CHAPTER 2

LOW LOAD COMPRESSION TESTING:

A NOVEL WAY OF MEASURING BIOFILM THICKNESS

INTRODUCTION

Biofilms are microbial communities consisting of microorganisms surrounded by an extracellular polymeric matrix (EPS). Biofilms are a preferred way of microbial existence as they provide protection against existing physical forces and chemical attack, if necessary. Formation of a biofilm is desirable in some cases (wastewater treatment, biochemical production), whereas in others it poses severe problems (marine equipment fouling, biomaterial-related infections). Being able to quantitatively describe biofilms, for instance in terms of volume, wet weight, number of species present, or thickness allows for better systems engineering, and reduction of damages and operational costs. Biofilm thickness is especially important for calculation of heat exchange or diffusion rates of antimicrobials or nutrients through a biofilm (4, 15) and evaluation of their mechanical properties (9, 10).

Several destructive and non-destructive methods are available for biofilm thickness measurements. Destructive methods like scanning electron microscopy (5) and cryoembedding (2) require extensive dehydration or freezing, leaving the biofilm unsuitable for any further measurements. Additionally, dehydration may lead to underestimation of the biofilm thickness due to shrinkage. Non-destructive optical methods available are light microscopy (1), scanner with image acquisition system (13), laser triangulation sensor (14), confocal laser scanning microscopy (CLSM) (16) and two-photon excitation microscopy (17), which uses visible, laser or infrared light to elucidate the 3D structure of a biofilm. For light microscopy, the refractive index of a biofilm is required, which is mostly assumed to be that of water (1). However, the accuracy of the method suffers when thick and dense biofilms with low water content are in question. The application of the scanner method is limited to biofilms of 100
µm or less because for thicker biofilms it is difficult to obtain reliable measurements without destroying them (13). Furthermore, thickness measurements obtained with light microscopic techniques were used to calibrate the scanner method introducing possible errors. The laser triangulation sensor is a fast and non-destructive instrument to evaluate biofilm thicknesses but significant errors in the measurements are possible due to the presence of a film of water on the biofilm surface leading to the occurrence of stray light from the deeper layers of the biofilm. Optical techniques like CLSM, requiring staining of the biofilm with fluorescent dyes, are limited by the (in)ability of the dyes to penetrate the depth of the biofilm, and are subject to a fluorescent bleaching of a sample. Good quality CLSM images are only possible for biofilms up to about 70 µm thick (7). Two-photon excitation microscopy allows imaging of thicker biofilms up to 350 µm, due to improved spatial localization, deeper sectioning of the samples and reduced fluorescent bleaching (8). However, two-photon excitation microscopy remains expensive. Magnetic resonance imaging (MRI) (12) is another non-destructive technique, but it also requires an elaborate setup and expertise. Therefore a need is felt for a simple, non-destructive, accurate, and inexpensive method to measure biofilm thickness.

In this study we describe a new mechanical method to measure the biofilm thickness non-destructively. The method is based on the principle of uniaxial compression and the device is called a low load compression tester (LLCT). The device is relatively simple, inexpensive, and can be assembled in-house. It consists of a linear positioning stage and electronic analytical balance fixed on a stable granite base and interfaced to a computer for control, signal acquisition and data analysis. During measurement, the biofilm is kept in its physiological, hydrated state, which is
one of the main advantages of the method, and the force induced on the biofilm while it is squeezed during uniaxial compression is recorded by the acquisition system.

We considered a wide variety of yeast and bacterial biofilms to measure thicknesses with the new LLCT method. The thickness values obtained with LLCT were compared with values obtained with CLSM. For CLSM analysis, bacterial and yeast biofilms were stained with LIVE/DEAD BacLight, FUN1, and CalcoFluor, and images were taken over the depth of the biofilm. Images were analyzed with COMSTAT software (7) to determine the biofilm thickness and maximum depth to which CLSM technique can be applied.

MATERIALS AND METHODS

Microbial strains, growth conditions and harvesting

*Streptococcus oralis* J22, *Pseudomonas aeruginosa* SG81, *Enterococcus faecalis* BS385 and BS1037 (3, 9, 18) grown on blood agar plates, were used to inoculate 10 ml growth medium (Table 1) and allowed to grow for 24 h at 37°C in ambient air. This culture was used to inoculate a 100 ml main culture which was grown for 16 h. Cells were harvested by centrifugation and washed twice with sterile buffer (Table 1). *S. oralis* J22 forms chains and aggregates and hence these bacteria were sonicated on ice for 30 s at 30 W (Vibra cell model 375, Sonics and Materials Inc., Danbury, Connecticut, USA). Cooling on ice was done to ensure that the cells did not lyse. Following centrifugation and sonication, bacteria were resuspended in buffer for further use.

*Candida albicans* MB02, MB10 (11), and SC5314 (ATCC MYA-2876) grown on TSB (OXOID, Basingstoke, UK) agar plates were used to inoculate a 10 ml batch culture, which was grown at 30°C for 16 h in ambient air while shaking at 120 rpm.
Table 1. Growth and harvesting conditions, suspending liquid and suspension density for the microbial strains used in the study.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Growth Medium</th>
<th>Centrifugation</th>
<th>Buffer</th>
<th>Suspension density</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. oralis</em> J22</td>
<td>Todd Hewitt Broth (THB, OXOID, Basingstoke, UK)</td>
<td>10,000 x g 3 x 5 min 10°C</td>
<td>Adhesion buffer (KCl 3.73 g/l, K₂HPO₄ 0.174 g/l, KH₂PO₄ 0.136 g/l, CaCl₂.2H₂O 0.147 g/l, pH 6.8)</td>
<td>3x10⁸ cells/ml</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> SG81</td>
<td>Pseudomonas Isolation Broth (PIB) (Bactopeptone 20 g/l, K₂SO₄ 10 g/l, MgCl₂.6H₂O 1.4 g/l, Triclosan 0.025 g/l, Glycerol 25.2 g/l, pH 7.0)</td>
<td>10,000 x g 3 x 5 min 10°C</td>
<td>0.14 M NaCl</td>
<td>2x10⁸ cells/ml</td>
</tr>
<tr>
<td><em>E. faecalis</em> BS385 and BS1037</td>
<td>Tryptic Soya Broth (TSB, OXOID, Basingstoke, UK)</td>
<td>6,500 x g 3 x 5 min 10°C</td>
<td>10 mM Potassium phosphate buffer (K₂HPO₄ 0.87 g/l, KH₂PO₄ 0.68 g/l)</td>
<td>3x10⁸ cells/ml</td>
</tr>
<tr>
<td><em>C. albicans</em> SC5314, MB02, MB10</td>
<td>Tryptic Soya Broth (TSB, OXOID, Basingstoke, UK) For biofilm growth: Yeast Nitrogen Base (YNB, Difco™ without amino acids, Becton Dickinson, Sparks MD, USA)</td>
<td>5,000 x g 1 x 10 min 10°C</td>
<td>Phosphate-buffered saline (PBS) (NaCl 8.76 g/l, K₂HPO₄ 0.87 g/l, KH₂PO₄ 0.68 g/l, pH 7.0)</td>
<td>1x10⁷ cells/ml</td>
</tr>
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Yeast were harvested by centrifugation, washed once with sterile buffer and resuspended in buffer. Growth medium, buffer, centrifugation forces, and resuspension densities are summarized in Table 1 as well.

**Biofilm growth**

Biofilms were grown using three different methodologies. First, *S. oralis* J22 and *P. aeruginosa* SG81 biofilms were grown at a solid-liquid interface in a parallel plate flow system under constant shear (3). A parallel plate flow chamber with dimensions of 17.5 cm by 1.6 cm by 0.075 cm was used to grow biofilms on glass slides (Menzel-Glaser, Germany). A microbial suspension was perfused through the system under hydrostatic pressure in order to create a pulse free flow, as described before in detail (3). Flow chamber and glass slides were washed with a detergent, 2% RBS 35 (Omnilabo, Breda, The Netherlands), thoroughly rinsed with tap water and demineralized water and sterilized by autoclaving. Flow started with passing of adhesion buffer (for *S. oralis* J22) or 0.14 M NaCl (for *P. aeruginosa* SG81) during half an hour at a shear rate of 7.3 s⁻¹ for temperature (37°C) and flow to stabilize. The bacterial suspension was then allowed to pass through the flow chamber till a surface coverage on the bottom glass plate of 1 x 10⁶ cells cm⁻² was achieved. The flow chamber was again rinsed for half an hour with adhesion buffer in order to remove non-adhering bacteria. Growth medium was then introduced to the system (10% THB in adhesion buffer for *S. oralis* J22; 10% PIB in 0.14 M NaCl for *P. aeruginosa* SG81) at the same shear rate of 7.3 s⁻¹. The cultures were allowed to grow at 37°C for 36 h (*S. oralis*) and 64 h (*P. aeruginosa*) to form biofilms. The flow chambers were rinsed with buffer before removing the glass slides with biofilms.

As a second way of growing biofilms at the solid-liquid interface, *E. faecalis* BS385, *E. faecalis* BS1037, and *C. albicans* biofilms were grown on 1.5 x 1.5 cm
polymethylmethacrylate (PMMA) slides in 12-well tissue culture plates. Prior to growing candida biofilms, the slides were coated with 50% fetal bovine serum for at least 30 min to enhance adhesion (11), washed once with PBS, and placed into the wells. Three ml microbial suspension was added to each well and cells were allowed to adhere at 37°C while rotating at 60 rpm. Microbial suspensions were removed after 1.5 h and the slides were washed with buffer. Biofilm growth was propagated by adding 3 ml per well of TSB with 0.5% glucose (w/v) (E. faecalis) or yeast nitrogen base with 50mM glucose pH 7.0 (C. albicans) and incubated at 37°C for 48 h (E. faecalis) or for 16-72 h (C. albicans), also under rotation (60 rpm). Afterwards, the medium was discarded and biofilms were washed once with buffer.

In the last methodology applied here to generate biofilms, P. aeruginosa SG81, E. faecalis BS385, E. faecalis BS1037, and S. oralis J22 biofilms were grown statically at the solid-air interface on a Millipore filter (HTTP04700) with a pore size of 0.45 μm. For P. aeruginosa SG81, 1 ml of bacterial solution with cell density of 1 x 10^8 cells ml^-1 was filtered through a sterile filter. The membrane filter covered with bacteria was placed on the surface of PIB agar plates. After incubation for 24 h at 37°C, a confluent and mucoid bacterial lawn was obtained on the surface of the membrane filter. For E. faecalis BS385, E. faecalis BS1037, and S. oralis J22, suspensions were diluted to 3 x 10^8 cells ml^-1 and 10 ml was filtered through the membrane filter. The filters covered with bacteria were placed on TSB agar plates and incubated for 72 h at 37°C.

**CLSM analysis**

All images were acquired with Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) with beam path
settings for FITC- and TRITC-like labels. Stacks of images were obtained with a 40x water objective lens. For imaging bacterial biofilms, biofilms were stained with LIVE/DEAD BacLight stain (Molecular Probes, Eugene, OR, USA) and incubated at room temperature in the dark for 15 min. Yeast biofilms were stained with FUN1 (Molecular Probes Eugene, OR, USA) viability stain and CalcoFluor (Sigma, MO, USA) and incubated at room temperature in the dark for 45 min according to the manufacturer's staining protocol. In addition to FUN1 and CalcoFluor, C. albicans SC5314 biofilms were also stained with LIVE/DEAD BacLight stain. The images were analyzed by COMSTAT software (7). For COMSTAT analysis, CLSM files were converted into TIF format and manually thresholded by the user in order to convert color images into black-and-white, which could be analyzed by COMSTAT software to yield the mean biofilm thickness from the stacks of images.

**LLCT analysis**

Biofilm thicknesses were measured in a low load compression tester (LLCT), as schematically shown in Fig. 1. The LLCT apparatus consists of a linear positioning stage (Intellistage M-511.5IM, Physik instrumente, Karlsruhe, Germany) connected to a cylindrical moving upper plate with a diameter of 2.5 mm. A bottom stationary plate is fixed to an automatic force compensating balance, shown in Fig. 1 as a load cell (SW 50/300, Wipotec, Kaiserslautern, Germany). Both the load cell and linear positioning stage were interfaced to a PC for data acquisition and control using LabVIEW™ 7.1 software. The movement of top plate and force registered by the load cell were stored in a text file for further analysis by MS Excel. During the measurements, the substratum with biofilm was carefully placed on the bottom plate and the top plate was moved downwards until it touched an area of the substratum that was cleaned from biofilm with a tissue. This height was recorded as the bottom
of the biofilm. In the second step, the top plate was moved laterally over an area of the substratum containing biofilm and then moved downwards until it touched the biofilm surface and this height was recorded as well. Subsequently, biofilm thickness was calculated from the difference between both heights. “Touch” of the biofilm surface was considered to occur during top plate’s downward motion, when the load had increased above a predefined value. To prevent drying out of biofilms, thicknesses were measured immediately after growth. Additionally, the apparatus was encased in a box to minimize evaporation.

Figure 1. Frontal view of the low load compression tester, showing the main components of the system.

RESULTS

During measurement, the upper cylindrical plate moves towards the bottom stationary plate, holding the substratum and biofilm, at a speed of 1 \( \mu \text{m s}^{-1} \). During
approach, the load on the plate is registered by the force compensating balance connected to the bottom plate, and is essentially zero until the plate touches the biofilm surface, at which point the load starts increasing. As soon as the predefined touch load value of 0.01 g (see below) is registered, movement of the upper cylindrical plate is stopped and the plate is withdrawn to avoid damaging the biofilm. Typical data output for the soft surface of a biofilm is shown in Fig. 2, and includes the load measured by the force compensating balance during the uniaxial compression and deformation inflicted on a biofilm. The biofilm thicknesses derived and their reproducibility depend on the correct definition of the touch load value.

**Figure 2.** An example of data output during biofilm thickness measurements, with load (black symbols, left axis) and deformation (gray symbols, right axis) values.

A touch load value of 0.01 g was chosen because at that load the interferences from the background noise and lateral displacement of water through biofilm could be avoided and the deformation of the biofilm due to compression, i.e. the fractional change in biofilm thickness from initial contact to the touch load value.
adapted, is generally less than 0.1%, as can be seen in Fig. 2. Moreover, a touch load of 0.01 g yielded good reproducibility of the heights measured and ten repeated measurements on the same spot of a cleaned substratum were identical within 0.08 µm. Based on the above, a touch load value of 0.01 g was used throughout the remainder of this study for biofilm thickness determination.

To demonstrate the applicability of LLCT to various types of biofilms, different growth conditions were applied. For all conditions, the biofilm thicknesses derived from LLCT as described above, were compared with CLSM evaluations of biofilm thicknesses. Fig. 3 shows CLSM images of bacterial and yeast biofilms used in this study. The images show heterogeneities in surface coverage and thickness of the biofilms grown under different conditions. From the biofilm cross-sections it can be seen, that biofilms grown under constant shear were carpet-like (Fig. 3a), whereas biofilms grown under rotation showed mushroom-like structures and flow channels (Fig. 3b). Bacterial biofilms were considerably thinner than yeast biofilms. Dye penetration was complete through bacterial biofilms, as shown in Fig. 3a and 3b, and, incomplete through yeast biofilms, as can be concluded from the absence of a defined border between the yeast biofilms and the substratum in Figs. 3c and 3d.

LLCT and CLSM thickness measurements were first compared for relatively thin bacterial biofilms of *S. oralis* J22, *P. aeruginosa* SG81 biofilms, grown in a parallel plate flow chamber, and biofilms of *E. faecalis* BS385 and BS1037 grown in tissue culture plate on PMMA under rotation. The average thicknesses of these bacterial biofilms by LLCT were between 23 µm and 117 µm, while by CLSM they ranged between 26 µm and 106 µm (Fig. 4a) which does not represent a statistically significant difference (p > 0.5, two-tailed Student t-test).
Figure 3. Confocal images of biofilms, scale bar represents 75 μm:

a) S. oralis J22 grown in a flow chamber stained with LIVE/DEAD BacLight bacterial viability stain with the arrow pointing to carpet-like structures,
b) E. faecalis BS1037 grown on PMMA stained with LIVE/DEAD BacLight bacterial viability stain with the arrow pointing to mushroom-like structures,
c) C. albicans SC5314 grown on PMMA stained with FUN1 yeast viability stain, and

d) C. albicans SC5314 grown on PMMA stained with CalcoFluor, demonstrating heterogeneous spatial distribution of the biofilms in x-z and y-z directions and limited stain penetration in yeast biofilms.

Secondly, thicknesses of yeast biofilms grown in tissue culture plates on PMMA under rotation were compared. Biofilm thicknesses for overnight and three-day old C. albicans SC5314, MB02, and MB10 ranged from 132 μm to 322 μm when measured with LLCT, but were significantly thinner (p < 0.00001, two-tailed Student t-test) between 25 μm to 40 μm when measured by CLSM after staining with FUN1 (Figs. 4b and c).
Finally, the thicknesses of biofilms grown at solid-air interfaces as opposed to solid-liquid interfaces as described above, were compared for *P. aeruginosa* SG81, *E. faecalis* BS385 and BS1037, and *S. oralis* J22. The thicknesses measured using LLCT ranged from 61 µm to 292 µm. CLSM analysis was only possible for the biofilms of *P. aeruginosa* SG81, as the solid-air grown biofilms disintegrated upon application of the fluorescent dye. CLSM measurements showed that the biofilms of *P. aeruginosa* SG81 were only 34 µm thick, which was significantly less than found by LLCT.

To determine the influence of fluorescent dyes and their penetration through yeast biofilms on CLSM biofilm thickness measurements, FUN1, LIVE/DEAD BacLight, and CalcoFluor (6), were applied. *C. albicans* SC5314, MB02, and MB10 biofilms grown for 3 days on PMMA under rotating fluid flow had a thickness by CLSM of 30 µm to 40 µm when stained with FUN1 and of 64 µm to 120 µm when stained with CalcoFluor. CLSM thicknesses of *C. albicans* SC5314 stained with LIVE/DEAD BacLight were on average 100 µm. LLCT measurements however, indicated significantly thicker biofilms (p < 0.05, Student t-test).

**DISCUSSION**

We have developed a new, non-destructive method for measuring biofilm thicknesses, based on Low Load Compression Testing, which has several advantages over currently available techniques, such as CLSM analysis. Fig. 5 compares all biofilm thicknesses measured using LLCT with those obtained by CLSM. For the bacterial biofilms less than 120 µm thick, there were no statistically significant differences in thicknesses measured and data points distributed close to the line of identity, but for yeast biofilms over 120 µm thickness, CLSM
underestimated the biofilm thickness as compared with LLCT, regardless of the fluorescent dye applied.

The major advantage of the new method over CLSM is that it has no depth limitation and can be used for measuring a wide range of biofilm thicknesses, making it a superior technique in comparison to a microscopy where dye penetration, depth of focus, and photobleaching limit the application. Additionally, LLCT is more reliable because microscopy based techniques suffer from observer-bias in image selection. LLCT allows for the analysis of an almost two-orders of magnitude larger area compared to microscopic methods, which leads to more accurate determination of biofilm thickness.

By comparison with other methods that are currently available for measuring biofilm thicknesses, LLCT also offers several advantages. First, the biofilm is kept in its physiological, hydrated state during measurements and is left intact for further studies because the compression during the tests is limited to less than 0.1% deformation. Second, the method can be used to measure thicknesses of the biofilms grown on solid-air interfaces where application of a dye destroys biofilm architecture, which impedes CLSM imaging. Third, the measurements are not as time and labor-intensive as other methods, such as cryoembedding or laser triangulation sensor, and the results are available almost instantaneously. Fourth, the measurements done with LLCT are highly reproducible, since differences between measurements on same spot were less than 0.08 µm, which is significantly smaller than the thickness of
Figure 4. (a) Biofilm thicknesses of bacterial biofilms measured with LLCT (black) and CLSM (white), after LIVE/DEAD BacLight staining for CLSM, (b) similar for yeast biofilms after FUN1 staining and (c) comparison of biofilm thicknesses of C. albicans strains measured with LLCT (black) against biofilm thicknesses obtained with CLSM after staining with FUN1 (white) and CalcoFluor (gray).
most of biofilms. The last major advantage is a relatively low cost of the system compared to other systems used, such as MRI or CLSM.

Figure 5. Biofilm thicknesses measured using LLCT as a function of their thicknesses derived from CLSM.
REFERENCES


