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Effect of Strain Rate on the Mechanical Properties of *Staphylococcus epidermidis* Biofilms

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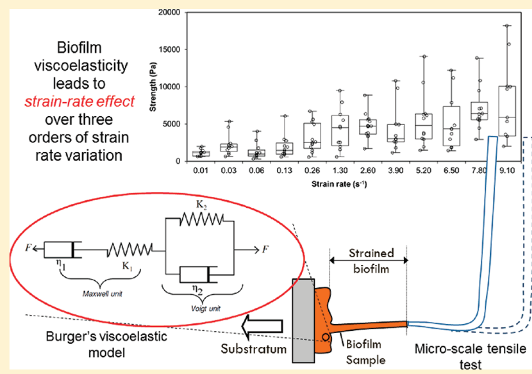
ABSTRACT: The quantification of biofilm mechanical properties can serve as a basis for understanding biofilm resilience and for developing biofilm control strategies. One aspect of tensile testing that is likely to be important for a viscoelastic material such as bacterial biofilm, but unfortunately is often overlooked (i.e., not controlled or reported), is the strain rate used during testing. Thus, we performed tensile testing on intact *S. epidermidis* biofilms using the microcantilever method at 12 strain rate values ranging over approximately 3 orders of magnitude (0.013–9.07 s^{-1}). Ultimate or cohesive strength, elastic modulus, and toughness increased with increasing strain rate and approached a plateau at approximately 1.3 s^{-1}. Failure strain, on the other hand, did not exhibit any trend with strain rate. Given that the mean values of some parameters increased by as much as 1 order of magnitude over the strain rate range used in this work, we suggest that the strain rate during tensile testing should be carefully controlled and reported to facilitate comparisons among different studies. Furthermore, the quantitative expressions developed in this work that relate mechanical property values with strain rate may be useful for modeling the deformation of bacterial biofilms under applied loads.



1. INTRODUCTION

As indicated by the number of publications over the past decade (>50), there is considerable interest in the measurement and manipulation of the mechanical properties of bacterial biofilms. This interest stems, at least in part, from the desire to control the accumulation of biofilms; especially to develop methods to enhance the removal of nuisance or problematic biofilms such as those occurring in pipelines, on human teeth, or on medical implant devices.[1−3] The ability of biofilms to withstand high mechanical forces can be attributed to their mechanical properties (e.g., elastic modulus and viscosity).[4] Thus, the availability of a method or methods for accurate and reproducible determination of biofilm mechanical properties is critical for experimental evaluation of the effectiveness of various treatments aimed at manipulating biofilm mechanical properties.

One consistent observation concerning the measurement of biofilm mechanical properties, noted by several researchers,[2,5,6] is the large variability in the results from a set of tests done in a given laboratory and the large differences in mean or median values produced by different laboratories. For example, various reports of biofilm strength values, from different laboratories in the literature, vary over 4 orders of magnitude (2–19 000 Pa)[7]

Certainly some of this variability, particularly for results from a given lab, can be attributed to the inherent heterogeneous nature of biofilms.[8,9] The wide range in reported mechanical property values between laboratories could be due to a variety of factors including differences in the bacterial strains used to establish the biofilms, the growth conditions (e.g., nutrient levels, fluid shear, and culture age), and the method used to test the mechanical properties. Recent studies in biofilm mechanics indicate that biofilms are viscoelastic materials.[10−13] A characteristic of viscoelastic materials is that the mechanical properties are rate/time dependent. Thus, another factor that could account for some of the variability in reported mechanical property values is differences in or lack of control of the strain rate during testing. Although a variety of methods have been employed by biofilm researchers to investigate the mechanical properties of biofilms and biofilm components including glass-capillary flow cells,[12−15] rotating disk rheometry,[2,16−19] holographic microrheology,[20] microbead force spectroscopy,[21] microfluidic rheometry,[22] and particle-tracking microrheology,[23] researchers typically do not report the strain rate used. Furthermore, we are unaware of any attempts to explore the effect of strain rate on the mechanical properties of biofilms.

We have recently developed a method for testing the mechanical properties of intact biofilms,[7,24] which we have employed here for investigating the effect of strain rate on the mechanical properties of biofilms. To our knowledge, this is the first study to directly explore the strain rate dependence of biofilm mechanical properties.

2. METHODS

2.1. Biofilm Inoculation and Growth. *Staphylococcus epidermidis* (ATCC strain 35984) biofilms were grown and used for tensile testing. *S. epidermidis* is a recognized biofilm former and has been previously

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used for studies of biofilm mechanical properties.[19,22,25] The culture was maintained on tryptic soy broth (TSB) agar plates, which were restreaked monthly. To prepare the inoculum for the biofilm reactor, a single colony was aseptically transferred into a 250 mL Erlenmyer flask containing 100 mL of TSB medium and was allowed to grow overnight at 37 °C on a shaker table at 100 rpm to a final optical density at 600 nm (OD_{600}) of 0.7–0.9. For biofilm growth, the inoculum was transferred to a 240 mL (liquid volume) continuous-flow rotating disk reactor (RDR) fed 10% TSB at a hydraulic retention time of 55 min. The disk was rotated at 170 rpm, resulting in estimated fluid shear stresses in the range of 1.08–1.76 Pa on the surfaces of the biofilm coupons. Details of the reactor operation were described previously.[7]

2.2. Effect of Strain Rate. To test the effect of strain rate, the biofilm was allowed to grow on coupons for ~3 days (74 h), after which the coupon was removed from the RDR for testing. Tensile tests were performed on the intact biofilm sample using the microcantilever method[24] over a range of separation (or retraction) velocities (1–3500 μm/s) using a motorized micromanipulator (LTA-HS, Newport Corporation, CA). Strain-rate was computed by dividing the separation velocity by the original biofilm thickness of the specimen.

Testing was conducted at 12 velocities (5, 10, 25, 50, 100, 500,1000, 1500, 2000, 2500, 3000,and 3500 μm/s). For an average biofilm thickness of 386 μm, these separation velocities result in strain rates ranging from 0.013 to 9.07 s^{-1}, which is within the range of strain rates used by other researchers investigating the mechanical properties of various biomaterials (10^{-4} to 10^{3} s^{-1}; Table 1). Because of the inherent heterogeneity in biofilm mechanical properties, at least 12 tests were performed at each of the twelve strain rate levels. A total of 154 tests were conducted.

Table 1. Comparison of Strain Rates Used in Tensile or Compression Testing of Biomaterials and Synthetic Polymers

material		strain rate (s^{-1})	ref(s)
skin	goat skin	8×10^{-5}–1.6×10^{-2}	Arumugam et al. 1994[33]
	rat skin	3×10^{-1}–6×10^{1}	Haut et al. 1989;[34] Dombi et al. 1993[35]
	pig skin	4×10^{-3}–4×10^{3}	Shergold et al. 2006[28]
polymers	nanocomposite foam	3×10^{-4}–1.6×10^{-2}	Jo and Naguib 2008[32]
	polypropylene	3×10^{-4}–3.3×10^{-2}	Ingram et al. 2008[31]
tendon	chicken tendons	10^{-4}- 10^{-1}	Wu 2006[30]
	rat tendon	10^{-4}- 10^{-2}	Cheng and Screen 2007[36]
bone	human cortical bone	8×10^{-2}–1.8×10^{1}	Zioupos et al. 2008[27]
rubber	silicone rubber	4×10^{-3}–4×10^{3}	Shergold et al. 2006[28]
biofilm	*S. epidermidis* biofilms	1.3×10^{-2}- 9.1×10^{1}	current study

2.3. Analysis of the Video Files. The 154 videos from the intact microcantilever testing were analyzed to derive stress–strain curves (Figure 1) which were used to extract various biofilm mechanical properties including strength, failure strain, toughness, and elastic modulus as described earlier.[7] All of the video image analysis was carried out using ImageJ (version 1.4 g). Cross-sectional area of separation (estimated from the width of the biofilm at the plane of separation) and equivalent diameter (of the detached biofilm fragment) were also dertermined as described previously.[24] Thus, a total of six parameters were derived from each test.

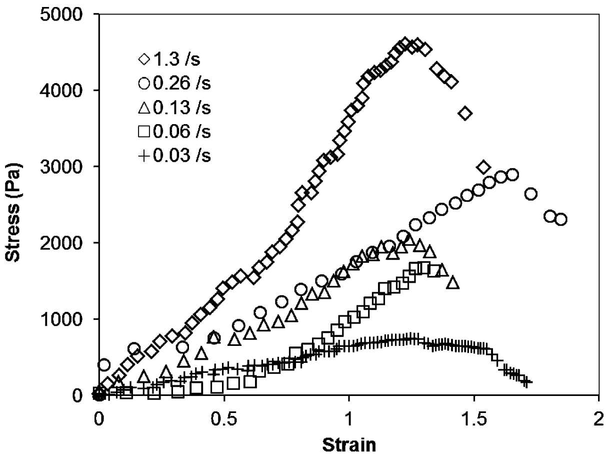

Figure 1. Representative stress–strain plots for *S. epidermidis* biofilm at different strain rates ranging from 0.03 to 1.3 s^{-1}. Each plot presents results from a single tensile test. Not all strain rates are represented in the graph for the sake of clarity.

2.4. Statistical Analyses. Kruskal–Wallis pairwise comparisons (a nonparametric test, with Bonferroni error protection) were used to examine a statistical difference with strain rate. These comparisons were performed for each of the six parameters. ANOVA was conducted with Fisher's LSD for posthoc pairwise comparisons. Kruskal–Wallis comparisons and ANOVA were performed using Analyze-it for Microsoft-Excel (version 2.12).

3. RESULTS

3.1. Data Distributions and Variability. Representative stress–strain plots for individual tensile tests (for strain rates ranging from 0.03 to 1.3 s^{-1}) are shown in Figure 1. Although the data for only a few tensile tests are shown, the results suggest that strength (i.e., the maximum stress value observed), elastic modulus (i.e., the slope of the stress–strain curve), and toughness (i.e., the area under the curve) increase with increasing strain rate. Given that biofilms, even those developed from a single bacterial species, exhibit significant heterogeneity, multiple tensile tests were performed at each strain rate. The results from an initial statistical analysis of the data generated are provided below.

The data sets for each parameter at each strain rate (n = 12–14 data points per parameter per strain rate) were examined to determine the underlying distributions. Specifically, normality tests were conducted on both the original data and the log-transformed data. Then, the number of strain rates (out of 12 strain rates total) for which normality was rejected ($p < 0.05$) and the number of strain rates for which normality could not be rejected ($p > 0.3$) were counted for each parameter (Table 2).

For elastic modulus, toughness, and strength, normality of the original data set was rejected in most cases (7–9 out of 12) while normality of the log-transformed data could not be rejected in most cases (7–10 out of 12). Thus, elastic modulus, toughness, and strength were, in general, log-normally distributed. For failure strain, normality of the original data set was infrequently rejected (1 out of 12), but log-normality could not be rejected in most cases (10 out of 12). For cross-sectional area, normality of the original and log-transformed data was infrequently rejected (0–2 out of 12 cases) and the number of cases where normality could not be rejected was similar for both the original and log-transformed data.

The coefficient of variation (CV) values ranged from 21% to 134% (Table 2). Out of six parameters, failure strain had the

Table 2. Summary of Results from the Tensile Testing of *S. epidermidis* Biofilm at Different Strain Rates

	range of measured parameter values			coefficient of variation (C.V.)			number of strain rate levels for which normality is rejected ($p < 0.05$)			number of strain rate levels for which $p > 0.3$	
parameter	min.	mean	max.	min.	mean	max.	as-obtained data	log-transformed data	as-obtained data	log-transformed data	distribution
elastic modulus (Pa)	201	4329	21092	48%	82%	115%	9	1	0	9	log-normal
strength (Pa)	312	4051	18209	35%	62%	82%	7	0	1	10	log-normal
toughness (J/m^3)	342	4029	31100	26%	66%	134%	7	1	5	7	log-normal
failure strain	1.7	5.4	12.3	21%	37%	53%	1	0	8	10	log-normal
equivalent diameter (μm)	48	157	430	32%	48%	68%	9	0	1	9	log-normal
cross-sectional area (μm^2)	728	8123	47525	43%	56%	77%	2	0	8	6	log-normal

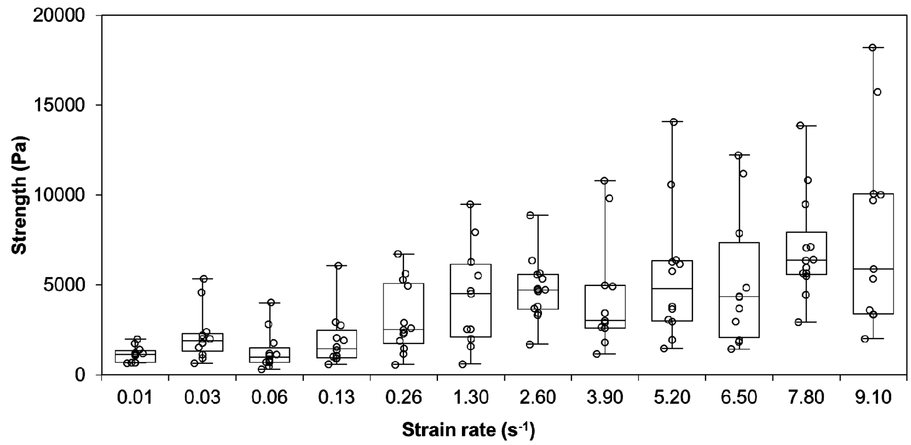

Figure 2. Box plot showing the distribution of measured strength values for *S. epidermidis* biofilm at different strain rates ranging from 0.013 to 9.07 s^{-1}. The bottom and top boundaries of the box represent the 1st and 3rd quartiles, respectively, while the horizontal line inside the box denotes the median. The whiskers extend to the minimum and maximum observations. The circles represent individual observations. Note that five extreme outliers (>3*interquartile range) were removed from this data set (out of a total of 146 data points) before plotting.

lowest CV values (21−53%), while equivalent diameter, elastic modulus, toughness, cross-sectional area and strength were more variable (mean CV > 50%). CV values for all of the parameters did not show any trend or correlation with strain rate (average R^2 = 0.1). To further illustrate the variability in our data, a box-plot for one of the parameters (strength) is presented in Figure 2.

3.2. Effect of Strain Rate. The effect of strain rate on each of the six parameters was examined and the results are shown in Figure 3. The data were fit with a variety of functions (e.g., logarithmic, power-law) and the best-fit curves are shown for correlations deemed significant (R^2 > 0.5). Elastic modulus, strength, and toughness were strongly correlated with strain rate (R^2 = 0.86−0.90), whereas the cross-sectional area of separation had a modest correlation coefficient value (R^2 = 0.61). The remaining two parameters (i.e., failure strain and equivalent diameter) did not correlate with strain rate (R^2 < 0.5). According to the plots in Figure 3, elastic modulus, strength, toughness, and cross-sectional area begin to plateau at a strain rate of approximately 1.3 s^{-1}.

To test the statistical significance of the apparent differences in measured parameters between the 12 different strain rate levels, pairwise comparisons were made. In general for elastic modulus, strength, and toughness, the mean parameter values for strain rate levels of 0.013, 0.026, 0.065, and 0.13 s^{-1} were statistically similar (p > 0.5) but different than those for strain rates ≥0.26 s^{-1}, which also were statistically similar among themselves. For the pairwise comparisons done of the mean values of failure strain, equivalent diameter, and cross-sectional area at different strain rates, nearly all (64 out of 66) resulted in no significant difference. Thus, strain rate appeared to have little or no effect on these parameters.

4. DISCUSSION

The mechanical properties of *S. epidermidis* biofilms were measured over approximately 3 orders of magnitude variation in strain-rate. Strong strain-rate dependence was noted for strength, elastic modulus and toughness and the variation was evident especially at lower strain-rates. The mean of these three parameters exhibited a 7×, 10×, and 4×, respectively increase in response to an increase in strain-rate from 0.013 to 9.07 s^{-1}. This suggests that the relatively wide disparity of biofilm mechanical property results between different laboratories, and possibly even within a given laboratory, could be due, at least in part, to the lack of control of and differences in the applied strain rate.

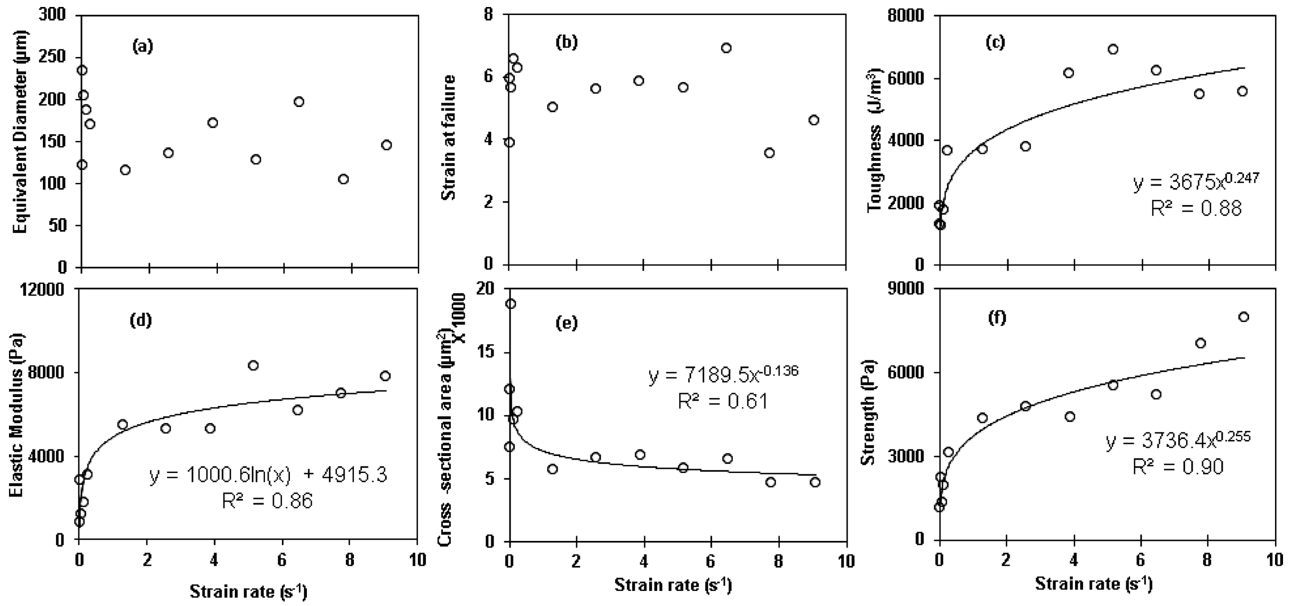

Figure 3. Scatter plots of mean values of the six parameters, namely: (a) equivalent diameter, (b) failure strain, (c) toughness, (d) elastic modulus, (e) cross-sectional area of separation, and (f) strength. Regression equations are shown for which the best fit R^2 was at least 0.5. Each data point represents means, from a set of at least 12 replicate tensile tests, one set for each separation velocity setting.

In this investigation, a significant effect of strain rate on biofilm mechanical properties was only observed at very low strain rates (<0.26 s^{-1}). Lau and co-workers[21] varied retraction velocity during AFM-based microbead force spectroscopy testing of biofilm adhesion and viscoelasticity. From the reported retraction velocities (1−4 μm s^{-1}) and biofilm thicknesses (0.5−3 μm), we computed strain rates ranging from 0.3 to 8 s^{-1}. The authors reported that the mechanical properties measured, including adhesion force and adhesion pressure, were not affected by retraction velocity (i.e., strain rate) over this range. Thus, this observation, albeit for different parameters than measured in this work, is consistent with the results reported herein. The emergence of strain rate (i.e., time) dependent mechanical properties can be explained by the ideal viscous element (i.e., dashpot) that is used in combination with the elastic spring element to develop constitutive models of viscoelastic materials. For a dashpot element, force (F) is related to displacement (denoted by Δ_d) by the following equation: $\Delta_d/dt = \eta F$, where η is the viscosity of the dashpot. Hence, the viscous term becomes more important at longer time scales or lower strain rates. In our experiments, increasing strain rate from 0.013 to 9.07 s^{-1} resulted in a decrease in the average testing time (i.e., time to specimen failure) from 8.1 to 0.67 s.

Failure strain did not correlate with strain rate. This result was somewhat surprising as it was expected that lower strain rates (i.e., longer testing times) would result in an increase in failure strain according to constitutive viscoelastic models. Our experimental observations are consistent with those for dorsal rat skin,[26] where tensile strength was dependent on strain rate but failure strain was not. On the other hand, Zioupos et al.[27] reported a decrease in failure strain with increasing strain rate (for strain rates higher than 1 s^{-1}) for human cortical bone.

The cross-sectional area at the location of biofilm failure, however, did show a significant decrease with strain rate, the reason for which was evident from the video files (not shown). With increasing strain rate the point of failure shifted away from the biofilm substratum and because the extended biofilm typically had a conical geometry, locations farther away from the substratum had a smaller cross section.

A strain-rate dependence of mechanical properties is a characteristic of viscoelastic materials and is thus commonly exhibited by various biomaterials and polymers. A comparison of elastic modulus values as a function of strain rate for different materials is presented in Figure 4. A clear positive trend of

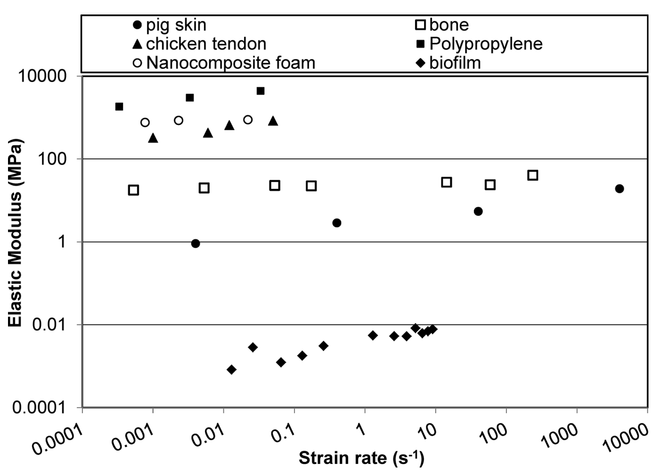

Figure 4. Comparison of variation of elastic modulus values with strain rate for pig skin,[28] bovine bone,[29] chicken tendon,[30] polypropylene,[31] nanocomposite foam,[32] and *S. epidermidis* biofilm (mean values from current study). Shear modulus values for pig skin from ref 28 were used to estimate the elastic modulus values shown here.

elastic modulus with strain rate is apparent for skin, tendon, polypropylene and biofilm. It is also noteworthy that biofilms are much weaker than the other biomaterials and polymers shown in the plot. In comparison to the literature, a unique aspect of the current study is the large number of strain rate levels employed (twelve) over a wide range. This allows us to derive correlations relating mechanical properties with strain rate (Figure 3), while most studies in Table 1 simply report a qualitative trend.

One limitation of the present work is that only results for *S. epidermidis* biofilms were reported and only for a fixed set of growth conditions (i.e., nutrients, ionic strength and fluid shear). We do not know how other single species or multispecies biofilms will behave. Therefore, more work is needed to investigate the effect of strain rate on the mechanical properties for a wide variety of bacterial biofilms and growth conditions.

5. CONCLUSIONS

Our results indicate that the mechanical properties of bacterial biofilms (especially strength, elastic modulus and toughness) show clear strain-rate dependence. Moreover, most of the properties exhibited an increasing log-linear or power law correlation with strain rate. Therefore, this work also provides a quantitative description of the variation of mechanical properties with strain rate and could aid in modeling the deformation of bacterial biofilms under applied loads. Furthermore, this research highlights the importance of controlling and reporting the strain rate used during biofilm testing in order to facilitate comparison of results among research groups.

In conclusion, we have highlighted upon an important aspect of the response of biofilms to an applied tensile load: the effect of strain rate. A better understanding of such key attributes of biofilm mechanical properties is essential for enhancing our overall understanding of biofilm surface attachment and survival mechanisms and should be helpful in developing better biofilm control strategies.

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