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Biofilm thickness measurements by light microscopy

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Summary

Light microscopy has been used to measure biofilm thickness. The vertical displacement of the sample required to focus from the biofilm-liquid interface to the biofilm-substratum interface is measured by the stage micrometer. Biofilm thickness is proportional, but not equal, to the measured vertical displacement. An expression for the proportionality constant, k_f, in terms of refractive indices is determined from a geometric analysis of the light path. k_f can be estimated as the ratio of the refractive index of the film to the refractive index of the media interfacing the film between the objective lens and the sample. The thickness of any transparent film may be determined by light microscopy when the refractive index of the film is known.

Key words: *Biofilm – Light microscopy – Refractive index – Thickness – Thin film*

Introduction

Several methods for thickness measurements of thin films are available [1, 2], but they are not well suited for biological films. Biofilm thickness should, ideally, be obtained in situ without influencing the sample. This may be accomplished by growing the biofilm in a transparent duct and determining the film thickness by light microscopy.

Biofilm thickness measurement by light microscopy has previously been applied in biofilm studies to determine film thickness of samples removed from biofilm reactors [3, 4]. It has, however, erroneously been assumed that the vertical displacement of the sample measured by the vertical stage micrometer of an optical microscope equals the vertical distance between the two surfaces (biofilm-liquid and biofilm-substratum interfaces) in focus. Due to differences in refractive indices for air, water, glass, etc., the proportionality constant, k_f, between the vertical stage

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displacement and biofilm thickness is not equal to unity, and in general will vary for different biofilm specimens. Bennett and Bennett [1] referred to this phenomenon as the difference between optical and mechanical thickness.

Biofilm thickness, L_f, is an important parameter in both theoretical analysis and practical application of biofilms due to its role in mass transfer. For example, diffusion of compounds into and out of biofilms depends on diffusion distance, which is a function of biofilm thickness. Diffusion rates also depend on biofilm density, which can be determined only when biofilm thickness is known. Transfer of biofilm mass such as cells and extracellular polymeric substances from the biofilm to the liquid phase is influenced by biofilm thickness, because fluid shear forces, which cause biofilm detachment, increase with biofilm thickness. Head loss due to biofilm formation (e.g., biofouling in pipelines) depends on biofilm thickness and, similarly, heat transfer resistance due to biofouling is a function of biofilm thickness.

The goal of this study was to determine the influence of specimen refractive indices on thickness measurements of transparent films (e.g., biofilms) by light microscopy.

Theory

The problem is illustrated in Fig. 1, which describes the method applied by Trulear and Characklis [3]. The light ray DO originating from point D on plane Σ_1, the air-biofilm interface, and ending in the plane of the microscope objective lens traverses a single medium, air, and hence is a straight line. The ray originating at point F on plane Σ_3, the biofilm-substratum interface, and ending on the the objective plane

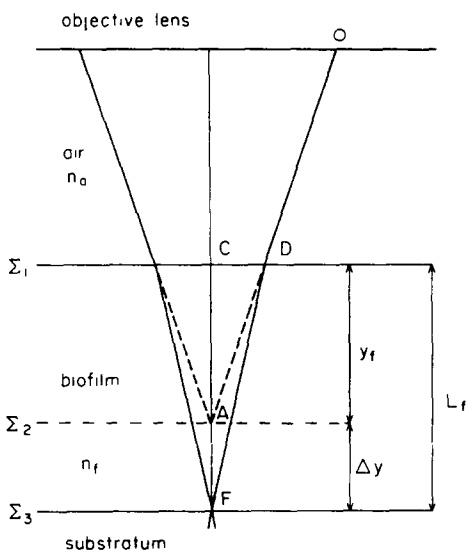

Fig. 1. Light paths from focal plane Σ_1 at the substratum-biofilm interface, through the biofilm sample and air to the objective lens.

traverses two media with different refractive indices; air with refractive index n_a and biofilm with refractive index n_f. Since the ray is not normal to the plane Σ_1, it is refracted (bent) at this air-biofilm interface and the resultant optical path FDO is not a straight line. For an observer focusing the microscope on Σ_3, point F appears to be at point A on the 'virtual' Σ_2 plane where no true interface exists, due to the refraction at the air-biofilm interface Σ_1. The stage movement necessary to change the microscope's focal plane from the air-biofilm to the biofilm-substratum interface is the distance between planes Σ_1 and Σ_2. This distance, y_f, is the optical thickness of the film. The actual (mechanical) thickness, L_f, is the distance from plane Σ_1 to plane Σ_3.

The problem, thus illustrated, was analyzed to derive the relationship between mechanical film thickness, L_f, and its optical thickness, y_f. We will further show that to close approximation the two thicknesses are proportional:

$$L_f \simeq k_f y_f$$

with the proportionality constant k_f a simple function of the refractive indices involved.

The coordinates and angles of the light path described in Fig. 1 are defined in Fig. 2. The solid line, FD, represents a light path with the top surface of the film being plane Σ_1, while the broken line AD, is the apparent optical path. Note that AB = CD. Therefore:

$$\frac{AB}{y_f} = \frac{CD}{y_f} = \tan \alpha_a$$

$$\rightarrow y_f \tan \alpha_a = AB \qquad (1)$$

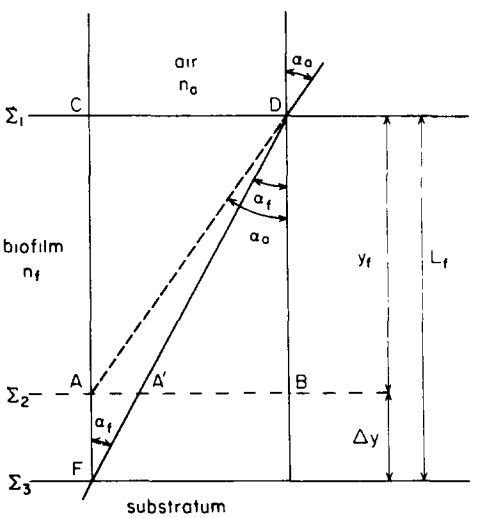

Fig. 2. Coordinates and angles for optical path within the biofilm. Σ_3 is the focal plane and Σ_2 is the apparent focal plane.

and

$$\frac{A'B}{y_f} = \tan \alpha_f$$

$$\rightarrow y_f \tan \alpha_f = A'B \tag{2}$$

Also:

$$AA' = AB - A'B \tag{3}$$

Substituting Equations 1 and 2 in 3 yields:

$$AA' = y_f (\tan \alpha_a - \tan \alpha_f) \tag{4}$$

Also:

$$\frac{AA'}{\Delta y} = \tan \alpha_f$$

$$\rightarrow \Delta y \tan \alpha_f = AA' \tag{5}$$

Combining Equations 4 and 5 and rearranging yields:

$$\Delta y = \frac{\tan \alpha_a}{\tan \alpha_f} y_f - y_f \tag{6}$$

Since:

$$L_f = y_f + \Delta y$$

$$\rightarrow L_f = \frac{\tan \alpha_a}{\tan \alpha_f} y_f \tag{7}$$

Note that:

$$\frac{\tan \alpha_a}{\tan \alpha_f} = \left(\frac{\sin \alpha_a}{\sin \alpha_f}\right) \left(\frac{\cos \alpha_f}{\cos \alpha_a}\right) \tag{8}$$

From Snell's law of refraction at an interface:

$$\frac{n_f}{n_a} = \frac{\sin \alpha^a}{\sin \alpha_f} \tag{9}$$

Combining Equations 7, 8, and 9 yields:

$$L_f = \frac{n_f}{n_a}\left(\frac{\cos \alpha_f}{\cos \alpha_a}\right) y_f \tag{10}$$

which is an exact expression for the mechanical thickness, L_f, in terms of the optical thickness, y_f.

A simpler and more applicable expression was derived based on the following approximation. Since:

$$\cos \alpha = 1 - \alpha^2/2! + \alpha^4/4! \ldots$$

we can see that:

$$\cos \alpha \simeq 1 \text{ for } \alpha \ll 1$$

(small angle approximation, α measured in radians).

Since light microscopes can be operated with $\alpha_a \ll 1$ and $\alpha_f < \alpha_a$ for biofilms:

$$L_f \simeq \frac{n_f}{n_a} y_f \tag{11}$$

so the proportionality constant between the mechanical and optical film thickness k_f, is:

$$k_f \simeq n_f/n_a \tag{12}$$

Multiple layers: When, for example, film thickness is measured in situ on films developed in glass ducts, the light path passes through three layers (film, glass, and air or oil) between the objective lens and the focal point. This complicates the exact analytical solution. The approximate value for k_f, however, is independent of α_a and α_f, and depends only on the ratio of refractive indices at the top interface of the film. Therefore, k_f can, in general, be estimated as:

$$k_f \simeq n_f/n_m \tag{13}$$

where n_m is the refractive index of the medium interfacing the film at its top surface (the film interface closest to the objective lens).

Test

The validity of the theory was tested by measuring the thickness of glass slides by light microscopy and with a micrometer. The results are presented in Table 1. The close comparison between thickness measurements of the same samples by the two methods supports the theory.

Conclusions

The following conclusions were derived from this study.

TABLE 1

COMPARISON OF THICKNESS MEASUREMENTS OF GLASS SLIDES (n_f = 1.5253 ± 0.0008) BY LIGHT MICROSCOPY AND WITH A MICROMETER (sample size was 5)

Sample	Microscopy		Micrometer L_1 [μm]
	y_1 [μm]	L_1 [μm]	
#1 Cover slip	93.6 ± 0.6	144 ± 1	150 ± 10
#1½ Cover slip	126 ± 3	193 ± 4	200 ± 15
Microscope slide	657 ± 5	1002 ± 8	1020 ± 21

1. The mechanical thickness of transparent films can be measured by light microscopy by measuring the vertical displacement of the sample required to move the focal point across the film thickness.

2. The mechanical thickness is proportional to the vertical displacement (optical thickness), but not equal to it.

3. The proportionality coefficient depends on the ratio of the refractive indices of the measured film to that of the medium interfacing the film between the film and the objective lens.

To determine biofilm thickness by light microscopy, knowledge of biofilm refractive indices is, therefore, required. Due to biofilm's high water content, it is reasonable to assume that its refractive index will be close to that of water (~1.33). Work is presently being conducted to determine index of refraction in biofilms.

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