The influence of fluid shear and AlCl$_3$ on the material properties of *Pseudomonas aeruginosa* PAO1 and *Desulfovibrio sp.* EX265 biofilms


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Abstract An understanding of the material properties of biofilms is important when describing how biofilms physically interact with their environment. In this study, aerobic biofilms of *Pseudomonas aeruginosa* PAO1 and anaerobic sulfate-reducing bacteria (SRB) biofilms of *Desulfovibrio sp.* EX265 were grown under different fluid shear stresses ($\tau_g$) in a chemostat recycle loop. Individual biofilm microcolonies were deformed by varying the fluid wall shear stress ($\tau_w$). The deformation was quantified in terms of strain ($\varepsilon$), and the relative strength of the biofilms was assessed using an apparent elastic coefficient ($E_{app}$) and residual strain ($\varepsilon_r$) after three cycles of deformation. Aluminium chloride (AlCl$_3$) was then added to both sets of biofilm and the tests repeated. Biofilms grown under higher shear were more rigid and had a greater yield shear stress than those grown under lower shear. The addition of AlCl$_3$ resulted in a significant increase in $E_{app}$ and also increased the yield point. We conclude that the strength of the biofilm is in part dependent on the shear under which the biofilm was grown and that the material properties of the biofilm may be manipulated through cation cross-linking of the extracellular polysaccharide (EPS) slime matrix.

Keywords Biofilm; cross-linking; *Desulfovibrio*; elasticity; EPS; extracellular polymeric substances; *Pseudomonas*; shear stress; strength

Introduction

Control of biofilms is important both in industry and in medicine. It is in our interests to be able to manipulate biofilms and predict how they may behave under different environmental conditions. Biofilms growing in flowing systems are constantly subjected to the shear exerted by the moving fluid. How a biofilm physically responds to this shear will be largely determined by the material properties of the biofilm. Stronger biofilms will be able to withstand shears that would cause weaker biofilms to break up and detach into the bulk liquid, resulting in biomass loss or product contamination. To understand how biofilms interact physically with their environment, we need to know something about their material properties. However, research on the material properties of biofilms has been underrepresented in the field.

Biofilms, like most biopolymers are viscoelastic materials meaning they can flow away from a force causing deformation either permanently or semi-permanently (Ohashi and Harada, 1994; Stoodley *et al*., 1999a). We have developed an *in situ* method by which we can relate the degree of deformation of individual cell clusters or microcolonies in response to a change in the bulk fluid flow rate to perform biofilm stress-strain and creep tests (Stoodley *et al*., 1999a). We found that pure culture *Pseudomonas aeruginosa* and mixed species biofilms growing aerobically behaved elastically until a critical (yield) fluid shear stress ($\tau$) was exceeded after which they began to flow. The apparent elastic modulus ($E_{app}$)
of these biofilms was in the range of 20 to 40 N/m². These findings were similar to those found by destructive biofilm sampling (Ohashi and Harada 1994). Additionally, time lapse imaging showed that a mixed species biofilm grown in turbulent flow formed ripples that appeared to flow along the wall of a glass flow cell (Stoodley et al., 1999b).

We wished to extend our investigations to include sulfate reducing bacteria (SRB) biofilms and investigate the influence of the shear under which both aerobic and anaerobic biofilms were grown (hereafter termed the growth shear, \( \tau_g \)), on the strength of the biofilm material. Liquid velocity has been shown to strongly influence biofilm structure (Stoodley et al., 1999c) and density (Melo and Vieira, 1999) and, it is reasonable to believe, may also influence material properties. Lastly, we wished to determine if the material properties of both aerobic and anaerobic biofilms could be manipulated by the addition of multivalent cations. In previous work we had hypothesized that the material properties of the biofilms we studied were determined largely by the extracellular polysaccharide (EPS) slime matrix. Uronic acids are a common component of bacterial EPS in many species (Lindberg, 1990) and mixed species waste water biofilms also have a significant uronic acid content (Nielsen et al., 1997). It has also been shown that the viscosity and gelation of purified microbial polymers can be influenced by the addition of calcium through cross-linking of the acidic sugars in the polymer strands (Mitchell and Blanshard 1974). We hypothesized that we could manipulate the material properties of biofilm in situ by changing the ionic environment of the biofilm through the addition of the trivalent cation, aluminium.

Methods

Inocula and media

For the anaerobic biofilms we used Desulfovibrio sp. EX265 a 16S rDNA typed strain (Genbank submission no. AF234891) isolated from a North Sea oil production facility. Desulfovibrio sp. EX265 was grown on a sea water medium consisting of: Instant Ocean™ (33g/l nanopure water), sodium pyruvate (0.6g/l), yeast extract (0.1g/l), and sodium acetate (0.07g/l).

For the aerobic biofilms we used Pseudomonas aeruginosa PAO1 grown on 1/50 strength Lauria Bertani (LB) broth (tryptone, 0.2 g/l, yeast extract, 0.1 g/l, and NaCl, 0.1 g/l).

Biofilm reactor systems

The basic reactor system consisted of two 20 cm long square (3 x 3 mm) glass tube flow cells (Friedrich & Dimmock, www.fdglass.com/CATALOG/spe-1.html) giving a hydraulic diameter \( (D_h) \) of 3 mm. The flow cells were positioned in parallel in a chemostat recirculation loop (Figure 1). One of the flow cells had an adjustable clamp at the inlet so the biofilms could be grown at different average flow velocities \( (u_{ave}) \). Media and sparge gas (filtered air for PAO1 or furnace/catalyst purified nitrogen for EX265), were added to the mixing chamber (chemostat) which also had an effluent overflow.

For the EX265 biofilm, a Reglo analog MS-416C (Ismatec, Glattbrugg-Zurich) pump was used to pump media through a flow break into the mixing chamber. A Micropump (Millipore Corporation, Bedford, MA) was used to recirculate media through the flow cells and the recycle loop. The volume of the entire system was 1l. Media was added at a flow rate of 0.5 ml/min. giving a residence time of 33 hours. For the PAO1 biofilm the recycle flow rate was controlled with a vane head pump (Masterflex, Cole Parmer, Niles, Il.). The volume of the system was 170 ml and media was added at 4 ml/min to give a residence time of 40 min.

Sterilization

The reactor systems were sterilized by autoclaving at 121°C for 15 min. or, for non-autoclavable components, by exposure to 70% ethanol for 15 minutes, 20% bleach solution for
15 minutes, and 70% ethanol for 15 minutes. Media was autoclaved separately at 121°C for 15 minutes per litre. For the SRB system sterile media was pumped through the system for 48 hours. No turbidity was observed after this time and the mixing vessel was inoculated with 1 ml of a stationary phase culture (10⁹ cells/ml by direct cell count) by injection into the mixing vessel. Restriction fragment length polymorphism (RFLP) analysis of the effluent at termination of the experiment was used to confirm culture purity. To confirm sterility in the PAO1 experiment media was pumped through the system for 24 h and two 0.1 ml aliquot samples were plated on Tryptic Soy Broth (Difco). Culture purity was confirmed by plating throughout the experiment.

Flow cell hydrodynamics
For the EX265 system the \( u_{\text{ave}} \) in the high flow cell was controlled by calibrating the pump setting by volumetric displacement. The \( u_{\text{ave}} \) in the clamped flow cell was calibrated by measuring the flow rate at various clamp settings (closed, \( \frac{1}{4} \) open, \( \frac{1}{2} \) open, \( \frac{3}{4} \) open, and 1 full revolution open (n = 4 calibration curves at each setting)). In the PAO1 system \( u_{\text{ave}} \) in the flow cells was continually monitored by in line flow meters (McMillan Flo-sensor model 101T # 3724 and 3835 supplied by Cole-Parmer, Niles, IL).

During biofilm growth the \( u_{\text{ave}} \) in each flow cell was kept constant and the corresponding shear stress was defined as the growth shear (\( \tau_g \)). The EX265 biofilm was grown under laminar flow for 7 days at \( \tau_g = 0.47 \text{ N/m}^2 \) and 0.028 N/m² in the high and low flow cells respectively. The PAO1 biofilm was grown for 6 days at 5.3 N/m² (turbulent) and 0.09 N/m² (laminar) in the high and low flow cells respectively. For details on flow cell hydrodynamics see Stoodley et al., 1999c. The wall shear stress (\( \tau_w \)) for laminar flows was calculated from:

\[
\tau_w = 8u_{\text{ave}} \eta / D_h
\]

(based on the assumption that \( u_{\text{max}} = 2u_{\text{ave}} \)) where \( \eta \) was the fluid dynamic viscosity. The \( \tau_w \) for turbulent flows was calculated using:

\[
\tau_w = f \rho_w u_{\text{ave}}^2 / 2
\]
where \( \rho_w \) = fluid density and \( f \) = friction factor from the Blasius equation (Characklis et al., 1990).

**Stress-strain experiments**

After the growth period individual microcolonies were microscopically selected for the stress-strain experiments. Where possible we tried to pick fields of view which contained at least two microcolonies to provide duplicate measurements. The biofilms were subjected to 3 deformation cycles in which each cycle consisted of a loading phase in which shear was incrementally increased at 10s intervals and an unloading phase in which the shear was similarly decreased. One minute was allowed for biofilm recovery between each cycle. For the SRB biofilms \( \tau_w \) was varied between 0.10 and 0.3 N/m². For the PAO1 biofilms \( \tau_w \) was varied between 0.40 and 7.5 N/m². A digital image using Scion Image analysis package (Scion Inc. Frederick, MD) was captured at each increment for subsequent measurement of engineering strain (\( \varepsilon \)):

\[
\varepsilon = \frac{\Delta l_c}{l_c}
\]  

(3)

where \( l_c \) = the original microcolony length measured in the direction of the bulk liquid flow and \( \Delta l_c \) was the change in length. The stress-strain curves were used to determine the apparent elasticity modulus (\( E_{app} \)) of the microcolonies and the residual strain (\( \varepsilon_r \)). \( E_{app} \) was analogous to Young’s modulus (E), which is a measure of stiffness, or resistance to deformation (Vincent, 1990) and was found from linear regression of the loading portion of the stress-strain curve. Aluminium chloride (AlCl₃) was then added to the mixing chamber (for a final concentration of 1g/l) and allowed to diffuse through the biofilm for 3 hours. Three sets of loading and unloading curves were then repeated.

**Statistical analysis**

One way ANOVA tests were performed (using Minitab 12.1 for Windows software) to compare \( E_{app} \) and \( \varepsilon_r \) of biofilm microcolonies grown at different \( \tau_g \) as well as before and after AlCl₃ addition. Differences were considered significant when \( P < 0.05 \). When the \( \Delta l_c \) of the microcolonies was so small that no strain could be measured a “greater than” value was calculated based on a detection limit of a change in length of 3 pixels divided by the original length of the monitored microcolony. For statistical analysis the “greater than” value was used to give the most conservative estimate of significant difference at the 95% level.

**Results**

**Biofilm structure as a function of growth shear stress (\( \tau_g \))**

As found previously the \( \tau_g \) had a significant influence on biofilm structure (Stoodley et al., 1999c). The microcolonies in the biofilms grown at lower shear were approximately circular in plan view, while those grown at higher shear were elongated in the downstream direction to form filamentous streamers (Figure 2).

**Material properties of biofilms grown under different \( \tau_g \)**

The shapes of the biofilm stress-strain curves (Figure 3) were similar to those measured previously in mixed culture and \( P. aeruginosa \) biofilms (Stoodley et al., 1999a). For the EX265 biofilm grown at \( \tau_w \) of 0.45 N/m² the loading and unloading portions of the curve were linear and followed the same path indicating that the biofilm was behaving elastically. The \( E_{app} \) was 1.93 ± 0.28 (mean ± 1 S.D., n = 6) N/m² and the \( \varepsilon_r \) was 0.006 ± 0.01 (n=6).

However, for the biofilm grown at \( \tau_w \) of 0.03 N/m² there was an evident hysteresis in the
curve indicating that the yield point had been exceeded and that the biofilm microcolonies had begun to flow. The $E_{\text{app}}$ was $1.06 \pm 0.04 \, \text{N/m}^2$ (n=6), approximately half that of the biofilm grown at higher shear. This difference was significant at the 95% level ($P=0.024$, n=12). The $\varepsilon_r$ was $0.085 \pm 0.058$ (n=6), approximately 15 times greater ($P=0.014$, n=12).

The PAO1 biofilm showed a similar trend. The $E_{\text{app}}$ of the microcolonies grown at a $\tau_w$ of 5.3 N/m² was $99.6 \pm 66.1 \, \text{N/m}^2$ (n=6), approximately 4 times greater than the $E_{\text{app}}$ of 26.5 $\pm 6.3 \, \text{N/m}^2$ (n=3) of the biofilm grown at 0.09 N/m². This difference was also significant at the 95% level ($P<0.000$, n=9). The $\varepsilon_r$ of the low $\tau_w$ PAO1 biofilm was $0.033 \pm 0.048$ (n=3), approximately 23 times greater than that of the biofilm grown at higher shear ($\varepsilon_r = 0.001 \pm 0.012$ (n=6)). However, because of the high variance associated with these measurements ANOVA showed that the $\varepsilon_r$ values were not significantly different between the biofilms grown at different $\tau_w$ ($P=0.226$, n=9).

Figure 2 Influnence of fluid shear on biofilm structure. A and B) SRB EX265 biofilm grown at $\tau_g$ of 0.03 and 0.45 N/m² respectively. Scale bars = 50 µm. C and D) P. aeruginosa biofilm grown at $\tau_g$ of 0.09 and 5.3 N/m² respectively. A circular shaped microcolony is indicated by small arrow in "C". The filamentous streamers which formed under higher shear were apparent in "D". Scale bars = 100 µm. Large arrows indicate bulk liquid flow direction.

Figure 3 Stress-strain curves for EX265 biofilms grown at 0.45 (6) and 0.03 (#) N/m². The loading and unloading portions of the curves are indicated by the "up" and "down" arrows respectively. Each strain value represents the mean of 6 measurements taken on two microcolonies, error bars = 1 S.D.
Influence of AlCl₃ on the material properties of the biofilm microcolonies

In all cases the addition of AlCl₃ resulted in a significant increase in $E_{\text{app}}$ (Figure 4). The rigidity of both EX265 and PAO1 biofilms was increased so that for some loading cycles no measurable deformation was observed. In these cases a “greater than” $E_{\text{app}}$ value was reported. There was no noticeable hysteresis in the stress-strain curves of the EX265 and PAO1 biofilms grown at low shear and the $\varepsilon_r$ decreased to $0 \pm 0$ (n=6) and $0.006 \pm 0.035$ (n=9) respectively indicating that the AlCl₃ had increased the yield shear stress point in both biofilms. Table 1 shows the $E_{\text{app}}$ and $\varepsilon_r$ results for biofilms before and after AlCl₃ addition.

Discussion

It is difficult to conduct precise stress-strain experiments on attached biofilms. Although we could accurately measure changes in microcolony length and, therefore, strain, we had no way of measuring the actual stresses acting on the individual biofilm microcolonies. However, we could relate the strain to the theoretical fluid wall shear stress to construct stress-strain curves and calculate an apparent elasticity modulus ($E_{\text{app}}$) which was analogous to Young’s elastic modulus ($E$). Although only a rough estimate of $E$, we found that $E_{\text{app}}$ was a useful parameter by which to compare the influence of the growth environment and the addition of multivalent cations on the material properties of biofilms at the microscopic scale. Indeed, it may be argued that the use of fluid shear to determine the mechanical properties and behavior of biofilms growing in flowing environments may be more

![Figure 4](https://iwaponline.com/wst/article-pdf/43/6/113/429625/113.pdf)

Figure 4 Influence of AlCl₃ on the stiffness of biofilm microcolonies. A) Loading portion of the EX265 biofilms grown at 0.45 ($) and 0.03 (#) N/m² before and after AlCl₃ addition (open symbols). Each data point was a mean of 6 measurements. B) Loading portion of the PAO1 biofilms grown at 5.3 ($) and 0.09 (#) N/m² before and after AlCl₃ addition (open symbols). Each data point was a mean of 6 measurements with the exception of (#) which was a mean of 3 measurements from 1 microcolony.

Table 1 $E_{\text{app}}$ and $\varepsilon_r$ before and after AlCl₃ addition (mean ± 1 S.D.). P values are from ANOVA comparison of the $E_{\text{app}}$ and $\varepsilon_r$ means before and after AlCl₃ addition. The means were considered significantly different when P < 0.05.

<table>
<thead>
<tr>
<th>Species</th>
<th>$\tau_g$ (N/m²)</th>
<th>AlCl₃</th>
<th>$E_{\text{app}}$ (N/m²)</th>
<th>$\varepsilon_r$ (-)</th>
<th>n</th>
<th>P ($E_{\text{app}}$)</th>
<th>P ($\varepsilon_r$)</th>
<th>n</th>
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<tbody>
<tr>
<td>EX265</td>
<td>0.03</td>
<td></td>
<td>1.06 ± 0.004</td>
<td>0.085 ± 0.058</td>
<td>6</td>
<td>&lt;0.000</td>
<td>0.026</td>
<td>12</td>
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<tr>
<td></td>
<td>0.45</td>
<td></td>
<td>1.93 ± 0.28</td>
<td>0.006 ± 0.035</td>
<td>6</td>
<td>&lt;0.000</td>
<td>0.211</td>
<td>12</td>
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<tr>
<td></td>
<td>After</td>
<td></td>
<td>&gt;17.5</td>
<td>0 ± 0</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>0.09</td>
<td></td>
<td>26.5 ± 6.3</td>
<td>0.033 ± 0.048</td>
<td>3</td>
<td>0.031</td>
<td>0.186</td>
<td>9</td>
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<tr>
<td></td>
<td>After</td>
<td></td>
<td>&gt;190</td>
<td>0 ± 0</td>
<td>6</td>
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<tr>
<td></td>
<td>5.3</td>
<td></td>
<td>99.6 ± 66.1</td>
<td>0.001 ± 0.012</td>
<td>6</td>
<td>0.005</td>
<td>0.828</td>
<td>12</td>
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<tr>
<td></td>
<td>After</td>
<td></td>
<td>&gt;336</td>
<td>0.003 ± 0.004</td>
<td>6</td>
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appropriate than using conventional testing apparatus designed for other materials behaving under different circumstances.

Structure and material properties
Qualitatively the shape of the stress-strain curves support our earlier findings on mixed species and pure culture *P. aeruginosa* biofilms in which we concluded that the biofilm microcolonies behaved elastically below a yield point but flowed when the shear stress was increased past the yield point (Stoodley *et al*., 1999a). Our results suggest that the hydrodynamic conditions during growth not only influence the morphology of the biofilm structures but also their material properties such as stiffness and yield point. In both SRB and *P. aeruginosa* biofilms the biofilms grown at a higher fluid shear had a significantly higher $E_{app}$ (were more rigid) and had a higher yield point than those grown at a lower shear. Taken together we conclude that the biofilms grown under high shear were mechanically stronger than those grown under lower shear. There are a number of possible explanations for this, some physical and some biological. Under higher shear (and better mixing) it is possible that lower molecular weight components were washed out of the EPS leaving a stronger matrix. Another possibility was that the higher shear environment selected for a subset of organisms which produced a stronger EPS. However, there may be a more active response in which biofilm cells can in some way “sense” shear and regulate the strength of their EPS accordingly. In this study we have shown that the strength of the biofilm can be significantly influenced by AlCl$_3$, presumably through increased cross linking of the EPS polymers. Biofilm bacteria may be able to similarly influence the strength of the biofilm matrix by influencing the local ionic environment through chelation mechanisms. The biofilm matrix could also be made stronger by producing a denser EPS with higher MW polymer strands. Proteins, which are also found in the EPS (Nielsen *et al*., 1997), may also play an as yet unidentified structural role in the mechanics of the EPS. This study presents the possibility that systems that operate under continual high shear may be selecting for stronger biofilms which may ultimately make them harder to remove.

Influence of AlCl$_3$ on biofilm strength
After the addition of AlCl$_3$ each biofilm tested became significantly more rigid and more resistant to deformation. Presumably this was due to increased cross linking between EPS polymer strands as we had originally hypothesized. Our assumption was based on the model of the cross linking of uronic acids in alginate by Ca$^{++}$ but it is likely that EPS contains other active groups capable of cross linking. It is also possible that in the anaerobic reactor the Al$^{+++}$ could have complexed with sulfide and other dissolved metals to form precipitates which may have contributed to the stiffening of the biofilm. Our results show that it is possible to manipulate biofilm strength through altering the ionic environment of the biofilm. This may have several important applications for biobarrier technology which has recently been proposed for treating contaminated groundwater plumes (Bouwer *et al*., 2000). One concern to using these barriers is that contaminated water could essentially ‘break through’ untreated. Our study suggests that it may be possible to stabilize the bio-barrier if required and conversely cause the biofilm to disrupt by reducing the cross linking by addition of monovalent cations or chelating agents.

Conclusions
1) Both *Pseudomonas* spp. and *Desulfovibrio* spp. biofilms had similar viscoelastic properties.
2) Hydrodynamic shear not only influenced biofilm structure but also the cohesive strength of the biofilm.
3) The addition of AlCl₃ increased the stiffness and the yield point of the biofilm.
4) The material properties of biofilm can be manipulated by changing the local ionic environment.

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References