High-throughput Assays Show the Timescale for Phagocytic Success Depends on the Target Toughness

Layla A. Bakhtiari[1,2*], Marilyn J. Wells[1,2*], Vernita D. Gordon[1,2,3,4 †]

† Vernita Gordon is the author to whom correspondence should be addressed: gordon@chaos.utexas.edu twitter: @Vernita.Gordon

* LAB and MJW contributed equally to this work.

1 Department of Physics, The University of Texas at Austin, 2515 Speedway, C1600, Austin, TX 78712-1192

2 Center for Nonlinear Dynamics, The University of Texas at Austin, 2515 Speedway, Stop C1610, Austin, Texas 78712-11993

3 Institute for Cellular and Molecular Biology, The University of Texas at Austin, North Hackerman Building, 100 East 24th St, NHB 4500, Austin, TX 78712

4 LaMontagne Center for Infectious Disease, University of Texas at Austin, Neural Molecular Science Building, 2506 Speedway, Stop A5000, Austin, TX 78712

ABSTRACT

Phagocytic immune cells can clear pathogens from the body by engulfing them. Bacterial biofilms are communities of bacteria that are bound together in a matrix that gives biofilms viscoelastic mechanical properties that do not exist for free-swimming bacteria. Since a neutrophil is too small to engulf an entire biofilm, it must be able to detach and engulf a few bacteria at a time if it is to use phagocytosis to clear the infection. We recently found a negative correlation between target elasticity and phagocytic success. That earlier work used time-consuming, manual analysis of micrographs of neutrophils and fluorescent beads. Here, we introduce and validate flow cytometry as a fast and high-throughput technique that increases the number of neutrophils analyzed per experiment by two orders of magnitude, while also reducing the time required to do so from hours to minutes. We also introduce the use of polyacrylamide gels in our assay for engulfment success. The tunability of polyacrylamide gels expands the mechanical parameter space we can study, and we find that high toughness and yield strain, even with low elasticity, also impact phagocytic success, as well as the timescale thereof. For stiff gels with low yield strain, and consequent low toughness, phagocytic success is nearly four times greater when neutrophils are incubated with gels for six hours than after only one hour of incubation. In contrast, for soft gels with high yield strain and consequent high toughness, successful engulfment is much less time-sensitive, increasing by less than a factor of two from one hour's to six hours' incubation.

INTRODUCTION

Immune cells serve critical roles in the body in fighting infection. One type of human immune cells, neutrophils, are the most numerous white blood cell and act as the first responders to sites of infection [1]. Neutrophils are phagocytic cells and a part of the innate immune defense. They are developed in the bone marrow and released into the bloodstream, where they use complex defenses to control the pathogens that they encounter on patrol in vasculature and upon recruitment into tissue [2, 3]. Neutrophils are professional phagocytes, and as such they can both recognize foreign objects as immune targets and subsequently attempt to engulf targeted foreign objects as a

way of clearing them from the body [4]. Phagocytosis begins with the neutrophil wrapping its membrane around the target until the target eventually is fully engulfed [5]. In suspension, each neutrophil is approximately 10 μm in diameter. The size of the phagocytic target is important for dictating the success of phagocytosis. *In vitro* studies show that neutrophils readily engulf stiff abiotic targets (polystyrene beads with Young's modulus ~3000 MPa) that are smaller than 10 μm, but any attempts to phagocytose such targets larger than 10 μm fail [6].

Similarly, neutrophils readily engulf free-swimming bacterial cells, which are each a few micrometers in size and thus smaller than neutrophils [7], but biofilm infections, composed of ~100 μm aggregates comprising many bacteria bound together in a polymeric matrix, are too large for neutrophils to engulf whole [8-10]. Indeed, biofilms are well-known to resist clearance by the immune system [11-14]. Images of biopsies from biofilm-infected lungs and wounds show neutrophils surrounding bacterial aggregates without entering them [15, 16]. From this we infer that, to clear biofilm infections by phagocytosis, neutrophils must detach pieces from the aggregate surface and engulf them.

The phenomenon of neutrophils breaking pieces from larger-than-self phagocytic targets is a relatively recent discovery of a mechanism neutrophils may use to clear pathogens. Neutrophils have been observed to break off and engulf piece from the pathogen *Trichomonas vaginalis*. Like most biofilms, cells of *Trichomonas vaginalis* are too large for neutrophils to engulf whole [17-20]. Neutrophils also have been observed to kill cancer cells using a similar process [21]. Such examples show that neutrophils have the ability to remove and engulf pieces from large phagocytic targets which are otherwise too large to clear whole. This work presents a similar phenomenon, where neutrophils remove pieces from target structures made of gels that contain fluorescent beads to act as tracers for engulfment.

Detaching pieces from large structures is a process that requires energy, and the amount of energy required for such a process can be quantified through mechanical testing of the material that is to be broken [22]. Thus, understanding both the mechanical properties of biofilms and the impact of mechanics on phagocytic success is important for understanding how neutrophils interact with biofilms. Such understanding could guide the development of treatments that reduce biofilms' resistance to the immune system through compromising biofilm mechanics [23]. Many studies have measured the mechanical characteristics of biofilms [24-28]. Our recent publication was the first to investigate the impact of the mechanical properties, of a target structure much

larger than a neutrophil, on phagocytic success [7]. In that publication, we incubated neutrophils with large agarose or alginate gels that were formed containing fluorescent beads, and measured phagocytic success as the fraction of neutrophils that engulfed beads from the gel [7]. This measurement was done by manual analysis of microscopy images for 100 neutrophils per sample in each experiment. This was sufficient to show that phagocytic success negatively correlates with gel stiffness, but it was far from high-throughput or fast. Here, we present a high-throughput approach that allows data from two orders of magnitude more neutrophils to be analyzed in less time than that required for image analysis of 100 neutrophils. This technique uses a flow cytometer to sort a population of 20,000 neutrophils collected from each gel sample into bead-positive or bead-negative categories. We validate these measurements by comparing them to measurements obtained by manual analysis of micrographs. Flow cytometers are widespread in research and clinical institutions, so we expect that the improved technique we present here will enable many more researchers to study the effects of the mechanics of biofilms and other large target structures on phagocytosis.

Furthermore, we have also developed polyacrylamide gels for use in engulfment studies. The tunability of these gels allows us to explore the effects of new mechanical parameters, namely toughness and yield strain, which can be studied independently from the elastic modulus. We find that toughness and yield strain impact both phagocytic success and the time-dependence of phagocytic success. This shows that the toughness and yield strain of biofilms, as well as their stiffness, should also be considered when developing treatments that aim at compromising biofilm mechanics to facilitate clearance by the immune system.

MATERIALS AND METHODS

Isolation of neutrophil cells from whole blood

This work was approved by the Institutional Review Board at the University of Texas at Austin (Austin, TX) as Protocol Number 2015-05-0036.

Human neutrophils from healthy adult volunteers were isolated from blood following the same protocols used in our previous work [7, 29]. In brief, blood was acquired by venous puncture from healthy adult human volunteers and collected into a 10mL heparin-coated tube (BD

Vacutainer, Cat. 367880) by a registered phlebotomist. Cells were isolated using dextran sedimentation, Ficoll-Paque density gradient, and centrifugation. We refer the reader to the methods of our previous work for further details on this standard neutrophil isolation protocol [7].

Following isolation, neutrophils were suspended in 1 mL of Hank's Balanced Salt Solution (HBSS) medium containing calcium and magnesium (Gibco Laboratories, Cat. 14025092) and 20% human serum (Gibco Laboratories, Cat. H4522). Each experimental gel sample needs 200uL of neutrophil suspension, so post-isolation one tube of blood provides enough cells to test at most 5 gel samples. For experiments where neutrophils were incubated for only 1 hour on gel samples, only one 10mL heparin-coated tube (BD Vacutainer, Cat. 367880) of drawn blood was sufficient to provide enough neutrophils. For experiments where neutrophils were incubated on gel samples for longer periods (1 hour, 3 hours, and 6 hours), two tubes of blood were drawn from the same donor to provide sufficient volume of cell suspension to apply to all samples without diluting the neutrophil concentration. These two tubes of blood were collected from the same venous puncture and isolated in tandem. After cells were isolated from each tube the cell suspensions were combined and mixed thoroughly via pipette before finally distributing the cells to all gel samples.

Each replicate experiment, from each donor, was done on a different day with an independent neutrophil isolation. N-values in figure captions indicate the number of replicates of each measurement.

Alginate gel preparation

Alginate gels were prepared following our previously published procedures [7]. In brief, 4% sodium alginate (catalog number 180947; Sigma-Aldrich) was dissolved in distilled water. A solution of 5% calcium carbonate (catalog number C4830; Sigma-Aldrich) and 5% D-(+)-gluconic acid δ-lactone (catalog number G4750; Sigma-Aldrich) was added to reach final concentrations of 10, 20, and 30 mM of $CaCO_3$.

Acrylamide gel preparation

Acrylamide gels were fabricated using a modification to published casting methods [30]. The bulk mechanical parameters of gels were tuned by changing both the amount of acrylamide monomer and the amount of bis-acrylamide (BIS) crosslinker. Gel A contains 8% acrylamide and .48% BIS (data for this gel is shown in orange in the figures). Gel D contains 3% acrylamide and .03 % BIS

(data for this gel is shown in blue in the figures). Large stock bottles of acrylamide monomer (Biorad, cat. 161-0140) and BIS (Biorad, cat. 161-0142) crosslinker solutions that were purchased from the manufacturer were stored for long-term storage at 4°C. Gels were made from small 40mL aliquots of these stock solutions, which were allowed to reach room temperature before gel preparation began. To prepare gels, acrylamide monomer and bis-acrylamide crosslinker were combined with DI water in a 15mL Falcon tube (Fisher Sci, cat. 352097). A freshly prepared solution of bovine serum albumin (BSA, GE life sciences, cat. SH30574.01) in DI water was added to the sample to reach a final concentration of 10 mg/mL in the gel. The lid was sealed, and the contents inverted at least 12 times to ensure the solution was well mixed. Tetramethylethylenediamine (TEMED, Biorad, cat. 161-0800) and a freshly prepared sample of 10% Ammonium Persulfate (Biorad, cat. 161-0700) in DI water was then added to the tube. The solution was capped and inverted again at least 12 times. 500uL aliquots of this solution was transferred into wells of a 24 well plate (Corning, purchased from VWR, cat. 15705-060) and 5uL of 1μm diameter fluorescent polystyrene beads (Bangs Labs, cat. FSDG004) were thoroughly mixed into the gel solution in each well. The well plate containing gel samples was then quickly transferred to a benchtop vacuum chamber (Fisher Scientific) and a Maxima C Plus drive pump (Fisher Scientific, cat. 0125778) was used to pump the chamber down to -0.06 MPa. The chamber was held at this pressure for one minute, then purged with Nitrogen gas. Three such vacuum-nitrogen purge cycles were used in succession to remove excess oxygen from the chamber. The reason for this is that oxygen is a free radical trap and as such has the ability to inhibit acrylamide polymerization [31]. The gels were then allowed to sit for at least 30mins at -0.06MPa vacuum until the gel completely polymerized. The gels used in this study were prepared the day before experiments and stored in sealed well plates at 4°C until use the next day. For tensile testing, the sample thickness varied from 1.3-1.8mm.

High-throughput cell assay

Following neutrophil isolation, 200uL of cell suspension containing 20% human serum was added to each gel sample and incubated for 1 hour at 37°C under static conditions (Fisher Scientific Isotemp Incubator). Solutions containing cells were then collected from each gel sample and placed in 5mL polystyrene tubes (Falcon, Ref. 352052). CD45 targeting antibodies conjugated with PE-Cyanine5 (ThermoFisher, Cat. 15-0459-42) were then added to each solution at a

concentration of 0.00015ug/uL of collected cell solution. Samples were then transported to the UT Austin Center for Biomedical Research Support Microscopy and Imaging Facility to be analyzed by a BD LSR Fortessa SORP Flow Cytometer. Controls were run initially to set the acquisition gates and experimental template utilized for all experiments; freshly isolated neutrophils labelled with fluorescently conjugated anti-CD45 were inserted into the machine and single cells were selected using side scatter area and side scatter height. A sample of only beads was later inserted into the flow cytometer to calibrate the appropriate fluorescence channel of the machine. During experiments data was collected for each sample until information on 20,000 individual neutrophils was collected. Fluorescent channels were then used to further sort the single cell population of 20,000 neutrophils into either bead positive (Q2) and bead negative (Q1) cell quadrants. FITC-A channel is associated with the bead fluorescence, while PE-Cy5-A channel is associated with the fluorescence of the fluorescently conjugated anti-CD45 antibodies that are bound to the neutrophils. Sample acquisition and reports were done using FACSDiva v6.1.3.

For time series experiments the protocol was identical to that listed above except neutrophils were incubated on three identical gel samples for each type of gel tested. Neutrophils were then collected from one gel sample after 1 hour, 3 hours, and 6 hours of incubation and analyzed with the same experimental template and gates by the flow cytometer.

To evaluate the phagocytic success of neutrophils collected from different subjects, we present results for cells donated by three healthy adult volunteers, referred to as volunteer 1, volunteer 2, and volunteer 3. Initial time-series experiments were performed with cells from volunteer 1 and volunteer 2 using both microscopy and flow cytometry assays. Cells from volunteer 1 were initially analyzed after 1 and 3 hours of incubation with gels, and cells from volunteer 2 were analyzed after 1, 3, and 6 hours of incubation with gels. We were able to collect data using volunteer 1's neutrophils only after 1 and 3 hours of incubation due to research restrictions resulting from the Covid-19 pandemic (when these data were being collected, cohort scheduling according to the policy of University of Texas did not allow the experimenter to stay in lab for six hours). The purpose of these initial experiments was to validate that the information given by flow cytometry was consistent with that given by microscopy. Following this initial validation, and after research restrictions were lifted, we performed additional flow cytometry measurements at 1, 3, and 6 hours for cells from volunteer 1 and from a third donor, volunteer 3.

Microscopy-based cell assay

The microscopy-based assay follows the same protocol as our previous work [7]. We briefly outline the protocol here: Following isolation of neutrophils, 200uL of neutrophil suspension containing 20% human serum was added to each gel sample and incubated statically for 1 hour at 37°C. Following incubation, the suspension containing neutrophils was collected from the gel surface by pipette and placed onto a microscope slide that was then sealed with an imaging spacer and glass coverslip (Thermo Fisher Scientific cat. 12-550-15 and 12-541-B; Sigma-Aldrich cat. GBL654004). Laser-scanning confocal microscopy was performed on the sample with an Olympus IX71 inverted confocal microscope, a 60X oil-immersion Olympus objective, and the confocal microscope and image acquisition were controlled by an instrument computer running FluoView FV10-AWS software, version 04.02, all from Olympus America. Objective oil (ThorLabs, Olympus IMMOIL-F30CC) was used between the coverslip and objective. Micrographs were then collected until images of at least one hundred cells were captured.

For time-series experiments the protocol was identical to that listed above except cells were incubated on three identical gel samples for each type of gel tested. Cells were then collected from one gel sample after each time marker (1, 3, and 6hrs).

For microscopy-based evaluation of the phagocytic success of neutrophils collected from different subjects, we present results of cells donated by two healthy adult volunteers, volunteer 1 and volunteer 2. Time-series experiments were performed with cells from both donors. Cells from volunteer 1 were analyzed after 1 and 3 hours of incubation with gels, and cells from volunteer 2 were analyzed after 1, 3, and 6 hours of incubation with gels. We were able to collect microscopy data using volunteer 1's neutrophils only after 1 and 3 hours of incubation due to research restrictions resulting from the Covid-19 pandemic (by policy of the University of Texas at Austin, the experimenter was not able to be in the lab for six hours when these experiments were being done). Microscopy experiments were not performed with cells from volunteer 3.

Assessing the impact of polyacrylamide gel chemistry on phagocytosis

To rule out potential effects of polyacrylamide gel chemistry on phagocytosis, the above microscopy-based assay was modified to test for any toxic effects of polyacrylamide gel chemistry on engulfment by neutrophils. Gels were prepared as described above, excepting without fluorescent beads. While neutrophils from volunteer 1 were being isolated, 200uL of HBSS with calcium and magnesium was added to each gel sample and incubated statically at 37°C. As a control, 200uL of HBSS with calcium and magnesium was also incubated separately, not in contact with any of the acrylamide gels. After each of two timepoints (1 and 3 hours), 100uL of the HBSS was collected from the gels and controls and placed in test tubes. Neutrophils were suspended in 200uL of human serum, and 25uL of this suspension was added to the HBSS solution removed from each of the gels and controls. Each sample was well-combined by pipette mixing, and 1uL of free fluorescent polystyrene beads (not embedded in a gel) was added to each test tube. All samples were incubated statically for 1hr at 37°C. After 1hr of incubation, cells were extracted from test tubes, placed on microscope slides, and sealed with an imaging spacer and coverslip. Each sample was subsequently analyzed with confocal microscopy as described in the above section. Due to institutional restrictions on research arising from the Covid-19 pandemic (see above), we were only able to perform these experiments at the 1 and 3hr timepoints.

Bulk mechanical characterization of gels by oscillatory rheology

The bulk mechanical properties of the hydrogels used in the study were measured using a stress-controlled AR 2000ex rheometer (TA instruments, New Castle, DE) and 8mm parallel-plate tool geometry. Hydrogel samples were removed from 4°C storage on the day of the experiment and incubated at 37°C with HBSS for 1 hour before characterization. An 8mm biopsy punch was then used to acquire a sample of each gel which could then be placed between the head and the plate of the tool. A custom-made solvent trap was used to prevent the gel samples from drying during characterization [24].

Once gels were loaded between the parallel plates of the rheometer, an oscillatory frequency sweep was performed at 1% strain with frequencies ranging from 0.1 to 600 rad/s. A strain sweep was subsequently performed at a frequency of 3.14 rad/s and strain ranging from 0.1 to 200%. The elastic modulus was taken to the midpoint of the G' plateau region when plotted against strain on a logarithmic scale. To determine the yield point, the plateau region of G' was fitted with a linear

trendline, and the nonlinear regime fitted with a power law. The yield point was taken to be the intersection of these regions. Toughness was taken to be the area under the stress-strain curve.

To confirm that the gel bulk mechanics do not significantly change in the time series experiments, additional gel samples that were incubated with HBSS at 37°C for 3 and 6 hours were characterized as well in the same manner.

Mechanical characterization of stiff acrylamide gels by tensile testing

Toughness in our oscillatory rheology measurements only provides information on the energy needed to start to cause plastic deformation, or permanent damage to a material. Tensile testing allows one to go beyond that and instead quantify the amount of energy needed to damage and fully break a material. A TA Instruments RSA3 Dynamic Mechanical Analyzer was used to perform tensile testing. Only the stiffest gel type that was used in the engulfment assays was able to be characterized by this method as the remainder of the gels lacked the rigidity and toughness needed to load samples into the machine without damaging the gel.

Samples of acrylamide gel were prepared in 60mm petri dishes (Fisher cat. FB0875712) at a depth of 1.3-1.8mm. The gel tested contains 8% acrylamide and .48% BIS. Stock mediums were stored in the fridge and allowed to reach room temperature before gel preparation began. Tetramethylethylenediamine (TEMED, Biorad, cat. 161-0800) and a freshly prepared sample of 10% Ammonium Persulfate (Biorad, cat. 161-0700) in DI water was then added to the tube. Each preparation of solution was capped and inverted again at least 12 times before transferring to petri dishes. Gels were incubated for 1 hour at 37 degrees Celsius with HBSS buffer before testing. Gels were then transferred to a flat surface and cut using a "dogbone" shaped metal diecut. This geometry yields reliable test runs as samples of this shape most often break in the uniformly narrow gauge section, and a good grip is insured with the wide ends, preventing slipping. Measurements from samples that did not break within the gauge region, or slipped during testing, were labelled invalid and thrown out of this study. Four pieces of sandpaper were placed on the gel sample ends to also prevent slippage at the grips of the machine. The thickness of each gel sample was measured using digital calipers. The thickness slightly varied for each unique sample but fit within a range of .5mm. Sample thickness varied from 1.3-1.8mm, and each thickness was entered into the software before testing. The rate of extension was fixed at 0.1mm/sec for all runs. Four valid tests were performed across two unique preparations of each gel type that was characterized.

The Young's modulus reported for each sample was acquired by performing a linear fit to each stress-strain plot at low strains from 0-.05. The slope from this linear fit equation is then reported as the Young's modulus for the sample. Ultimate elongation is reported as the maximum percent strain each sample underwent before breaking. Ultimate tensile strength is the maximum value of stress measured for each sample. Toughness is taken as the area under the curve in the stress-strain plot given from the strain sweep.

Statistics

All statistical analysis done for this work was performed in Microsoft Excel. Where appropriate, horizontal bars and accompanying asterisks in figures indicate p-values from Student two-tailed T-test between groups they connect: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

RESULTS

Initial validation of high-throughput measurements by flow cytometry

In our previous publication, we defined phagocytic success as neutrophils containing engulfed tracer beads that had initially been embedded within the gel. Calcium crosslinks alginate and increasing crosslinking increases elasticity – thus, varying the concentration of calcium (to 10mM, 20mM, or 30mM, with elastic moduli 60±20 Pa, 1400±440 Pa, and 2800±50 Pa, respectively) results in the formation of alginate gels with different mechanical properties. We validate high-throughput, flow cytometry measurements of phagocytic success by comparing with previously-published measurements obtained using microscopy and manual image analysis, in which we found a negative correlation between the stiffness of alginate gel target structures and the likelihood of phagocytic success (figures 4 and 7 of our prior publication [7]). Flow cytometry measurements show a very similar trend in measurements of the association of fluorescent tracer beads with neutrophils (Figure 1). Eight replicate experiments were performed for each gel type, each on a different day with an independent neutrophil isolation, with each sample within an experiment counting the number of bead-positive and the number of bead-negative neutrophils in a population of twenty thousand neutrophils. These experiments were performed with cells from volunteer 2. The percent of bead-positive cells within each sample population is shown. ANOVA significance testing yields a p-value for the group of $p=0.00019$.

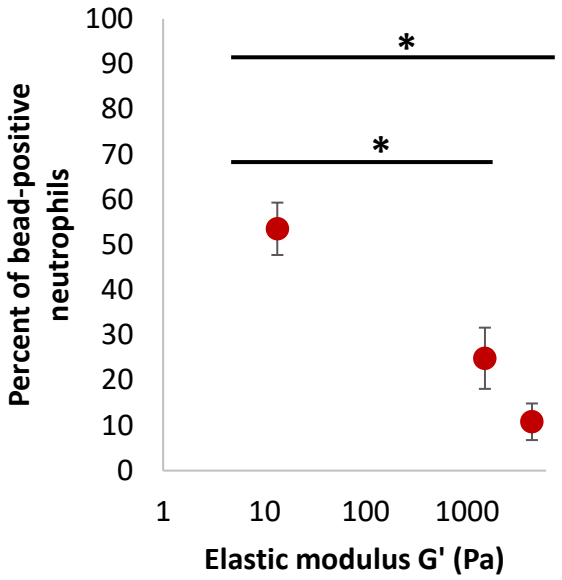

Figure 1

High-throughput measurements of phagocytic success using flow cytometry preserves previously-published trends. The elastic modulus of alginate hydrogels negatively correlates with neutrophil association with beads that were initially embedded in gels, as expected from our previous study [7]. ANOVA significance testing yields a p-value for the group of p=0.00019. Horizontal bars and accompanying asterisks indicate p-values from Student two-tailed T-test between connected pairs of data: * $p < 0.05$ (N=8 experiments per data point, with 20,000 neutrophils per sample in each experiment).

Notably, this p-value, though still well below the significance threshold, is higher than the p-value we obtained previously using ANOVA significance testing of engulfment success on the same three types of alginate gel, with 4 replicate experiments per gel type and manual counting of 100 neutrophils per experiment [7]. Furthermore, the bead-positivity percentages measured for neutrophils exposed to the medium (20 mM calcium) and stiff (30 mM calcium) alginate gels here, using flow cytometry, are 5 and ten times higher, respectively, than the corresponding percentages of neutrophils containing beads that we measured previously using manual image analysis [7]. Unlike manual image analysis, the flow cytometer we use cannot distinguish between internalized and surface-associated beads. We suspect that flow cytometry measurements show higher bead-positivity percentages than their microscopy-based counterparts due to the inability to discriminate false-positive neutrophils from bead-positive neutrophils. False-positive neutrophils are those

which possess tracer beads bound to the cell surface, which are not fully internalized (Supplementary Figure 1). Based on this understanding, we interpret flow cytometry measurements as a ceiling on the amount of phagocytic success actually present.

Quantifying gel mechanical properties

Our previous work examined only the impact of gel elasticity on phagocytic success [7]. This is not sufficient to yield complete understanding of how the mechanical properties of biofilms impact their susceptibility to clearance by neutrophils, since biofilms also vary in other mechanical properties, notably including yield strain and energy to yield [24]. Therefore, to extend the parameter space we can explore using neutrophil engulfment assays, we developed and characterized abiotic polyacrylamide gels for such use. The mechanical properties of these gels were quantified by oscillatory rheology and tensile testing.

Comparison of the mechanical properties across samples of polyacrylamide and alginate hydrogels

Using oscillatory bulk rheology, frequency and strain sweeps were performed to assess the elastic and viscous moduli of two different polyacrylamide gel types. Frequency sweeps were done at 1% strain, which is well within the plateau region found by the strain sweep. Strain sweeps were done at 3.14 rad/s, which is well within the plateau region found by frequency sweeps. We previously estimated a frequency characterizing deformations that can be imposed by neutrophils of about 0.4 rad/s [7], which is also well within the plateau region found by frequency sweeps. Representative frequency and strain sweeps for each gel type are shown in Figure 2. The mechanical properties shown for all alginate gels come from new analysis of previously-published rheological data [7]. Three replicate gels of each type were made, measured, and analyzed. The results for all replicates were then averaged to obtain a value for each mechanical parameter. In both gel types, the viscous modulus is one order of magnitude lower than the elastic modulus, indicating that the elastic properties dominate, and gels behave primarily as viscoelastic solids. Rheological characterization of biofilms reveal that most biofilms also behave primarily as viscoelastic solids [23, 24]. Therefore, we focus on the solid-like properties of target gels.

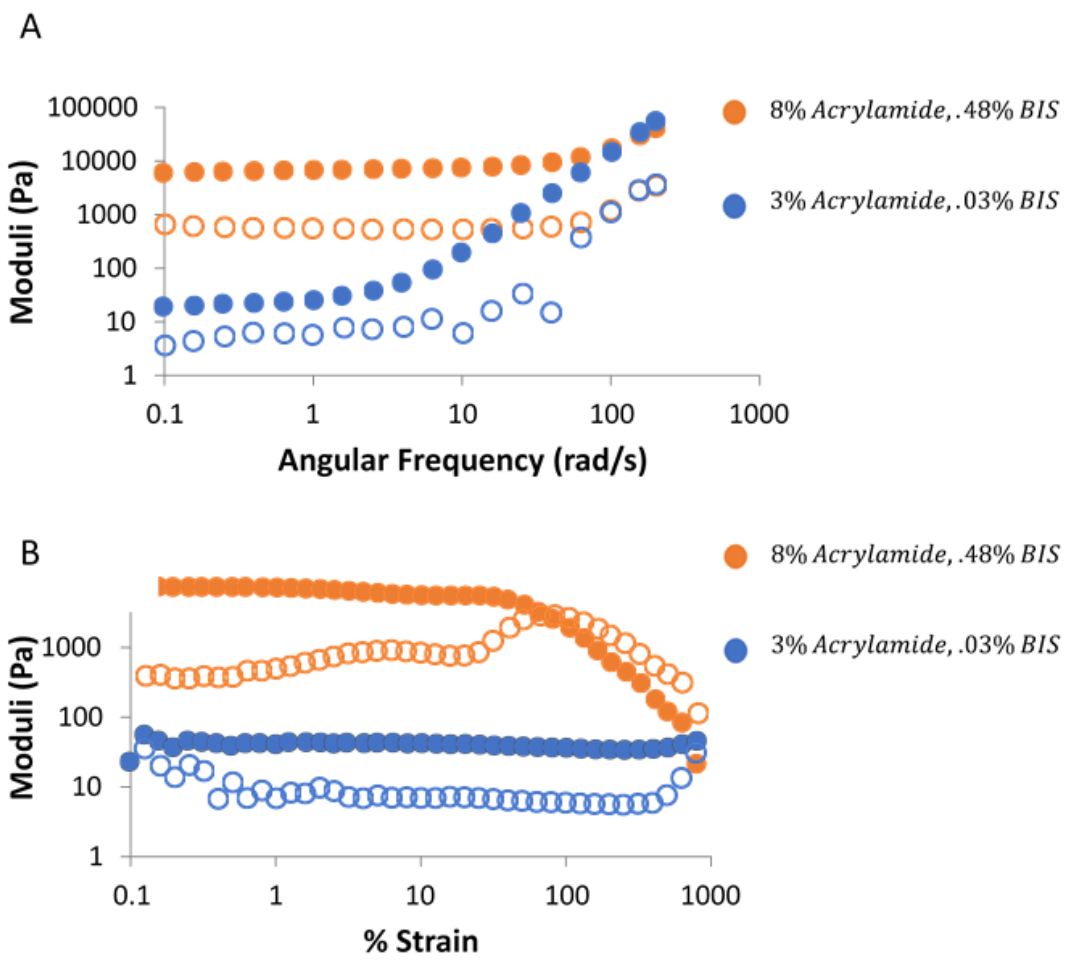

Figure 2

Representative (A) frequency and (B) strain sweeps from bulk oscillatory rheology measurements on polyacrylamide gels. Elastic moduli for each sample are shown with a solid marker, while viscous moduli are shown with hollow markers. Gels made with 8% acrylamide have an average elastic modulus of 5320 Pa, while those made with 3% acrylamide are two orders of magnitude softer with an average elastic modulus of 43 Pa. Elastic and viscous moduli are values corresponding to the measurement at 1% strain within the plateau region of each gel. In both gel types, the viscous modulus is one order of magnitude lower than the elastic modulus, indicating that the elastic properties dominate, and gels behave primarily as viscoelastic solids. We note that the differences in mechanical properties for polyacrylamide gels are attributed to not just the acrylamide concentration displayed in the legend, but also the crosslinker concentration (see methods for details).

For polyacrylamide gels, the concentration of acrylamide monomer as well as the concentration of BIS crosslinker affects the mechanical properties of the gels. Gels made with 8% acrylamide and .48% BIS have an average elastic modulus of 5320 Pa, while those made with 3%

acrylamide and .03% BIS are two orders of magnitude softer with an average elastic modulus of 43 Pa (Figure 3A). However, gels made of 3% acrylamide and .03% BIS have much higher yield strain than those made of 8% acrylamide and .48% BIS and those made of alginate (Figure 3B). As a result, although the yield stresses of all gels studied are similar (Figure 3C), gels made of 3% acrylamide and .03% BIS are significantly tougher than those made of 8% acrylamide and .48% BIS and those made of alginate (Figure 3D). ($T = 258\ \frac{J}{m^3}$ as compared to $= 62\ \frac{J}{m^3}$). The mechanical differences between the two polyacrylamide gels are attributed to both the acrylamide and the crosslinker concentration. Gels containing 3% acrylamide did not yield during testing, and therefore analysis was performed as if the yield strain were 100% to provide a lower-bound estimate of the gel's yield stress, yield strain, and toughness.

The large difference in yield strain and toughness between the two types of polyacrylamide gels makes it possible to assess experimentally the effects of these properties on phagocytic success and on the timescale required for phagocytic success. Specifically, *the development of our assay to use polyacrylamide gels allows us to investigate the effects of toughness independent of elastic modulus*, which our previous work, using agarose and alginate gels, did not allow us to do.

To confirm that gel mechanics are not sufficiently affected as they are incubated with neutrophils in the neutrophil medium, we incubated gels with the same liquid medium used for isolated neutrophils, at 37 C, for one, three, and six hours. We find no significant change in any measured mechanical property with varying incubation times (Table 1).

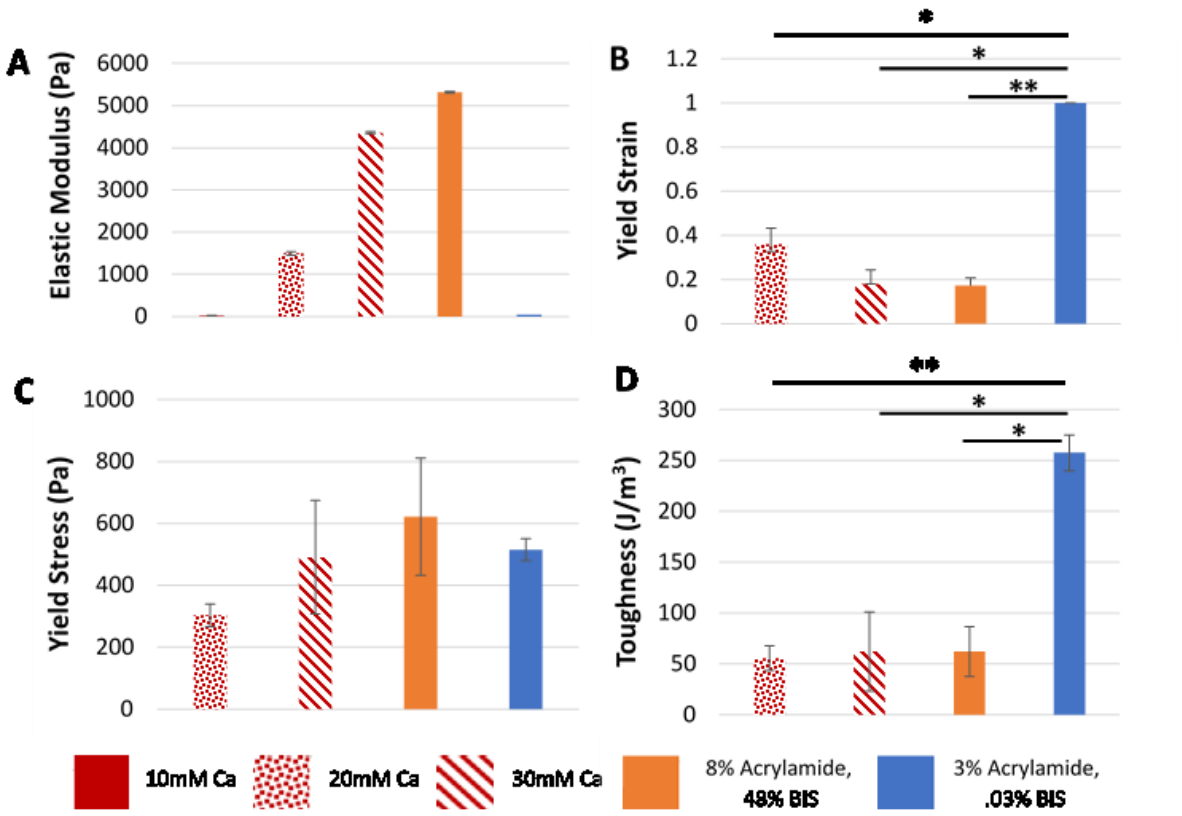

Figure 3

Comparison of gel mechanics across various polyacrylamide and alginate hydrogels. Each gel is characterized by oscillatory rheology to obtain the material's elastic modulus (A), yield strain (B), yield stress (C), and toughness (D). Gels containing 3% acrylamide are significantly tougher than both those containing 8% acrylamide and those containing alginate ($T = 258\ \frac{J}{m^3}$ as compared to $= 62\ \frac{J}{m^3}$). The high toughness arises from very high yield strain. The differences in mechanical properties for polyacrylamide gels are attributed to not just the acrylamide concentration, but also the crosslinker concentration (see methods for details). Gels containing 3% acrylamide did not yield during testing, and thus this analysis provides a lower bound estimate of the gel's mechanical properties. Horizontal bars and accompanying asterisks indicate p-values from Student two-tailed T-test between groups they connect: * $p < 0.05$, ** $p < 0.005$ (N=3).

Table 1

Table of mechanical measurements of gels acquired using oscillatory rheology show that gel mechanics do not statistically differ over longer incubation times. The mechanical properties of gel

samples do not greatly change over the longer incubation times utilized in time series experiments and remain within the same order of magnitude. Standard error of the mean is shown in parenthesis for each measurement. ANOVA significance testing (not shown) reveal no statistically significant difference over time for all parameters. (N=3)

Gel type	**Incubation time (hours)**	**G' Pa(sem)**	**G" Pa(sem)**	**yield strain**	**yield stress Pa(sem)**	**Toughness ($\frac{J}{m^3}$)**
8% acrylamide, .48% BIS	1	6120(499)	834(111)	0.173(.034)	621(189)	62(24)
	3	7671(226)	819(28)	0.135(.026)	700(128)	52(16)
	6	8265(309)	765(29)	0.116(.007)	678(32)	40(4)
3% acrylamide, .03% BIS	1	28(4)	4.6(1)	1	515(35)	258(18)
	3	32(1)	2.5(0.4)	1	449(15)	225(7)
	6	35(3)	4.7(1)	1	485(22)	242(11)

Tensile testing

Tensile testing of gels provides information about the mechanical properties of the gel that is not obtainable by oscillatory rheology. Toughness in general is a material's ability to absorb energy and undergo plastic deformation without fracturing. Toughness in our oscillatory rheology measurements only provide information on the energy needed to start to cause plastic deformation, or permanent damage to a material. This is not to be confused with the amount of energy per volume required to completely break a material into two pieces (as might be the case if a neutrophil pulls off and engulfs a piece of biofilm). Tensile strength testing, a technique wherein a sample is pulled apart at constant rate of extension until complete rupture occurs, can provide this information. In order to perform tensile tests, gel samples must be cut to shape, handled, and loaded into the dynamic mechanical analyzer. Gels made of 8% acrylamide and .48% BIS were able to undergo tensile testing, however the other gel types used in our engulfment assays could not be fully prepared for such testing without being damaged. A representative data set from tensile testing is shown in Supplementary Figure 2. The tensile toughness is the area under the line on the graph. Four samples were tested and the measurements averaged to obtain each result (Supplementary Table 1). We found that the tensile toughness of 8% acrylamide gels to be 4.4 kPa (standard error of the mean (SEM) 0.28 kPa).

From such tests, one can also gain a measure of the ultimate tensile strength, ultimate elongation, and tensile Young's Elastic modulus, E. The ultimate tensile strength is the maximum stress the material can endure before rupture and is thus the highest recorded point on the stress-strain curve. The ultimate elongation is the percentage increase in length the material undergoes just before the material breaks. For gels made of 8% acrylamide, we found ultimate tensile strength to be 17.6 kPa (SEM 0.09 kPa), the ultimate elongation to be 52.1 % (SEM 1.0%), and the Young's modulus to be 3714 Pa (SEM 1054 kPa).

Although we could not directly measure the tensile properties of 3% acrylamide gels, formulas derived from fits to experimental data can be used to estimate the Young's modulus of acrylamide gels using only the concentration of acrylamide and crosslinker [32]. Using this formula we predict the Young's modulus for 3% acrylamide gels to be around 9 Pa. The same formula predicts the Young's modulus for 8% acrylamide gels to be 3018 Pa, which agrees well with our experimental measurement on this gel type (Supplementary Table 1). Nonetheless, we note that this formula is only reported to be applicable for BIS crosslinker concentrations between 0.02% and 0.20%, which range does not include the 8% acrylamide and 0.48% BIS gel.

For a perfectly elastic solid, the Poisson's ratio, ν, can be used to relate the Young's modulus, E, of a material to the elastic shear modulus, G', which we measured for gels using oscillatory rheology:

$$E = 2(1+\nu)G'.$$

The Poisson's ratio is used to quantify the expansion (or contraction) of a material in directions that are perpendicular to the direction of mechanical loading and for polyacrylamide gels the Poisson's ratio has been measured to take on a value between 0.45 and 0.5 [33]. While our gels have loss moduli in oscillatory rheology data and thus are not perfectly elastic, this formula along with our measurements of G' from rheology do allow estimates of a Young's modulus of 15963 Pa and 129 Pa for gels composed of 8% acrylamide and .48% BIS, and 3% acrylamide and .03% BIS, respectively (Table 1). Measurements on polyacrylamide gels of the same concentrations have been performed using AFM indentation, and the Young's moduli reported are within the same order of magnitude as this estimation [34]. Therefore, while not obtainable by tensile testing, from such forms of estimation we expect the Young's modulus of gels containing 3% acrylamide and .03% BIS to fall within the order of $10^0 – 10^2$ Pa.

Testing for inhibition of phagocytosis by polyacrylamide chemistry

To rule out the possibility that the chemistry of polyacrylamide gels may inhibit phagocytic success, we tested neutrophil engulfment of fluorescent beads floating freely in buffer solutions which had been incubated on each gel type. Using microscopy-based analysis, we find no significant change in phagocytic success compared to the control experiment for either gel type (8% acrylamide and .48% BIS or 3% acrylamide and .03% BIS) after incubation of the buffer solution on gels for 1 and 3hrs (Supplementary Figure 3).

Phagocytic success for large gel target structures

Microscopy-Based Analysis

After one hour of incubation, microscopy of cells from both volunteer 1 and volunteer 2 found more successful engulfment of beads from gels made of 3% acrylamide and .03% BIS than from gels made of 8% acrylamide and .48% BIS (Figures 4A and 5A). Gels made of 8% acrylamide and .48% BIS have an elastic modulus comparable to that of alginate gels made with 30mM Ca (Figure 3A); for both alginate and agarose gels with similar moduli, we previously found near-zero phagocytic success [7], so it is unsurprising that comparably-stiff polyacrylamide gels are associated with very low phagocytic success in the same microscopy-based assay, in which neutrophils are exposed to gels for one hour (Supplementary Figure 4). However, gels made of 3% acrylamide and .03% BIS are of similar stiffness to alginate gels made with 10mM calcium, and for alginate gels with a comparable modulus and for agarose gels with a higher modulus, we previously found significant phagocytic success [7]. In contrast, soft polyacrylamide gels are nevertheless associated with very little phagocytic success in the microscopy-based, one-hour assay (Supplementary Figure 4). This is surprising, and it suggests that the stark difference in yield strain and/or toughness between these two gels, which are unlike anything seen for alginate or agarose gels (Figure 3 B, D), may be playing a role.

To exclude the possibility that polyacrylamide gel chemistry limits all phagocytic success, we increased the time that neutrophils were incubated with polyacrylamide gels to three and six hours. We expect that if gel chemistry per se were the limiting factor, then phagocytic success would remain equally poor for both gels over increasing time. Averaging the phagocytic success at each timepoint across multiple days and experiments, as was done in our previous work [7], shows an

increase in phagocytic success as the time neutrophils are incubated with the 8% acrylamide and .48% BIS gel increases from one to six hours, but no significant change with time for neutrophils incubated with the 3% acrylamide and .03% BIS gel (Figure 4A and Figure 5A). However, taking averages like this does not account for significant day-to-day variation in successful engulfment, shown by the dashed lines in Figure 4A and Figure 5A. Between four and six replicates were done for each experiment using neutrophils from Volunteer 2; three replicates were done for each experiment using neutrophils from Volunteer 1. Each replicate experiment was done on a different day, with an independent neutrophil isolation. Microscopy experiments using cells from Volunteer 1 were done only at the 1 and 3 hour time markers due to research restrictions arising from the Covid-19 pandemic.

Additionally, we report findings for cells taken from two donors, whose immune systems are inherently unique and may be affected on a day-to-day basis by stress, diet, sleep, etc. To account for this, for each day's experiments we calculated the fold change in successful engulfment from one to three or six hours' incubation time. Thus, fold changes are separately calculated for the three hour and six hour time markers for each experiment done using Volunteer 2's neutrophils, and for the three hour time marker for each experiment using Volunteer 1's neutrophils. This is similar to the analysis our group has performed on rheology data from bacterial biofilms; in such work, overall trends were also lost in significant day to day variation and recovered upon comparative fold change analysis [23, 24]. The calculated fold changes, rather than the percent of bead-containing neutrophils, are then averaged across all days. The resulting analysis shows statistically-significant increase in phagocytic success associated with both types of gel but with larger and more statistically significant increases associated with the 8% acrylamide and .48% BIS gel (Figure 4C and Figure 5E), which has a similar toughness to 20mM and 30mM calcium gels from our previous study [7]. These overall trends are observed for neutrophils isolated from both volunteers, despite day-to-day variation in phagocytic success and intrinsic biological differences between the two blood donors.

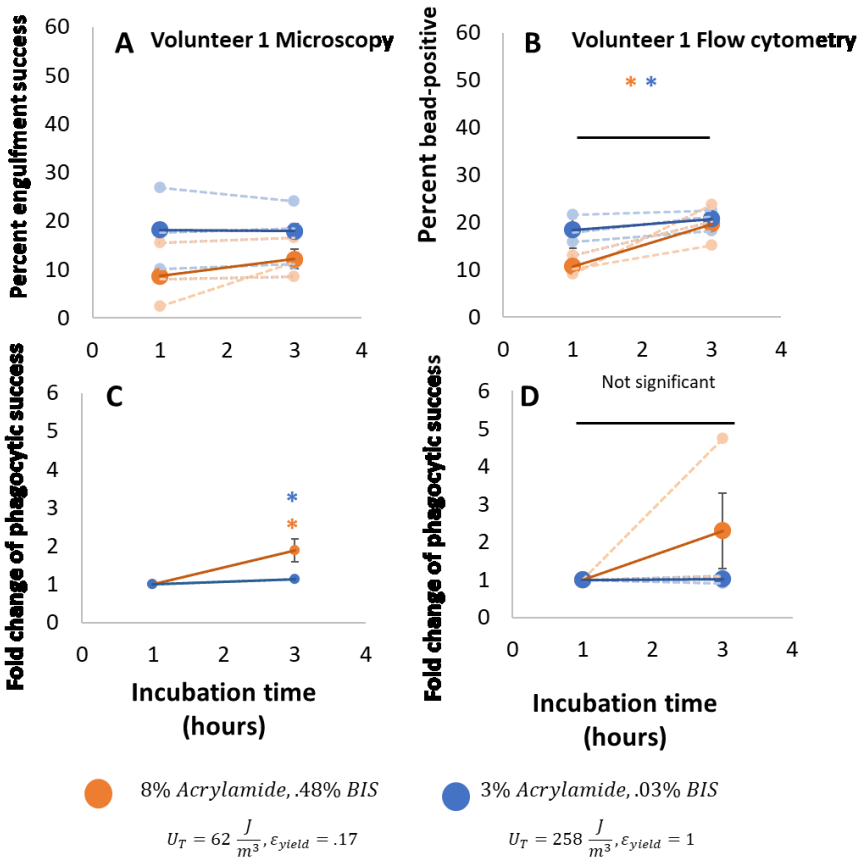

Figure 4

Phagocytic success and fold change for microscopy-based assay (A,C) and high-throughput assay (B,D) with cells from volunteer 1. Data for individual experiments is shown with a dashed line, and the average from each gel group is shown with a solid line. Initial high-throughput measurements demonstrate similar trends to the microscopy-based assay after 1 and 3 hours incubation. Cells incubated on gels with 8% acrylamide experienced a larger increase in phagocytic success after extended incubation times than cells incubated on gels with 3% acrylamide. A significant fold change at the 3 hour time marker was, however, not observed for cells from volunteer 1 with either acrylamide gel using the high-throughput method. This highlights the need for more extensive studies involving neutrophils isolated from different healthy donors. ANOVA significance testing yields a p-value for the group of all gels of (A) p=.01. Horizontal bars with accompanying asterisk indicate p-values from using a Student two-tailed T-test to compare results from the same type of gel at 1 and 3 hours; gel type is indicated with asterisk color. Asterisks without horizontal bars indicate that data for one gel type at one timepoint is being tested with a null hypothesis that the fold change is unity. * $p < 0.05$. N=3 for all measurements.

High-throughput assays using flow cytometry

To continue validating our high-throughput flow cytometry approach, we want to confirm that it measures similar trends for phagocytic success, associated with different polyacrylamide gel types and different times of incubation with gels, as that measured using microscopy (Figures 4 and 5). Flow cytometry allowed us to measure, in each experiment, the fraction of bead-positive neutrophils out of a total population of 20,000 neutrophils.

To allow neutrophils to be confidently distinguished from any cellular or extraneous debris, fluorescently conjugated anti-CD45 antibodies were added to the suspension of isolated neutrophils (Supplementary Figure 5A). CD45 is a transmembrane protein present on all immune cells; therefore antibodies which target CD45 bind to neutrophils. The population of neutrophils thus indicated was further restricted, using side-scatter area and height, to include only single cells and exclude doublets or other large structures (Supplementary Figure 5B). Only these single cells were included in our count of total neutrophil population. Fluorescence from tracer beads was used to determine whether neutrophils were bead-positive or bead-negative (Supplementary Figure 5C).

After one hour of incubation with acrylamide gels, flow cytometry showed no difference in the percentage of bead-positive neutrophils harvested from each gel type (Supplementary Figure 6). This mirrors one-hour microscopy-based measurements (Supplementary Figure 4). Upon increasing incubation time to three and six hours, flow cytometry measured an increase in the percentage of bead-positive neutrophils from low-toughness gels (Figure 4 B, D and Figure 5 B, F). This mirrors the stronger trend seen in the corresponding microscopy-based measurements (Figure 4 A, C and Figure 5 A, E). However, the weaker trend over time for neutrophils from high-toughness gels that was statistically significant in our microscopy measurements (Figure 4 C and Figure 5 E) was not apparent in flow cytometry measurements (Figure 4D and Figure 5 F).

Furthermore, as for the alginate cases discussed earlier, flow cytometry often measures higher percentages of bead positivity than the percentages of internalized beads measured using microscopy and manual image analysis (Figure 4 A, B and Figure 5 A, B). After one hour of incubation with polyacrylamide gels, the microscopy-based assay measured statistically-indistinguishable internalized-bead percentages of 5% and 7% (Supplementary Figure 4) and flow cytometry measured statistically-indistinguishable bead-positivity percentages of 25% and 20%

(Supplementary Figure 6). As for the earlier alginate cases, we suspect high throughput analysis show higher "success" values than the microscopy-based counterparts because flow cytometry identifies both neutrophils with internalized beads and neutrophils with surface-bound beads as bead-positive (Supplementary Figure 1). The latter are false positives that are identifiable and excluded from true positive results in the microscopy-based assay, but not in the high-throughput, flow cytometry assay.

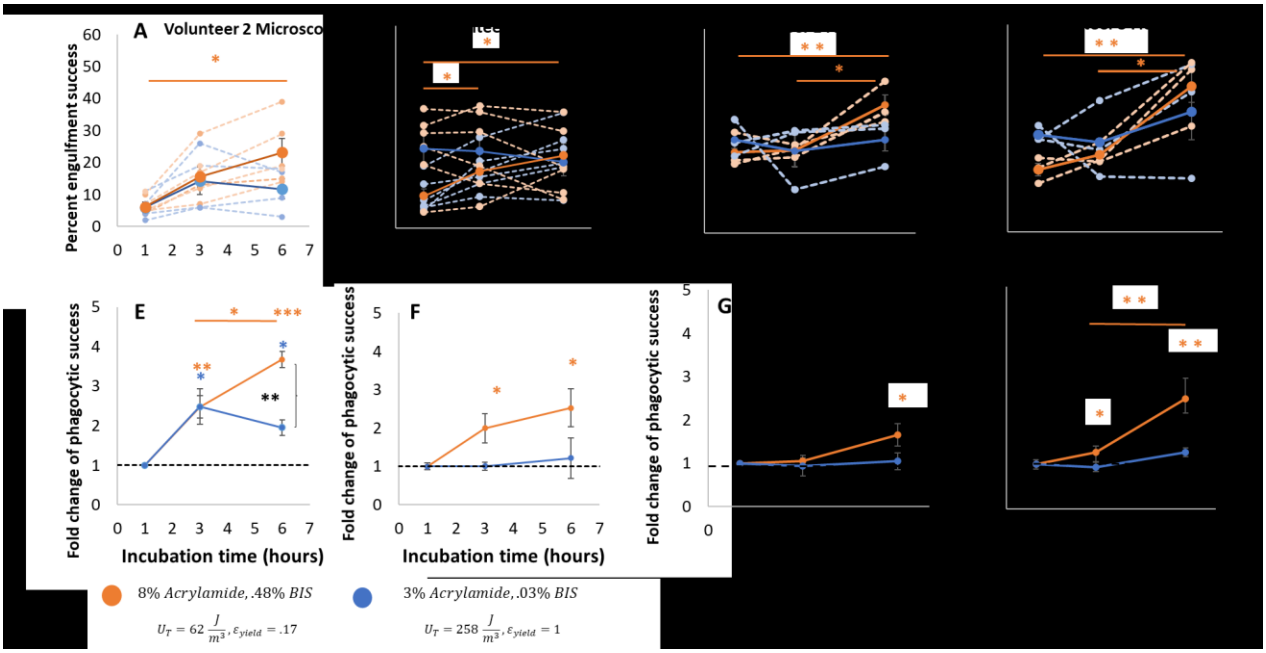

Figure 5. **Phagocytic success (top row) and fold change (bottom row) for microscopy-based assay (A,E) and high-throughput assay (B,F) with cells from Volunteer 2, and additional high-throughput measurements with cells from Volunteers 1 (C,G) and 3 (D,H).** Data for individual experiments is shown with a dashed line, and the average from each gel group is shown in bold. Using the microscopy-based assay, we see some successful engulfment from 8% acrylamide gels (orange markers) after extending the time neutrophils are incubated on gel samples for up to 6 hours. Such a strong change in phagocytic success after extending incubation time to 6 hours is not seen for neutrophils incubated on 3% acrylamide gels (blue markers). ANOVA significance testing yields a p-value for the group of all gels of (A) p=.02, (E) p=.01, and (H) p=.05. Horizontal bars and accompanying asterisk indicate p-values from Student two-tailed T-test for gel samples shown with the corresponding color. Horizontal bars indicate two datasets from the same gel type and different timepoints are being compared. Asterisks without horizontal bars indicate that data from one gel type at one timepoint is being compared with the null hypothesis of unity. Black asterisks (with a vertical line) indicate that data from both gel types, at the same timepoint, are being compared with each other. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. For Volunteer 1: for microscopy-based data, N=4 for

data displayed in blue and N=5 for data displayed in orange; for high-throughput data, N=6 for all measurements. For Volunteers 2 and 3, N=3 for all measurements.

DISCUSSION

Detaching pieces from a structure is a process that requires energy; how much energy required for such a process can be quantified through mechanical testing of the material that is to be broken. This paper presents a high-throughput method to assess the phagocytic success of large populations of neutrophils challenged with targets of diverse mechanics. Target stiffness, yield strain, yield stress, and toughness were characterized by oscillatory rheology (Figure 2, Figure 3, and Table 1); these bulk measurements provide quantification of each target material's stiffness, as well as how far the material can be sheared, and how much energy is required per unit volume, before permanent damage begins to occur to the material. Tensile testing provides measurements of Young's modulus, percent elongation, and tensile toughness; tensile toughness provides further insight into this work as it is the quantification of the amount of energy needed to fully break the material (Supplementary Table 1 and Supplementary Figure 2). Such information allows for estimation of energy barriers, per unit volume, that neutrophils would need to overcome to break and subsequently engulf pieces of large targets. Assessment of what ranges of mechanical values will hinder or allow phagocytic success is pertinent beyond the breakup of the gels presented in this work, and should provide insight into the phagocytosis of other large structures such as bacterial biofilms present in chronic infection. The results from our previous work [7], combined with the new data presented within this work, show that while target stiffness can affect phagocytic success, additional properties such as yield strain and toughness may also have an impact on engulfment and on the timescale required for successful engulfment.

In this work we found that polyacrylamide gels that have similar stiffness but greater toughness (Figure 3A) than alginate gels that allow high phagocytic success (Figure 4 of our previous study[7]) are associated with poor initial engulfment after neutrophils are incubated with gels for one hour (Supplementary Figure 4). However, extending the incubation time up to six hours increases phagocytic success (Figures 4 and 5). Therefore, we posit that high gel toughness

requires neutrophils to exert more energy per unit volume, and given extended time to attack such surfaces, neutrophils are able to gain some successful clearance of this material.

Phagocytic success increases more strongly with increasing incubation time for gels of low toughness (Figures 4 and 5, data displayed in orange), than for gels of high toughness (Figures 4 and 5, data displayed in blue). This may suggest that such a high toughness value may be near an energy barrier that neutrophils cannot easily overcome. An additional interpretation is that high yield strain may be limiting phagocytosis due to physical constraints of neutrophil extension, yet with repeated attempts of clearance these surfaces may undergo failure over time. Future studies investigating whether neutrophil-secreted products are present at longer incubation times, and whether such products degrade the mechanical integrity of the gels of this study, or biofilms, would provide insight into the mechanisms with which neutrophils might be able to overcome such a barrier and achieve some phagocytic success.

While overall trends appear to be preserved for neutrophils isolated from different volunteers, there will of course be variation between the immune systems of donors. Our work with neutrophils from volunteer 1 was initially limited due to research restrictions during the Covid-19 pandemic (the experimenter was not allowed to be in lab for 6 hours at a time), and therefore we were initially unable to observe engulfment trends (either for microscopy or for flow cytometry) over 6 hours of incubation. Our flow cytometry measurements for cells isolated from volunteer 1 did not show a significant fold change at the 3 hour time marker (Figure 4D), though this was indeed observed with the microscopy method (Figure 4C). Upon easing of institutional research restrictions so that experiments of longer duration were allowed, a significant fold change was later measured at the 6 hour time marker for cells from volunteer 1 and an additional donor, volunteer 3, using the flow cytometry method (Figure 5 G, H). It is possible that false positives from cells which had not fully internalized beads may be convoluting flow cytometry data trends. It is also possible that rate of phagocytosis could vary enough between volunteers (due to the physiological conditions of the subjects and strength of their immune systems) that the time-series trends do not directly correlate between donors. To further generalize the impacts of gel mechanics on phagocytic success, future studies should be performed with an extended group of blood donors, with the use of high-throughput flow cytometry making data collection far more efficient.

In its current form, the high-throughput flow-cytometry method presented here is good for fast measurement of strong trends in phagocytic success. This is seen in comparing the engulfment

trends seen of alginate gels in microscopy (Figure 4 of our previous study [7]) and flow cytometry (Figure 1) measurements. However, as shown in our observation of phagocytic success of polyacrylamide gels over time, the fast, flow cytometry, method may fail to detect weaker trends. This is observed when comparing the time dependence of phagocytic success of polyacrylamide gels analyzed with microscopy (Figure 4A, C and Figure 5A, E) with those analyzed with flow cytometry (Figure 4B, D and Figure 5B, F). The strong increase in engulfment was identifiable in both microscopy- and flow cytometry- based measurements for gels composed of 8% acrylamide and .48% BIS, however the weak increase in engulfment of 3% acrylamide .03% BIS gels that we observed by microscopy was not observed in high-throughput measurement. Thus, the flow cytometry assay is valuable for studying strong trends in phagocytic success due to its rapid, high-throughput nature – two orders of magnitude more neutrophils than in the microscopy assay are analyzed within minutes, rather than hours. However, in its current form, the flow-cytometry method may fail to detect weaker trends.

Currently in the flow cytometry assay, neutrophils with beads bound to the cell surface cannot be distinguished from neutrophils that have engulfed beads (Supplementary Figure 1). The resulting false-positive results provide "noise" which is particularly harmful to measurements of weak trends. Our current flow cytometry method could be improved upon, and weaker trends perhaps recovered, by introducing an additional fluorophore before assessing phagocytic success to quench the fluorescence from cell-surface associated beads [35, 36]. Trypan blue has been used to quench emission from FITC-labeled bacteria[35]. (*Salmonella typhimurium*) and zymosan particles[36] attached to the outside of phagocytes without affecting emission from bacteria that had been engulfed. Another possible approach, if experiments were done on biofilms instead of non-living gels, would be to treat samples with antibiotics after the neutrophils had time to engulf bacteria. The appropriate antibiotic treatment can cause constitutively-fluorescent bacteria to lose their fluorescence, so that bacteria not shielded by the neutrophil, and therefore exposed to antibiotic, to become "dark" to flow cytometry. Antibiotic treatment that kills bacteria outside neutrophils could also form the basis of a protection assay [37-39] in which phagocytic cells are lysed, the resulting suspension plated on nutrient agar, and colonies counted to reveal the number of bacteria that were inside neutrophils. An imaging flow cytometer, which can take individual small images of each cell, is yet another alternative solution which can also remove such false positives from analysis in the event such a machine is accessible to the researcher.

CONCLUSION

Here we present a new method of assaying how the mechanics of a large target structure impact the phagocytic success of immune cells. The speed of data acquisition through the implementation of this high throughput analysis has decreased the amount of time needed for analysis from several hours to a matter of minutes, and the number of neutrophils assessed using this method is increased by two orders of magnitude. We have also discovered through this study that there is a strong increase in phagocytic success over time for stiff targets of low yield strain that also shows up in high throughput analysis, yet the same strong trend is not observed for soft targets of high toughness.

It is reasonable to expect that phagocytic success of any group of neutrophils would be highly dependent on the strength of the donor's immune system, in addition to a number of other physiological factors which vary between subjects. Future studies which include a larger number of donors across broad demographics would lead to a greater understanding of how phagocytic trends depend on physiology of the volunteer. The high-throughput method presented here provides the ability to efficiently collect data from a large population of neutrophils, and could be extended to studies involving larger groups of volunteers, which was previously unrealistic using the time-consuming microscopy-based method.

Pathogenic biofilms cause chronic infections that resist antibiotics and the immune system, cause amputations and deaths, and increase healthcare costs. Knowledge of how mechanical properties of biofilms impact their resistance to phagocytic clearance could guide the development of treatments that reduce biofilms' resistance to the immune system by compromising biofilm mechanics. This work advances such knowledge by showing that the timescale required for phagocytic success depends on the toughness of the target structure. We also validate a flow-cytometry-based technique for high-throughput studies of phagocytosis, which make feasible future studies of phagocytic cells from many different people and of biofilms or other targets spanning a large mechanical-parameter space, including studying the effects of toughness independent from elastic modulus. Such studies will be necessary for translating our basic-science understanding into medical application.

Supplementary Material

See supplementary material for a microscopy image of neutrophils and fluorescent beads, representative data from tensile testing, additional engulfment data obtained using microscopy and flow cytometry, and a table of the mechanical properties of acrylamide gels.

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Author Declarations

This work was approved by the Institutional Review Board at the University of Texas at Austin (Austin, TX) as Protocol Number 2015-05-0036. Informed content was obtained from all participants. The authors have no conflicts to disclose.

Data availability: The data that support the findings of this study are available within the article and its supplementary material.

REFERENCES

1. Amulic, B., et al., *Neutrophil function: from mechanisms to disease.* Annu Rev Immunol, 2012. **30**: p. 459-89.
2. Borregaard, N., *Neutrophils, from Marrow to Microbes.* Immunity, 2010. **33**(5): p. 657-670.
3. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
4. Silva, M. and M. Correia-Neves, *Neutrophils and Macrophages: the Main Partners of Phagocyte Cell Systems.* Frontiers in Immunology, 2012. **3**(174).
5. Richards, D.M. and R.G. Endres, *The mechanism of phagocytosis: two stages of engulfment.* Biophysical journal, 2014. **107**(7): p. 1542-1553.
6. Herant, M., V. Heinrich, and M. Dembo, *Mechanics of neutrophil phagocytosis: experiments and quantitative models.* Journal of Cell Science, 2006. **119**(9): p. 1903-1913.
7. Davis-Fields, M., et al., *Assaying How Phagocytic Success Depends on the Elasticity of a Large Target Structure.* Biophysical Journal, 2019. **117**(8): p. 1496-1507.
8. Flemming, H.-C. and J. Wingender, *The biofilm matrix.* Nature Reviews Microbiology, 2010. **8**(9): p. 623-633.
9. Gordon, V., L. Bakhtiari, and K. Kovach, *From molecules to multispecies ecosystems: the roles of structure in bacterial biofilms.* Physical Biology, 2019. **16**(4): p. 041001.
10. Mann, E.E. and D.J. Wozniak, *Pseudomonas biofilm matrix composition and niche biology.* FEMS Microbiology Reviews, 2012. **36**(4): p. 893-916.
11. Donlan, R.M. and J.W. Costerton, *Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms.* Clinical Microbiology Reviews, 2002. **15**(2): p. 167-193.
12. Jones, C. and D. Wozniak, *Psl Produced by Mucoid Pseudomonas aeruginosa Contributes to the Establishment of Biofilms and Immune Evasion.* mBio, 2017. **8**(3): p. e00864-17.
13. Mishra, M., et al., *Pseudomonas aeruginosa Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization.* Cellular Microbiology, 2012. **14**(1): p. 95-106.
14. Bhattacharya, M., et al., *Staphylococcus aureus biofilms release leukocidins to elicit extracellular trap formation and evade neutrophil-mediated killing.* Proceedings of the National Academy of Sciences, 2018. **115**(28): p. 7416-7421.

15. Kragh, K.N., et al., *Polymorphonuclear Leukocytes Restrict Growth of Pseudomonas aeruginosa in the Lungs of Cystic Fibrosis Patients.* Infection and Immunity, 2014. **82**(11): p. 4477-4486.
16. Kirketerp-Moller, K., et al., *Distribution, Organization, and Ecology of Bacteria in Chronic Wounds.* Journal of Clinical Microbiology, 2008. **46**(8): p. 2717-2722.
17. Mercer, F., et al., *Neutrophils kill the parasite Trichomonas vaginalis using trogocytosis.* PLoS Biol, 2018. **16**(2): p. e2003885.
18. Li, K.J., et al., *Membrane Transfer from Mononuclear Cells to Polymorphonuclear Neutrophils Transduces Cell Survival and Activation Signals in the Recipient Cells via Anti-Extrinsic Apoptotic and MAP Kinase Signaling Pathways.* PLoS One, 2016. **11**(6): p. e0156262.
19. Valgardsdottir, R., et al., *Human neutrophils mediate trogocytosis rather than phagocytosis of CLL B cells opsonized with anti-CD20 antibodies.* Blood, 2017. **129**(19): p. 2636-2644.
20. Taylor, R.P. and M.A. Lindorfer, *Fcgamma-receptor-mediated trogocytosis impacts mAb-based therapies: historical precedence and recent developments.* Blood, 2015. **125**(5): p. 762-6.
21. Matlung, H.L., et al., *Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis.* Cell Reports, 2018. **23**(13): p. 3946-3959.e6.
22. Pelleg, J., *Mechanical properties of materials.* 2013.
23. Kovach, K.N., et al., *Specific Disruption of Established Pseudomonas aeruginosa Biofilms Using Polymer-Attacking Enzymes.* Langmuir, 2020. **36**(6): p. 1585-1595.
24. Kovach, K., et al., *Evolutionary adaptations of biofilms infecting cystic fibrosis lungs promote mechanical toughness by adjusting polysaccharide production.* npj Biofilms and Microbiomes, 2017. **3**(1): p. 1.
25. Boudarel, H., et al., *Towards standardized mechanical characterization of microbial biofilms: analysis and critical review.* npj Biofilms and Microbiomes, 2018. **4**: p. 17.
26. Lieleg, O., et al., *Mechanical robustness of Pseudomonasaeruginosa biofilms.* Soft Matter, 2011. **7**(7): p. 3307-3314.
27. Stoodley, P., et al., *Biofilm material properties as related to shear-induced deformation and detachment phenomena.* Journal of Industrial Microbiology and Biotechnology, 2002. **29**(6): p. 361-367.
28. Peterson, B.W., et al., *Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges.* FEMS microbiology reviews, 2015. **39**(2): p. 234-245.
29. Mark T. Quinn, F.R.D., *Neutrophil Methods and Protocols*. 2014: Humana Press.

30. Tse, J.R. and A.J. Engler, *Preparation of hydrogel substrates with tunable mechanical properties.* Curr Protoc Cell Biol, 2010. **Chapter 10**: p. Unit 10.16.
31. Menter, P., *Acrylamide polymerization: a practial approach.* Bio-Rad.
32. Boudou, T., et al., *An extended relationship for the characterization of Young's modulus and Poisson's ratio of tunable polyacrylamide gels.* Biorheology, 2006. **43**(6): p. 721-8.
33. Boudou, T., et al., *An extended relationship for the characterization of Young's modulus and Poisson's ratio of tunable polyacrylamide gels.* Biorheology, 2006. **43**: p. 721-728.
34. Tse, J.R. and A.J. Engler, *Preparation of Hydrogel Substrates with Tunable Mechanical Properties.* Current Protocols in Cell Biology, 2010. **47**(1): p. 10.16.1-10.16.16.
35. Golenkina, E.A., et al., *The Potential of Telomeric G-Quadruplexes Containing Modified Oligoguanosine Overhangs in Activation of Bacterial Phagocytosis and Leukotriene Synthesis in Human Neutrophils.* Biomolecules, 2020. **10**(2): p. 249.
36. Bjerknes, R. and C.-F. Bassøe, *Phagocyte C3-mediated attachment and internalization: Flow cytometric studies using a fluorescence quenching technique.* Blut, 1984. **49**(4): p. 315-323.
37. Subashchandrabose, S. and H.L.T. Mobley, *Gentamicin Protection Assay to Determine Bacterial Survival within Macrophages.* Bio-protocol, 2014. **4**(18): p. e1235.
38. Subashchandrabose, S., et al., *Genome-Wide Detection of Fitness Genes in Uropathogenic Escherichia coli during Systemic Infection.* PLOS Pathogens, 2013. **9**(12): p. e1003788.
39. Elsinghorst, E.A., *Measurement of invasion by gentamicin resistance*, in *Methods in Enzymology*. 1994, Academic Press. p. 405-420.