Assaying how phagocytic success depends on the elasticity of a large target structure

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Running head: Structure stiffness impacts phagocytosis

Abstract

Biofilm infections can consist of bacterial aggregates that are an order of magnitude larger than neutrophils, phagocytic immune cells that densely surround aggregates but do not enter them. Since a neutrophil is too small to engulf the entire aggregate, it must be able to detach and engulf a few bacteria at a time if it is to use phagocytosis to clear the infection. Current research techniques do not provide a method for determining how the success of phagocytosis, here defined as the complete engulfment of a piece of foreign material, depends on the mechanical properties of a larger object from which the piece must be removed before being engulfed. This paper presents a step toward such a method. By varying polymer concentration or crosslinking density, the elastic moduli of centimeter-sized gels are varied over the range that was previously measured for *Psuedomonas aeruginosa* biofilms grown from clinical bacterial isolates. Human neutrophils are isolated from blood freshly drawn from healthy adult volunteers, exposed to gel containing embedded beads for one hour, and removed from the gel. The percentage of collected neutrophils that contain beads that had previously been within the gels is used to measure successful phagocytic engulfment. Both increased polymer concentration in agarose gels and increased crosslinking density in alginate gels are associated with decreased success of phagocytic engulfment. Upon plotting the percentage of neutrophils showing successful engulfment as a function of the elastic modulus of the gel to which they were applied, it is found that data from both alginate and agarose gels collapse onto the

same curve. This suggests that gel mechanics may be impacting the success of phagocytosis and demonstrates that this experiment is a step toward realizing methods for measuring how the mechanics of a large target, or a large structure in which smaller targets are embedded, impact the success of phagocytic engulfment.

Statement of Significance

Bacterial biofilms are viscoelastic materials made of bacteria embedded in a matrix of extracellular polymers and proteins. Biofilm infections resist clearance by the immune system and have been shown to consist of multiple discrete aggregates, each ~100μm in diameter, that are densely surrounded by neutrophils that are ~10μm in diameter; neutrophils do not enter inside the aggregates. Neutrophils are phagocytic immune cells that readily engulf bacteria that are not part of a biofilm. By physical reasoning, for a cell attempting phagocytosis of a target much larger than itself, or of small targets embedded in a structure much larger than itself, the success of phagocytic engulfment must depend on both the force exerted by the cell and on the mechanical properties of the large target or of the material in which smaller targets are embedded. There has not been a way to measure how such mechanics affect phagocytosis until our presentation of such a method in this work. Our measurements also show that the success of phagocytic engulfment may be impacted by elastic modulus for the range of moduli that we previously measured for biofilms re-grown from clinical isolates (~0.05 – 10 kPa).

Introduction

Neutrophils are short-lived phagocytic cells that are an integral part of the innate immune system and the most abundant white blood cell in humans (1-4). Phagocytosis is one of a neutrophil's main ways of clearing pathogens (5). Single bacteria, approximately 1μm in size, are readily engulfed by neutrophils. However, biofilm infections in soft tissue are made up of many bacteria bound together in a polymeric matrix to form aggregates that cannot be cleared by neutrophils (6-8). Imaging studies of chronic biofilm infections show ~100μm-diameter aggregates that are densely surrounded by neutrophils that nevertheless do not enter the aggregates (9, 10).

Neutrophils are ~10μm in diameter and are unable to engulf rigid polystyrene particles (with Young's modulus ~3,000 MPa) that are over ~10μm in diameter (11). Size and other geometric characteristics, like shape, also influence phagocytosis by macrophages, another type of phagocytic immune cell (12-15). A rigid bead 100μm in diameter would be far too big to be phagocytosed by neutrophils (9-11, 16). However, biofilms are not rigid solids but rather are composite viscoelastic materials. Such materials are often characterized by measuring their response to oscillatory shear. The elastic modulus G´ measures the energy-storing, solid-like response of the material, while the viscous modulus G´´ represents the energy-dissipating, liquid-like response. We have shown that biofilms re-grown from strains of *Pseudomonas aeruginosa* that were isolated from the lungs of Cystic Fibrosis (CF) patients at different points in time typically have

elastic moduli in the range ~1-10 kPa and loss moduli in the range ~0.1-1 kPa (17). For most of these, G´ was at least an order of magnitude bigger than G´´, indicating that these biofilms are dominantly solid-like. Both of these findings are consistent with prior work by others measuring different types of *P. aeruginosa* biofilms (18-20). Furthermore, we found that *in vivo* evolution in chronic CF infections changed the production of matrix polymers in a way that promoted mechanical toughness (17).

An earlier study that combined numerical modeling with micropipette-based experiments found that neutrophils can exert a stress of about 1kPa while phagocytosing a small, rigid target (11); macrophages and blood granulocytes also exert stresses of about 1kPa during phagocytosis (12, 21-23). Thus, the mechanical stress exerted by phagocytosing neutrophils (and other immune cells) is near the midpoint of the range of stiffnesses, or elastic moduli, of biofilms regrown from CF clinical isolates. Therefore, it is possible that variation in biofilm stiffness could impact biofilms' resistance to clearance by neutrophils and that the mechanical robustness promoted by evolutionary changes in polymer production could increase the infection's ability to evade phagocytic clearance by neutrophils. We expect that any phagocytosis of biofilm bacteria must take the form of small pieces of the biofilm being detached for subsequent engulfment. Indirect support for this idea comes from a recent finding that neutrophils can detach and engulf fragments of *Trichomonas vaginalis,* which is a pathogen too large for neutrophils to engulf whole (24-27). Neutrophils can also kill cancer cells using a similar

"nibbling" process (28). These examples show that neutrophils have the ability to remove small pieces from a large target before engulfing those pieces. Phagocytosing *Dictyostelium* and *Entamoeba* can also break off and ingest small pieces of a target (29, 30).

We expect that how successful neutrophils are at detaching pieces of biofilm or individual constituent bacteria for subsequent engulfment must depend on the mechanics of the biofilm material. Prior studies examining physical limitations of phagocytosis have focused on the size and shape of rigid targets, which are not good mechanical representations of biofilms (11-15). One earlier study using macrophages from mice showed that the stiffness of target particles could act as a cue for phagocytosis, with stiffer particles being more likely to be engulfed – however, the particles used were 6 μm or less in diameter, and therefore well below the limiting size for phagocytosis (31). There have not been protocols for determining how mechanics impact the success of phagocytic engulfment when either the target itself is much bigger than the cell attempting phagocytosis or the target is embedded in a material much larger than the phagocytic cell. This hinders both fundamental understanding of neutrophil phagocytosis and the development of new types of treatments that could weaken biofilms to make them more susceptible to clearance by the immune system.

To be able to elucidate the mechanical limitations on phagocytic engulfment, we developed a method to determine the impact of mechanical properties on neutrophils' ability to engulf micron-sized beads that were initially

embedded in centimeter-sized gels. Biofilms can produce chemical factors that kill neutrophils (32, 33). This is an important component of biofilms' evasion of the immune system that we think is likely to interplay with biofilm mechanics. However, in this early stage of research, active killing of neutrophils by biofilm bacteria would be a confounding factor that could obscure the effects of mechanics *per se*. Therefore, to isolate the effects of mechanics, we use abiotic gels to re-create specific aspects of biofilm mechanics. Although in this initial development we focus on solid-like stiffness, the gels we use are, like biofilms, viscoelastic; therefore, we anticipate that this method will be extensible to future study of how viscoelastic properties impact the success of, and the timescale for, phagocytic engulfment.

For two different hydrogel chemistries, alginate and agarose, we find that increasing either polymer concentration or cross-linking density is associated with lower success of phagocytic engulfment. We tentatively attribute this to a change in gel mechanics. As the elastic modulus of the gel varies across the range we previously measured for biofilms, the success of engulfment changes, from about 30% success at low stiffness to essentially 0% success at high stiffness. This is the first hint of a mechanical limit on phagocytosis. Thus, we demonstrate that a mechanical property is correlated with the degree of successful engulfment, and that this experimental design is appropriate for probing the effects of stiffness, or elasticity, on phagocytosis by neutrophils. Therefore, this work constitutes a methodological development that can be used to advance the field and may

constitute a step forward in basic understanding, pending further studies with additional gels.

METHODS

Microscopy

Laser-scanning confocal microscopy was done with an Olympus IX71 inverted confocal microscope, a 60× oil-immersion Olympus objective and Fluoview FV10-AWS Ver 04.02. Except when stated otherwise, epifluorescence and phase contrast imaging was done using an inverted phase contrast microscope with 60X objective (both from Olympus), QImaging Exi Blue CCD camera, and QCapture Pro 6 software. For microscopy of alginate gels on coverslip chambers (Matsunami, product D35-14-1-U), the coverslip was pretreated with 100μL of 0.1% Poly-l-lysine (Sigma-Aldrich, catalog number P9155) for 30 mins to facilitate adhesion of the gel to the glass surface. The alginate gels made with 10 mM calcium consistently detached from the coverslip during imaging and therefore were not used for these experiments.

Hydrogel preparation

Alginate gels were made by dissolving 4% sodium alginate (Sigma-Aldrich, catalog number 180947) in distilled water and then adding a solution of 5% calcium carbonate ($CaCO_3$, Sigma catalog number C4830) and 5% D-(+)-Gluconic acid δ-lactone **(**GDL, Sigma catalog number G4750). Final concentrations of 10, 20, and

30mM $CaCO_3$ were used. 5% GDL was added to cause internal gelation by slowly lowering the pH of the gel, causing calcium ions to be released from $CaCO_3$ into solution (34).

We used pH paper (Cardinal Health, product P1119-1C) to measure pH before and after the addition of $CaCO_3$ and GDL (Figure S1). The initial alginic acid solution had a pH of 8. For 10mM calcium, the pH reduced to 6 or 7; for 20mM or 30mM calcium, the pH reduced to 6. Two replicates of each measurement were done.

Agarose gels were made using low-gelling temperature agarose (Sigma-Aldrich, catalog number A9414) at 0.3%, 0.5%, 1%, 1.5%, and 2%.

The synthesis of poly(ethylene glycol) diacrylate (PEGDA) was adapted from a published protocol (35). All materials for this synthesis were purchased from Sigma-Aldrich unless stated otherwise. In brief, acryloyl chloride was added dropwise to the mixture solution of poly(ethylene glycol) (PEG) diol (35 kDa) in dichloromethane (DCM) with triethylamine (TEA). The molar ratio of PEG diol, acryloyl chloride, and TEA was 1:2:4. After 24 hour of reaction, 8 molar excess of 2M potassium carbonate solution was added to neutralize the unreacted acryloyl chloride. The reaction product was then precipitated with cold ether after the removal of residual water with sodium sulfate. After drying under ambient conditions for 24 hours, the final PEGDA product was dried under vacuum for an additional 24 hours.

PEGDA hydrogel slabs were fabricated by dissolving the PEGDA in deionized (DI) water at 4% and 10% polymer concentration. The UV initiator, Irgacure 2959 (1:100 weight ratio to the polymer), was added and precursor solutions were then injected between 0.75mm glass spacers and exposed to ultraviolet light (Intelli Ray Shuttered UV Flood Light, Integrated Dispensing Solutions, Inc., 365 nm, 4 mW/cm^2) for 6 minutes per side to initiate radical crosslinking.

When used for engulfment assays, all three gels contained 10mg/ml bovine serum albumin (BSA) (HycClone brand, purchased from GE Lifesciences, catalog number SH30574.01).

Alginate and agarose gels used for engulfment assays contained fluorescent beads (polystyrene Dragon Green beads, diameters 0.955μm, Bangs Laboratories, catalog number FSDG004), which were diluted volumetrically by a factor of 100 when they were added to the solution before gelling. According to data supplied by Bangs Laboratories, the original suspension of beads contained about 1.8×10^{11} beads per milliliter, so the dilution results in about 1.8×10^{9} beads per milliliter of gel. Each bead has a volume of less than 10^{-12} milliliters. Thus, the volume fraction of beads in the gel is approximately 10^{-3}. While the inclusion of rigid beads or powders by soft materials can result in changes in material properties, such as an abrupt increase in brittleness or stiffness, the volume fraction of embedded particles required to achieve the associated percolation transition is between 0.4 and 0.6 (36, 37).

PEGDA gels used for engulfment assays did not contain beads but were prepared with a modified protocol so that gels would be fluorescent. PEGDA precursor solutions in HBSS were prepared as described above with the addition of 0.01 mg/mL BSA and 200 µg/mL FITC-o-acrylate, both purchased from Sigma-Aldrich. The acrylate group on the FITC reacts with the end of the PEG chain and thus becomes conjugated to the gel. Hydrogel slabs were swollen in 200 µL HBSS overnight and then rinsed with HBSS three times to remove unconjugated FITC-o-acrylate prior to incubating with neutrophils.

Hydrogels used for microscopy experiments were stored overnight at 4˚C in 500µl aliquots in 24-well flat-bottom plates (Corning, purchased from VWR, catalog number 15705-060). Alginate and agarose hydrogels used for rheological measurements were poured into petri dishes (60mm diameter) at 1000-2000µm deep and stored overnight at 4˚C.

Rheology

Oscillatory bulk rheology was done using a stress-controlled AR 2000ex rheometer (TA Instruments). Hydrogel samples were taken out of 4˚C storage on the morning of the experiment and placed at 37˚C for one hour. Then, alginate and agarose gel sections were placed on the rheometer and cut down to the size of the 8mm parallel plate head. PEGDA gels for rheological measurements were swollen

in DI water overnight before specimens were punched from the slab with an 8 mm biopsy punch.

The height of the tool gap varied between 1 and 2mm; this was used to determine the depth of the gels. Oscillatory frequency sweeps from 0.1 to 600 rad/s at 1 % strain and strain sweeps from 0.1 to 200 % at 3.14 rad/s were performed on each sample on each day of measurement. Elastic modulus was taken as the midpoint of the G' plateau region for strain sweeps. Three replicate rheology measurements were done on bead-free gels for each concentration of agarose, calcium, and PEGDA used. In addition, two more replicate rheology measurements were done for each type of bead-containing agarose and alginate gels, at the bead concentration used for engulfment measurements.

Bracketing gel pore size

100 μL solutions of alginate (made with 20mM and 30mM calcium) and .3% agarose were deposited atop and allowed to gel on coverslip-bottomed dishes. The gel covered half of the coverslip so that the leading edge could be easily visualized. Solutions containing either a 1:100 dilution of 200nm fluorescent polystyrene beads (Bangs Laboratories, catalog number FSDG002) or 2000K molecular weight Dextran (ThermoFisher, catalog number D7137) were then deposited next to the gel; dextran of this size has a hydrodynamic radius of 35 nm (38). Confocal z-stack micrographs were acquired.

Neutrophil Isolation

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

Human neutrophils were isolated from two adult volunteer blood donors following a published protocol (39). In brief, blood was collected in lithium heparin-coated tubes (BD Vacutainer, catalog number 367880), mixed with a filter-sterilized (filters from VWR, catalog number 28145-501) 3% dextran and 0.9% sodium solution (Sigma, catalog numbers 31392 and S9888). Red blood cells fell out of solution. The resulting supernatant was centrifuged (Eppendorf 5810R, A-4-62 Rotor, with swinging buckets with 15mL conical tube adapters) for 10 minutes at 500g and the resulting pellet was re-suspended in 10ml of Hanks Buffered Salt Solution (HBSS, Gibco purchased from Sigma, catalog number 14175095) without calcium or magnesium. Cells were separated using a Ficoll-Paque density gradient solution (GE Healthcare, purchased from Sigma, catalog number 17-1440-02), spun at 400g for 40 minutes. This resulting pellet was re-suspended in dionized water for 30 seconds to lyse any remaining red blood cells. Then, the isotonicity of the solution was restored using a filter-sterilized 1.8% NaCl solution (Sigma, catalog number S9888). Cells were centrifuged for 5 minutes at 500g and the final neutrophil pellet was re-suspended in 1ml HBSS with calcium and magnesium (Gibco, purchased from Sigma, catalog number 14025092) and 20% human serum (Sigma, catalog number H4522). This medium was used for all neutrophil experiments.

Neutrophil Engulfment Assay and Microscopy for Agarose and Alginate Gels

On the day of phagocytic engulfment experiments, neutrophils were isolated as described above. While neutrophils were being isolated, 100µl of rabbit anti-BSA antibody (Invitrogen brand, purchased from ThermoFisher Scientific, catalog number A11133) diluted 1:1000 in Dulbeccos' phosphate-buffered solution (DPBS) (Gibco brand, purchased from Thermo-Fisher Scientific, catalog number 14190144) was added to each hydrogel and incubated at 4˚C for 30 minutes. Hydrogels were then washed three times with DPBS and 200µl of freshly-isolated neutrophil suspension was added to each well. The hydrogel plate with neutrophils was then incubated at 37˚C for one hour; 37˚C is human body temperature. After one hour, the solution containing cells was collected from off the top of the hydrogel. In some cases, to increase the number density of cells in the microscope field of view, collected neutrophils were concentrated and re-suspended in 50µl of HBSS. The collected cells were put on a microscope slide with a coverslip and Grace labs imaging spacer (Fischer Scientific, catalog numbers 12-550-15 and 12-541-B, and Sigma, catalog number GBL654004). Before using with cells, coverslips had been coated in 0.1% poly-l-lysine (Sigma-Aldrich, catalog number P9155) for 30 minutes and then triple rinsed. Cells were imaged using phase contrast microscopy and beads were imaged using epifluorescence microscopy with a filter for green fluorescent protein. At least 100 neutrophils from every sample, from fields of view chosen at random, were counted as either containing or not containing fluorescent beads, which served as indicators of gel engulfment.

Replicate numbers were as follows: 5 for 0.3% agarose, 4 for all other agarose concentrations and for all alginate gels.

Engulfment assay with PEGDA gels

8 mm punches of PEGDA gel were placed in a 24 well plate and each swelled overnight with 200 μL of HBSS buffer. 100 μL of anti-BSA antibodies at 1:1000 dilution were applied to the gels for 30 minutes after which each gel was rinsed three times with HBSS buffer medium. 200 μL of freshly-isolated neutrophil suspension was added to each gel and incubated for 1 hour. Cells were then collected from the gel substrate, concentrated, and counted from confocal micrographs. Three replicate experiments were done for each PEGDA gel concentration used.

Growth of Bacterial Aggregates

Overnight shaken cultures of *P. aeruginosa* naturally contain both multicellular aggregates and single cells (40). To promote the formation of more and larger aggregates, we used a lab strain of bacteria that over-expresses the extracellular polysaccharide Psl, Δ*wspF* Δ*pel* (41). The background for this lab strain is the University of Washington version of PAO1 (42, 43). A ΔwspF Δpel mutant of PAO1 previously developed by other researchers (44) was later modified to express green fluorescent protein (41) and subsequently used in this work.

We grew aggregates using the same process we have described previously (45, 46). Frozen bacterial stock was streaked onto a Lysogeny Broth (LB) agar petri plate and incubated at 37 °C overnight. From this plate, one colony was dispersed into 4mL of LB liquid growth medium and left to overgrow for approximately 20 hours at 37 °C on a rotating shaker (Labnet Orbit 1000, 242 rotations per minute), inside a Fisher Scientific Isotemp Incubator. Overgrowth to stationary phase increased the number of large multicellular aggregates present.

Microscopy of Neutrophils and Bacterial Aggregates

Freshly isolated human neutrophils at a concentration of ~10^6 cells/mL were mixed with bacterial culture (diluted to OD_{600} = 0.2 measured using a Spectronic 20 Genesys spectrophotometer) and placed on a coverslip chamber for imaging using timelapse phase contrast microscopy. As described in our previous work, the stage region of this microscope is enclosed in an incubator chamber, custom-built by Precision Plastics (41, 47, 48). During image acquisition the microscope stage incubator was set to 37 °C, human body temperature. Images were analyzed using Fiji, a software distribution of ImageJ. A total of 810 (Supplemental movie 1), 199 (Supplemental movie 2), and 4500 (Supplemental movie 3) frames were acquired for each video.

Microscopy videos of neutrophils excluded by gels

100 μL solutions of alginate (with 20mM or 30mM calcium) and of .3% and 1% agarose were deposited atop and allowed to gel on coverslip-bottomed dishes. Agarose gels made with 0.3% and 0.5% agarose were used because they were the softest agarose gels used in our engulfment assays, for which we measured the greatest success of phagocytic engulfment. The gel covered half of the coverslip surface so that the boundary with solution could be easily visualized. Timelapse phase contrast micrograph videos were then acquired at the gel boundary to visualize whether neutrophils were able to penetrate each gel. Videos of neutrophils on alginate gels were acquired with a Hamamatsu Orca-Flash 4.0 (C11440) camera and MetaMorph Advanced Version 7.7.6.0.

Height profile of neutrophils excluded by gel

A solution of .3% agarose containing BSA and 1μm fluorescent beads was gelled on top of the coverslip portion of a coverslip dish. The dish was stored at 4 °C for approximately 1 hour before use. A water soaked paper towel piece was left inside the dish, and the lid tightly sealed with parafilm to prevent the gel from drying out before use. 100μL of freshly-isolated neutrophil suspension were deposited on the top surface of the gel and confocal microscopy z-stacks imaging both gel and neutrophils were collected for two hours. The fluorescence images for each time point were analyzed using the Fiji distribution of ImageJ. A threshold was applied to better visualize beads and the built-in ImageJ Analyze Particles function was

used to filter out-of-focus particles from each frame. The reported bead count from this function was then plotted in Excel as a function of slice, and thus height. The number of neutrophils in focus at the mid-section of each cell per frame was counted manually and recorded as a function of z-slice, and thus height.

Statistics

Statistical analysis was done using Microsoft Excel.

Results

Measurements of Neutrophil Interactions with Biofilms

To assess whether neutrophils could remove individual bacteria from biofilms, and to estimate the timescale for such a process, time-lapse phase contrast microscopy was used to image neutrophils interacting with biofilm-like bacterial aggregates that were an order of magnitude bigger than the neutrophils. On several occasions, we saw neutrophils remove and engulf one or two bacteria out of an aggregate (Supplementary Movies 1-5; Figures S2-S4).

For many viscoelastic materials, including the gels used in this study, the elastic and viscous moduli measured depend on the frequency of oscillatory shear. Estimating the frequency characterizing the strain imposed by neutrophils will allow determination of what frequency regime is relevant for this study. For this, five protrusions of different sizes, from four different attacking neutrophils, were observed and the time that elapsed during each retraction of a protrusive

appendage was measured. The inverse of this time was taken to be the frequency characterizing the strain that could be imposed by the retraction of that particular protrusion. On average, this frequency was 0.06 Hz, with a standard error of the mean of 0.01 Hz; 0.06 Hz is approximately equivalent to an angular frequency of 0.38 radians/sec. The average observed distance covered by protrusions at their maximum extension was 2.97μm (standard error of the mean (SEM) 0.28μm), and the estimated average speeds were 0.26 μm/s (SEM 0.13 μm/s) for extension and 0.18 μm/s (SEM 0.10 μm/s) for retraction. Others have measured the leading edge of a neutrophil protrusion wrapping around a bead at 0.1 μm /sec, which is comparable to the speeds we measure (11).

Mechanical Properties of Hydrogels

The mechanical properties of the hydrogels used in this study were measured using oscillatory bulk rheology. Before rheology, the gels were placed in a 37˚C incubator for one hour to ensure the mechanics being measured were the same as those in the engulfment experiments in which we exposed gels to neutrophils at 37˚C (49). Two primary hydrogel types, agarose and alginate, were used so that the elastic moduli could be varied across comparable ranges using two different chemical compositions. Alginate is an important component of the biofilm matrix for many strains of *P. aeruginosa*.

Agarose gels

Agarose gel is a linear polymer with alternating D-galactose and 3,6-anhydro-L-galactose units. Increasing the concentration of polymer in a gel will increase the gel's elastic modulus (50). Using agarose concentrations from 0.3% to 2%, the resulting range of elastic moduli, ~0.1 to ~10 kPa (Figure 1), roughly covered the range of elastic moduli that we measured previously for biofilms grown from clinical isolates of *P. aeruginosa* (17). Adding bovine serum albumin (BSA) and polystyrene beads to the gels, as for engulfment measurements, did not significantly impact the mechanics of the gels (Figure S5A; Table 1). The value of the elastic modulus at the midpoint of the plateau region in strain sweeps is the value used herein to characterize these gels' mechanics, unless the material was already yielding for the lowest strain used – in that case, the value of the elastic modulus that was measured at the lowest strain was used (Figure 1B). For each concentration of bead-free agarose, three replicates were made and the measured elastic moduli were averaged (Table 1). For gels containing BSA and beads, two replicates each were made and measured (Table 1).

	Agarose Gels (N=3)				
Agarose concentration	0.30%	0.50%	1.0%	1.5%	2.0%
Elastic Shear Modulus (SEM) in Pascals	125 (3)	370 (60)	2,250 (70)	5,600 (300)	10,500 (700)
	Agarose Gels containing BSA and polystyrene beads (N=2)				

Agarose concentration	0.30%	0.50%	1.0%	1.5%	2.0%
Elastic Shear Modulus (SEM) in Pascals	120 (20)	320 (190)	1,700 (370)	5,200 (600)	8,700 (1,800)

Table 1: Moduli measured for gels made of different concentrations of agarose.

Alginate

Alginate polymers are made up of β-D-mannuronate (M) and α-L-guluronate (G) residues. Divalent calcium ions crosslink the G residues of alginate polymers, and increasing this crosslinking density is known to increase the elastic moduli of alginate gels (51). This is consistent with what we found using bulk rheology (Figure 2). Adding BSA and polystyrene beads did not impact the mechanics of the gels (Figure S5B; Table 2). Calcium concentrations of 10mM, 20mM, and 30mM resulted in elastic moduli of the alginate gels ranging from 0.1kPa-4.5kPa. The elastic modulus used herein is the value at the midpoint of the plateau region of the strain sweep. For bead-free gels, for each concentration of calcium used, three replicate alginate gels were made and the measured values averaged to determine the elastic modulus (Table 2). For gels containing BSA and beads, two replicate gels were made and measured for each calcium concentration (Table 2).

	Alginate Gels (N=3)		
Calcium concentration	10 mM	20 mM	30 mM

Elastic Shear Modulus (SEM) in Pascals	13 (1)	1500 (500)	4500 (300)
	Alginate Gels with BSA and polystyrene beads (N=2)		
Calcium concentration	10 mM	20 mM	30 mM
Elastic Shear Modulus (SEM) in Pascals	60 (20)	1400 (440)	2800 (50)

Table 2: Moduli measured for alginate gels made with different concentrations of cross-linking calcium ions.

At the low frequencies characterizing neutrophil-imposed deformations of *in vitro* biofilm aggregates, the elastic moduli have no significant dependence on frequency for all gels used (Figure 1A; Figure 2A).

Measurement of the Success of Phagocytic Engulfment

As an indicator of the success of phagocytic engulfment, we used phase contrast and epifluorescence microscopy to determine the percentage of neutrophils that, after one hour's incubation with a gel, contained fluorescent beads that had originally been embedded within the hydrogel. For each sample, at least 10 fields of view under the microscope were chosen at random, and at least 100 neutrophils were counted. Four or five replicate experiments were done, on different days, for each agarose or calcium concentration. The hydrogels were in the centimeter-sized bottoms of 24-well plates and much larger than the neutrophils themselves, so that the shape of the gel presented to neutrophils was

a flat surface, approximating the surface of a spheroid much larger than a neutrophil.

Alginate

For alginate gels cross-linked with 10 mM , 20 mM, and 30 mM calcium, on average, 77%, 5%, and fewer than 2%, respectively, of neutrophils internalized beads that had been initially embedded in a gel (Figures 3 and 4).

Agarose

The fraction of neutrophils containing beads that had originally been contained in agarose gels decreases sharply upon changing agarose concentration from 0.3% to 0.5% (Figure 5). For 0.3% agarose gels, on average 28% of neutrophils had internalized fluorescent beads. For gels at 0.5% agarose and above, on average less than 2% of neutrophils internalize fluorescent beads.

Gel Mesh Size and Neutrophil Exclusion

To assess the degree to which different amounts of successful engulfment arise from different gel mesh sizes, we use, as tracers, diffusible fluorescent molecules and beads, applied to the fluid medium outside gels. Alginate gels made with 20mM and 30mM calcium and 0.3% agarose gels all exclude 200nm fluorescent beads (Supplementary Movies 6-11). This indicates that pores are smaller than 200nm for all three of these gels and that ~10 μm neutrophils are unlikely to migrate through gels. We find that 35nm dextran enters the 0.3% agarose gel readily, but not alginate gels made with 20 mM and 30 mM calcium

(Supplementary Movies 12-16). This indicates that the lowest-concentration agarose gel we use has larger pores than the two more-crosslinked alginate gels. The pore sizes we bracket using diffusible tracers are reasonably consistent with pore sizes reported for similar alginate and agarose gels (52-57).

To directly assess whether neutrophils or their visible (~1μm) protrusions enter gels, we took time-lapse microscopy movies of neutrophils crawling along one edge of bead-containing agarose and alginate gels (Supplementary Movies 17-22). In no case was a neutrophil or protrusion seen to enter a gel.

Finally, for the 0.3% agarose gel, for which we measured the greatest successful engulfment, we applied neutrophils to the top of a gel and imaged them under the confocal microscope over two hours, which is twice the duration of our engulfment experiment (Supplementary Movies 23-25). We identified the volume of the gel using embedded beads, and the top of the gel as the z-position where the bead density abruptly began to drop. Over two hours, the neutrophils were consistently localized at the top of the gel (Figure 6).

Therefore, we conclude that neutrophils do not migrate through the alginate and agarose gels we use for engulfment experiments. This parallels findings that neutrophils do not enter biofilms *in vivo* (9, 10). The pore sizes we bracket for alginate and agarose gels are also consistent with previous measurements of biofilm pore sizes (38).

Successful Engulfment Correlates Negatively with the Elastic Modulus of the Gel

For this biofilm-mimicking regime, using both alginate and agarose gels allows a preliminary disentangling of the effects of stiffness from any effects arising from specific chemical composition. For both hydrogels, the percent of neutrophils that successfully engulfed beads varies with the stiffness in such a way that data from the two types of gel lie very nearly along the same curve (Figure 7).

Engulfment of bead-free gel

To probe whether phagocytosis would happen in the absence of beads, and thereby assess whether the gel itself can be a target for phagocytosis, as opposed to the beads *per se* being targets embedded in a larger, non-target structure, we require a gel that can be labeled for visibility under the microscope. For this, we use PEGDA gels that were labeled by covalently-bound fluorescein isothiocyanate (FITC), and neutrophils that have engulfed part of such gels are identifiable by confocal fluorescence microscopy (Figure 8). PEGDA gels containing 4% and 10% 35 kDa PEGDA have elastic moduli of ~200 kPa and ~2000 kPa. On average, 7% of neutrophils exposed to PEGDA gels contained internal FITC (SEM 1.2%). This is roughly twice the percentage of neutrophils that were determined to have achieved successful engulfment, using tracer beads, for agarose and alginate gels with about the same range of elastic moduli.

Discussion

Interpreting Measurements of Successful Engulfment

Alginate and agarose gels are advantageous for this study because they are chemically similar to the extracellular polysaccharides that scaffold biofilm matrices; indeed, alginate is an important component of many *P. aeruginosa* biofilms (58-60). However, alginate and agarose gels are not readily distinguishable from aqueous culture medium under the microscope and therefore we use embedded beads as tracers to allow us to determine whether engulfment has occurred. The use of tracer beads means that at least two interpretations of our engulfment measurements (Figures 4 and 5) are possible. In one view, the gel itself is the target for phagocytosis, and beads are engulfed when, by happenstance, they are contained in the piece of gel that is detached. In another view, the beads themselves are the target for phagocytosis. Neutrophils might sense rigid beads that contrast mechanically with the surrounding, soft gel, and pluck out beads on or near the gel surface. The latter interpretation is indirectly supported by work showing that the stiffness of target particles can act as a cue for phagocytosis by macrophages, with stiffer particles being more likely to be engulfed (31). In our case, this might involve infiltration of sub-optical filipodia (~0.2 μm in width) into the top of the gel, along the surface of an embedded bead, and subsequent formation of a phagocytic cup over the bead, leading to engulfment; steps of such a process have been shown using electron microscopy (61, 62).

There are at least three possible reasons that FITC-labeled PEGDA gels may give rise to higher measurements of successful engulfment than do bead-containing alginate and agarose gels. First, to avoid perturbing gel mechanics, we use only a low concentration of beads. This leaves regions of bead-free gel with sizes comparable to or larger than neutrophils; in contrast, the labeled PEGDA gels contain FITC throughout. Therefore, pieces of agarose and alginate gel may be engulfed without tracer beads; if so, the actual rate of successful engulfment is being under-counted in those experiments. Second, when PEGDA gels are formed containing beads, as for the agarose and alginate gels, the beads rapidly diffuse out of the PEGDA gel (for this reason, PEGDA gels containing beads were not used for engulfment measurements). This indicates that the pore sizes for the PEGDA gels used are comparable to or greater than the 1 μm bead diameter, compared with pore sizes less than 200nm for alginate and agarose gels. It is plausible that larger pores might impact the interaction of neutrophils with gels. Finally, we cannot discount the possibility that gel chemistry may be playing a role in immunogenicity or in adhesion to the surface and/or subsequent phagocytic engulfment.

Thus, the FITC-dyed PEGDA gels have significant structural and chemical differences from the bead-containing alginate and agarose gels. Therefore, while our results on PEGDA gels suggest that the gel itself can be a target for phagocytosis, this does not exclude the possibility that, in bead-containing agarose and alginate gels, it is actually the rigid embedded beads that are the only or

primary targets for phagocytosis. Both of these possibilities are consistent with gel mechanics impacting the success of engulfment.

Correspondence with biofilms

P. aeruginosa cells have a Young's modulus in the low tens of MPa and thus are at least three orders of magnitude stiffer than the biofilm in which they are embedded (63). The tracer beads we use are made of polystyrene, which has a Young's modulus in the low thousands of MPa, and thus are at least five orders of magnitude stiffer than the agarose or alginate gel in which they are embedded. Thus, there is a somewhat-similar mechanical contrast between beads and embedding gel, and biofilm bacteria and embedding matrix.

Notably, both the chemical composition and the mesh sizes of alginate and agarose gels are much closer to what is found in biofilm matrices than are the chemical composition and mesh sizes of PEGDA gels.

Other Potential Influences on Neutrophil Engulfment

The studies in this paper focus on the elastic modulus of large, gel targets. However, the yield strain and stress, toughness, and compliance, as well as other mechanical properties, could influence the success of phagocytosis as well. Failure strengths of several thousand up to nearly 20,000 Pa have been measured for biofilms, but at strain rates much greater than those applied by neutrophils; this

suggests that new measurements of biofilm failure, at low strain rates, are needed (64, 65). Furthermore, the strength of the adhesion of the phagocytic cell to the target could also impact the success of phagocytosis.

To engulf particles, neutrophils must bind to the target as it extends its cell membrane to engulf the target. The more binding sites, the stronger a neutrophil can adhere and wrap itself around the target (11, 66). Here, to make the abiotic gels recognizable to the neutrophils, bovine serum albumin (BSA) was added into the gel and was then incubated with an anti-BSA antibody before exposure to the neutrophils. Previous studies of the mechanical limitations of neutrophil phagocytosis have been measured only with antibody-mediated phagocytosis (11, 66). This method has been used to study neutrophil mechanics when the target is a stiff, polystyrene bead (11, 66). In the studies presented here, the presence or absence of antibodies did not affect the success of engulfment when neutrophils were applied to bead-containing alginate gels. BSA was still added to the gels, but as engulfment occurs without the antibodies present, this phagocytosis may not be mediated through the Fc receptors.

Work on macrophages, another type of phagocytic immune cell, indicates that the stiffness of the culturing substrate or of the target itself may impact phagocytosis by promoting phagocytosis or related phenotypic changes through macrophage mechanobiology (67-70). How such effects may interplay with the mechanical limitation we study here are not known.

Implications for biofilm disease

The range of elastic moduli re-created by agarose and alginate gels includes the range of elastic moduli that we previously measured for biofilms grown from clinical bacterial isolates, ~0.05 – 10 kPa (17). This range is indicated by the grey box in Figure 7. For gels with moduli spanning this range, the measured success of neutrophils at detaching and engulfing parts of the gel falls from about 30% success to about 0% success. This suggests that the elastic modulus of biofilm infections might impact their resistance to the immune system, and that the evolutionary trend toward promoting biofilm toughness that we showed in our earlier work (17) may reflect a selective advantage of higher elasticity for biofilms.

The increased resistance to phagocytosis conferred by increased elastic modulus of biofilms could result in worse outcomes for infected patients if it causes frustrated phagocytosis, in which neutrophils release reactive oxygen species that damage host tissue as well as bacteria (71, 72). Indeed, damage from the patient's own inflammatory response and associated release of reactive oxygen species is the primary cause of lung failure in cystic fibrosis patients (73, 74). Mechanical resistance to phagocytosis could also allow more time for bacterial virulence factors, such as pyocyanin and rhamnolipids produced by *Pseudomonas aeruginosa*, to be produced and to damage neutrophils (32, 33).

Conclusions and Future Work

We have made significant progress toward developing a method for determining how the mechanics of a large target, or a large structure in which targets are embedded, impacts the phagocytic success of an attacking cell. Future work could extend this method to other gel chemistries to examine the effect of other mechanical properties of the target, such as yielding, and of the mechanical forces binding the phagocytic cell to the target. The use of gels with specifically-tunable yield strains and surface chemistries, as well as elastic moduli, would be desirable for this. Our study here measures phagocytic success only after a fixed time, but our method is readily extensible to time-varying studies to determine how mechanics impacts the timescale for successful engulfment; we speculate that truly viscoelastic properties, such as compliance, may strongly impact this timescale.

As we and others continue to develop the method presented here, and extend these studies as outlined in the preceding paragraph, we expect to be able to determine what mechanical properties render a biofilm most susceptible to phagocytic clearance. We expect that this will guide us and other researchers as we work to develop new, non-antibiotic approaches to biofilm treatment, which we hope will circumvent biofilm's innate, phenotypic resistance to antibiotics, reduce damage to patients' health by the harmful side effects of antibiotics, and slow the evolutionary development of antibiotic resistance.

Author Contributions

M. D.-F. and L. A. B. performed experiments, analyzed data, and wrote the paper. M. D.-F. and L.A.B. contributed equally to this paper. Z. L., L. W., K. K. performed experiments. E. C.-H. designed research. V. D. G. designed research and wrote the paper.

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Figure captions

Figure 1. Representative (A) frequency and (B) strain sweeps, all from the same day of measurement, for agarose gels ranging in concentration from 0.3% agarose to 2% agarose. Frequency sweeps were done at 1% strain and strain sweeps were done at 3.14 radians/s. The elastic moduli (G') are shown with solid symbols and the viscous moduli (G") are shown with hollow symbols of corresponding shape and color.

Figure 2. Representative (A) frequency and (B) strain sweeps, all from the same day of measurement, for alginate gels made with 10, 20, and 30 mM calcium. Frequency sweeps were done at 1% strain and strain sweeps were done at 3.14 radians/s. The elastic moduli (G') are shown with solid symbols and the viscous moduli (G") are shown with hollow symbols of corresponding shape and color.

Figure 3. False-colored phase contrast images of neutrophils (blue) combined with fluorescence images of beads (red) after incubation with alginate gels cross- linked with 10mM calcium (A) and 30mM calcium (B). Neutrophil populations which were incubated with gels of lower elasticity (cross-linked by 10mM calcium) have a larger proportion of neutrophils with internalized beads than those populations that were incubated with gels of higher elasticity (cross-linked by 30mM calcium).

Figure 4. Neutrophils more successfully engulf beads when the embedding alginate gels are cross-linked with lower concentrations of calcium. As the concentration of calcium increases and therefore calcium cross-linking increases, the ability of neutrophils to successfully engulf beads decreases. Error bars are standard error of the mean. Single-factor ANOVA gives a p-value of 5×10^{-8}.

Figure 5. Neutrophils more successfully engulf beads when the embedding agarose gels are made with low concentrations of agarose. At 0.3% agarose, an average of 28% of neutrophils had internalized beads. For all other agarose concentrations, fewer than 2% of neutrophils had internalized beads. Error bars are standard error of the mean. Single-factor ANOVA gives a p-value of 0.002.

Figure 6. Neutrophils were applied to the top of a bead-containing 0.3% agarose gel. The gel+neutrophils were imaged on the confocal microscope immediately after neutrophils were added, one hour later, and two hours later. The number of beads visible in each frame (solid symbols) were counted to determine where the gel top was located, since the liquid medium was free of beads. Neutrophils (hollow symbols) were counted for the frames where their mid-planes were in focus. Dashed lines connecting neutrophil counts are a guide to the eye.

Figure 7. Percent engulfment decreases as elastic modulus of agarose or alginate hydrogel increases. Elastic moduli are from measurements of gels without beads. Error bars are standard error of the mean. The grey shaded region indicates the range of elastic moduli that we measured earlier for biofilms re-grown from clinical isolates taken from patients with Cystic Fibrosis.

Figure 8. Confocal z-slices of neutrophils that had been exposed to FITC-labeled (A) 4% and (B) 10% PEGDA gels for one hour. In each frame, a FITC-containing neutrophil is circled and a neutrophil that does not contain FITC is indicated with an arrow. 30 μm scalebars.

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