had an OSDI score of over 21. The inset shows a receiver operating characteristic (ROC) plot indicating ability to predict rapid thinning from lipid thickness; area under the ROC curve was 0.805 ($P = 0.0017$, Mann-Whitney test). The average of sensitivity and specificity reached a maximum of 0.82 (sensitivity, 0.83; specificity, 0.82) at a criterion thickness of $30.5 \mu\text{m}$.

Effect of Instillation of Lipid Emulsion

Figure 8 is a plot of lipid thickness 15 minutes after the instillation of lipid emulsion (Soothe; Bausch & Lomb) as a function of lipid thickness before instillation; pre-instillation

FIGURE 8. Lipid thickness after instillation of lipid emulsion as a function of thickness before instillation (averages of two measurements per subject; $n = 50$). *Filled circles*: seven eyes with OSDI scores ≥ 21 . *Dashed line*: equality of thickness before and after instillation.

and post-instillation values are averages of two measurements for each subject. Significant correlation was found between pre-instillation and post-instillation values ($R = 0.709$; $P < 0.0005$). Lipid thickness was significantly increased by instillation ($P = 0.003$; Wilcoxon signed rank test). Lipid thickness increased from (mean $\pm$ SE) 42.1 ± 3.2 to 48.1 ± 3.0 nm.

Figure 9 is a plot of thinning rate 15 minutes after instilla-

FIGURE 9. Thinning rates 15 minutes after instillation of lipid emulsion as a function of thinning rate before instillation ($n = 50$). *Filled circles*: seven eyes with OSDI scores ≥ 21 . *Dashed line*: equality of thinning rates before and after instillation. *Horizontal and vertical lines*: boundary between slow and rapid thinning rates of $4 \mu\text{m/min}$. Numbers of observations in the four resultant regions are indicated.

tion of lipid emulsion (Soothe; Bausch & Lomb) as a function of thinning rate before instillation; both pre-instillation and post-instillation values are averages of two measurements for each subject (except for one pre-instillation rate based on a single measurement). Horizontal and vertical lines indicate the boundary between slow and rapid thinning rates of $4 \mu\text{m}/\text{min}$; numbers of observations in the four resultant regions are indicated. Significant correlation was found between post-instillation and pre-instillation thinning rates ($R = 0.611$; $P < 0.0005$). As in Figure 6, the dichotomous slow/rapid classification after instillation was correlated with the classification before instillation; of 12 subjects who experienced rapid thinning before instillation, 11 still had rapid thinning after instillation (sensitivity, 92%). Of 38 who experience slow thinning before instillation, 33 had slow thinning after instillation (specificity = 87%). Instillation of lipid emulsion (Soothe; Bausch & Lomb) had no significant effect on thinning rates ($P > 0.05$, Wilcoxon signed rank test) nor did it significantly change the number of subjects in the rapid thinning category ($P > 0.05$, McNemar's test).

DISCUSSION

Analysis of lipid thickness was based on the assumption that lipid thickness was uniform within the measurement spot. Better fits to the reflection spectra could generally be obtained by assuming that the lipid thickness in the measurement spot was variable, which is consistent with high-resolution images of the lipid layer.¹⁶ It is unclear, however, what probability distribution of lipid thickness in the measurement spot should be assumed, so we preferred to use the simpler uniform thickness assumption. This gives thickness values that are highly correlated with the variable thickness assumption. The mean thickness reported here, 42 nm, was similar to that found by Olsen,¹² who also measured absolute reflectance, but it was smaller than found in some other studies.^{19–21} The minimum thickness observed was 15 nm, which, given a molecular length of 2 to 2.5 nm,²² corresponds to approximately six to eight layers of lipid molecules. Given the variability of lipid thickness within the measurement spot, it was expected that some regions of the lipid layer would contain fewer layers than this, perhaps only a monolayer or no layer.

The histogram of thinning rates (Fig. 4) was analyzed in terms of a bimodal distribution—the sum of a narrow distribution of slow thinning rates together with a broad distribution of rapid thinning rates. The intersection between slow and rapid thinning distributions occurred at a thinning rate of $4 \mu\text{m}/\text{min}$; this value was, therefore, taken as the boundary between slow and rapid thinning. Repeatability of thinning rate measures, given by the correlation between the first and second thinning rate measurements (pre-instillation), was modest (Fig. 6; $R = 0.325$; $P = 0.022$). However, analyzing the same data in terms of the slow/rapid dichotomy showed much more significant repeatability (Fisher exact test; $P = 0.00003$). Comparison of thinning rates before and after instillation of lipid emulsion (Soothe; Bausch & Lomb) showed similarly good repeatability when analyzed in terms of the slow/rapid dichotomy (Fig. 9). These findings support the proposal that the slow/rapid dichotomy is meaningful.

The correlation between thinning rate and lipid thickness was also modest (Fig. 7; $R = -0.385$; $P = 0.006$). Again, analysis of results in terms of the slow/rapid dichotomy shows a more obvious relationship between these two measurements; for example, the five subjects with the thinnest lipid experienced rapid thinning, whereas 31 of the 33 subjects with the thickest lipid experienced slow thinning. This relationship between thinning rate and lipid thickness supports

the proposal that the main contribution to thinning rate comes from evaporation (rather than tangential flow or inward flow). Slow thinning may correspond with a relatively good evaporation barrier, whereas rapid thinning may correspond with a relatively poor evaporation barrier. The barrier may be poor because there may be not enough of some component of the lipid layer (e.g., insufficient polar lipid to form a well-structured interface between the outer nonpolar lipids and the aqueous layer).²³ Polar lipids make up only a small fraction (probably $<10\%$) of total meibomian lipid.²³ Given a molecular length of polar lipids of 2 to 2.5 nm,²² lipid layers thinner than approximately 20 to 25 nm are likely to have insufficient polar lipids to form a good interface between nonpolar lipids and the aqueous layer; this may help to explain the observed result that the five subjects with lipid thickness <24 nm experienced rapid thinning (Fig. 7).

Craig and Tomlinson²⁴ studied the relation between evaporation rate and lipid layer patterns (and, hence, estimated lipid thickness) observed with a tear scope (Keeler, Windsor, UK). They found no significant differences in evaporation rates between different lipid patterns corresponding to medium lipid thickness, but evaporation rates were significantly increased, by about four times, in patients with no detectable lipid layer. This marked increase for very thin or absent lipids may roughly correspond to our rapid thinning classification, whereas the slower evaporation for moderate thickness may correspond to slow thinning. Unexpectedly, subjects with thick but abnormal lipid layers also experienced significantly increased evaporation, perhaps corresponding to the subject in Figure 7, who experienced rapid thinning despite a relatively thick lipid layer of 85 nm.

As previously demonstrated,²¹ the lipid layer was significantly thickened after instillation of lipid emulsion (Soothe; Bausch & Lomb; Fig. 8). However, no significant effect was observed on thinning rate. This is a further indication that thinning rate is dependent on other factors in addition to lipid thickness; the composition and structure of the lipid layer are also likely to be important, together with external factors such as air currents, relative humidity, and room temperature.

In conclusion, our results support the proposal that tear thinning is attributed primarily to evaporation, which, in turn, is controlled by the lipid layer. Evidence for a slow/rapid dichotomy of thinning rates includes an apparently bimodal distribution of rates (Fig. 4). Repeatability of thinning rate measures is more evident when rate is classified in this way. Although the correlation between thinning rate and lipid thickness is only modest, the relation between thinning rate and lipid thickness becomes more striking when thinning rate is analyzed using the slow/rapid dichotomy. Finally, correlations exist among four quantities: lipid thickness, thinning rate, evaporation, and BUT. The correlation between lipid thickness and thinning rate is reported in this study, whereas correlations between lipid thickness, evaporation, and BUT have been previously reported.²⁴ The methodology described here is novel in that it is capable of measuring outcomes at the identical time and location, whereas previously reported correlations between outcomes, such as evaporation and lipid thickness, were performed using different measurement methods (e.g., evaporimeter and thickness-dependent interferometer) at different times.²⁴

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