**Incorporation of collagen into *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms impedes phagocytosis by neutrophils**

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**Abstract**

Biofilms are communities of microbes embedded in a matrix of extracellular polymeric substances (EPS). Matrix components can be produced by biofilm organisms and can also originate from the environment and then be incorporated into the biofilm. For example, we have recently shown that collagen, a host-produced protein that is abundant in many different infection sites, can be taken up into the biofilm matrix, altering biofilm mechanics. The biofilm matrix protects bacteria from clearance by the immune system, and some of that protection likely arises from the mechanical properties of the biofilm. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common human pathogens notable for forming biofilm infections in anatomical sites rich in collagen. Here, we show that the incorporation of Type I collagen into *P. aeruginosa* and *S. aureus* biofilms significantly hinders phagocytosis of biofilm bacteria by human neutrophils. However, enzymatic treatment with collagenase, which breaks down collagen, can partly or entirely negate the protective effect of collagen and restore the ability of neutrophils to engulf biofilm bacteria. From these findings, we suggest that enzymatic degradation of host materials may be a potential way to compromise biofilm infections and enhance the efficacy of the host immune response without promoting antibiotic resistance. Such an approach might be beneficial both in cases where the infecting species is known and also in cases wherein biofilm components are not readily known, such as multispecies infections or infections by unknown species.

## Introduction

Biofilms are complex microbial communities that are embedded in a matrix of extracellular polymeric substances (EPS) and other materials; this matrix provides physical and chemical protection against antibiotic treatments and immune clearance [1-7]. Biofilm formation poses a significant clinical challenge as the cause of up to 80% of chronic infections [8], costing the healthcare system millions of dollars annually and impacting millions of lives [9, 10]. The most common bacteria isolated from chronic wound infections are *Staphylococcus aureus* and *Pseudomonas aeruginosa* [11].

Biofilm-forming microbes can produce EPS, and in addition EPS components can also originate from the environment [6, 12, 13]. Using an *in vitro* and an *in vivo* experimental model for chronic wounds, we have recently shown that *P. aeruginosa* biofilms incorporate collagen into their matrices, resulting in the change of the biofilms' mechanical properties to be more homogeneous, more relatively elastic, and less compliant [14, 15].

Type I collagen is the most abundant protein in all vertebrates [16], including humans, and it is the key structural component in major tissues and a mechanical barrier to invasion [17-19]. Type I collagen is commonly found in a wide range of infection sites such as chronic wounds, dental plaque, cystic fibrosis lungs, urinary tract infections, repetitive ear, sinus, and bladder infections, and so on [20-25]. Thus, it is plausible that the incorporation of type I collagen into biofilms, and consequent changes in biofilm composition and mechanics, may be widespread well beyond *P. aeruginosa* and chronic wounds specifically. However, how the incorporation of collagen into biofilms impacts the efficacy of the host immune response remains entirely uninvestigated.

In our previous work, we used gels, of sizes much larger than neutrophils, to re-create biofilm mechanics. We used these gels to assess the degree to which mechanical properties might impact the success of human neutrophils at removing and engulfing biofilm bacteria. Indeed, we found that the success rate and timescale required for successful phagocytosis depend on the elasticity and toughness of the gel target structure [26, 27]. Given the increase in relative elasticity and decrease in compliance arising from the incorporation of collagen into biofilms [14, 15], we hypothesize that the incorporation of collagen in biofilms should reduce the success of immune cells at phagocytosing biofilm bacteria [28].

Here we investigate the impact of collagen incorporation by *P. aeruginosa* and *S. aureus* biofilms on the success rate of phagocytosis by neutrophils. *P. aeruginosa* and *S. aureus* are common pathogens that form biofilms in a wide range of clinical diseases, including bacteremia, infective endocarditis, and infections of soft tissue and skin [11, 29, 30]. For *S. aureus*, adhesion to collagen has been correlated to the development of osteomyelitis and septic arthritis [31], but the interaction between collagen and other EPS components has not been shown. Both *P. aeruginosa* and *S. aureus* cells are negatively charged at near-neutral pH values, which should tend to promote electrostatic binding with cationic collagen. Both *P. aeruginosa* and *S. aureus* biofilms can have extracellular DNA (eDNA) as an EPS component of their matrices [32, 33]. eDNA is anionic, which should tend to promote electrostatic binding with cationic collagen. Among other materials, *P. aeruginosa* matrices can also contain Psl, which is a net-neutral polysaccharide with charge separation, opening up the possibility of dipole-like attraction with collagen. Other components of the *S. aureus* biofilm matrix include teichoic acids [34], which have a net negative charge [35] that could promote electrostatic interactions with positively-charged host material such as collagen. Unlike *P. aeruginosa*, *S. aureus* has proteins that specifically bind

to host materials that are important in wound healing. Proteins bound to the bacterial cell wall of *S. aureus* can also adhere to human matrix proteins such as fibrinogen and collagen [36].

We grew biofilms in media containing different concentrations of Type I collagen and subsequently incubated biofilms with freshly isolated human neutrophils. Bacteria were stained with a pH-dependent fluorescent dye so that bacteria that were internalized into a neutrophil's highly acidic phagosome could be identified by color. We imaged neutrophils using confocal laser fluorescence scanning microscopy, and quantified phagocytic success by measuring the percentage of neutrophils that contain engulfed bacteria. We found that when either *P. aeruginosa* or *S. aureus* biofilms are grown in a collagen-containing medium, the phagocytic success rate is consistently less than for biofilms grown without collagen, and that this effect increases with increasing the concentration of collagen concentration in the medium.

We have previously shown that treatment with enzymes specific to a dominant matrix material will compromise biofilm mechanics and microstructure [37]. Given the relationship between mechanical parameters and phagocytic success [26, 27], we posit that the enzymatic breakdown of collagen in biofilms may increase the ability of neutrophils to phagocytose biofilm bacteria. Therefore, we treated biofilms with collagenase, a protease commonly used in wound healing therapy [38, 39]. We found that, for biofilms grown in the presence of collagen, treatment with collagenase increases the phagocytic success rate. The enhancing effect of collagenase on phagocytic success was greater in cases where the anti-phagocytic effect of collagen was greater.

## Materials and Methods

### *Preparation of nutrient media for biofilm growth*

Sterile liquid and agar growth media were prepared by dissolving 30 g of LB broth powder (Sigma-Aldrich, Cat.# 154154, NJ, USA) or 30 g of LB agar powder (Sigma-Aldrich, Cat.# 213981, ON, USA), respectively, into 750 mL deionized water. Both forms of media were autoclaved on a liquid cycle at 121 °C for 20 minutes. Agar media was allowed to cool, stirring, for 30 minutes at room temperature before being poured into sterile Petri dishes (Fisher Scientific, Cat.# FB0875712, USA) and allowed to solidify overnight, then stored at 4 °C.

### *Preparation of collagen (type I collagen) solution*

All the pH-balanced collagen solutions in this research were made with refrigerated 4.39 mg/mL Collagen I solution (Collagen I, Rat, Corning 354236, MA, USA). To prepare 10 mL of this solution, 7 mL of collagen solution was mixed with 800 µL Phenol Red (Sigma-Aldrich, ACS Cas.# 143-74-8) to act as pH indicator, 200 µL Phosphate-Buffered Saline (PBS, Fisher Scientific, Cat.# BP3991, USA), and 200 µL 0.1M NaOH (BDH, ACS Cas.# 1310-73-2) to neutralize the pH of the solution. Deionized water was then added to bring the volume to 10 mL.

### *Growth of biofilms with collagen*

The bacterial strains used for biofilm growth in this study were *P. aeruginosa* strains PA01 Wild Type (WT) [40], PA01 Δ*pel* Δ*psl* [41], and *S. aureus* strain SA pJY209 [42]. The *P. aeruginosa* strains constitutively express green fluorescent protein (GFP), and the *S. aureus* strain constitutively expresses yellow fluorescent protein (YFP).

The frozen bacterial stock was streaked onto sterile LB agar plates and grown overnight at 37°C. One colony was selected and cultured in 4 mL of LB broth liquid media and grown shaking overnight at 37°C. Biofilms were grown by combining 200 µl of overnight culture with 50 µl of 3 mg/mL pH-balanced collagen solution, spread onto LB agar plates with an L-shaped cell spreader (VWR# 220412261B, PA, USA), and incubated statically overnight at 37°C.

*Treatment of biofilms with collagenase*

Solutions of collagenase were prepared by dissolving the enzyme in its powdered form (EMD Millipore Corp., Cat.# 3818971, USA) into phosphate-buffered saline (PBS) at concentrations of 4 mg/mL. Biofilm samples were collected from agar plates gently with a spatula and then transferred to 24-well plates (Fisher Scientific, Cat.# FB012929). Before the phagocytosis assay, each sample was treated with 100 µL of collagenase solution and incubated at 37 °C for 1 hour before staining with pH-dependent dye (see below). All collagenase treatments were of the same dose and duration. For all the control groups, samples were treated with equal volumes of PBS buffer. All other procedures between the control and experimental groups were the same.

*Staining biofilms with pHrodo*

A stock solution of pHrodo Red succinimidyl ester (ThermoFisher Scientific, Cat.# P36600, USA) was prepared at a concentration of 1 mg/150 µL in dimethyl sulfoxide (DMSO, Sigma, ACS Cas.# 67-68-5, USA). A working solution was prepared by diluting 1 µL of the stock into 1 mL of PBS. After the enzyme treatment, 500 µL of the working solution was added to each sample and incubated in the dark at room temperature for 30 minutes. After incubation, the samples were washed three times with PBS to remove excess stains and planktonic bacteria.

*Isolation of human neutrophils*

Work with neutrophils was approved by the Institutional Review Board at the University of Texas at Austin (Austin, TX) as Protocol No. 2021-00170.

Human neutrophils from two adult volunteer blood donors were isolated following a previously-published protocol [26, 27]. In brief, freshly drawn whole blood was mixed with an equal volume of a solution containing 3% dextran (Sigma-Aldrich, ACS Cas.# 9004-54-2, Denmark) and 1.8% sodium chloride (Fisher Scientific, ACS Cas.# 7647-14-6). After 20 minutes undisturbed, most red blood cells formed aggregates and dropped to the bottom of the tube. The supernatant was centrifuged (Eppendorf 5810R, A-4-62 Rotor) for 10 minutes at 500 x g to separate the remaining blood cells from the plasma. The supernatant, mainly containing plasma, was discarded, and the remaining pellet was suspended in 10 mL Hanks Buffered Salt Solution (HBSS, Gibco Laboratories, Cat.# 14175095, Frederick, USA) without calcium or magnesium. This suspension was slowly added to the top of 4 mLFicoll-Paque density gradient (GE Healthcare #17-1440-02, Uppsala, Sweden) and centrifuged for 40 minutes at 400 x g. The pellet was mixed with 4 mL of deionized water for 30 seconds to lyse the residual red blood cells, then stabilized by mixing with 4 mL of filter-sterilized 1.8% NaCl solution. Following 5 minutes of centrifugation at 500 x g, a white pellet of neutrophils was resuspended in 800 µL HBSS with calcium and magnesium and 200 µL human serum. Each lithium-heparin-coated tube of blood drawn yielded 1 mL of neutrophil-only solution. In experiments where more than one tube of blood was drawn, the resulting neutrophil solutions were thoroughly mixed into one tube by pipette.

For internal consistency within data sets, all *P. aeruginosa* and *E. coli* experiments were performed with blood from volunteer 1. All *S. aureus* experiments were performed with blood from volunteer 2.

*Phagocytosis assay*

Each sample was mixed with 200 µL of the isolated neutrophil solution (*see Isolation of human neutrophils*) in the 24-well plate and incubated at 37°C for 30 minutes. The supernatant in the wells was discarded by pipetting. The pellet at the bottom of each well was washed with 200 µL of HBSS with calcium and magnesium, then transferred and resuspended in a centrifuge tube. The tube was spun down for 3 minutes with a Mini centrifuge (Bioexpress, Max. speed 6,000 rpm). 150 µL of the resulting supernatant was removed, and the pellet was resuspended with the remaining supernatant. The resuspended liquid was transferred to an image spacer on a slide (Fisher Scientific, Cat.# 12-544-1, PA, USA) and covered with a coverslip (Fisher Scientific 12542B, PA, USA). The sample was then observed using confocal laser scanning microscopy (Olympus IX71 inverted confocal microscope, 60X oil-immersion objective), and analyzed with FluoView FV10-AWS version 04.02 from Olympus America. The fluorescence was visualized by overlaying bright-field image, red, and green fluorescence channels. Red fluorescence within a neutrophil indicates that one or more bacteria have been engulfed in the highly acidic phagosome (Fig. S1). We defined successful phagocytosis as a neutrophil containing at least one engulfed, and therefore red, bacterium. The phagocytic success rate was defined as the percent of neutrophils in a sample that contained at least one engulfed, red bacterium. At least 100 neutrophils were counted per sample and assessed for phagocytic success. The numbers of biological replicates and total cells counted are shown below in Table 1.

*Table 1. Numbers of biological replicates and neutrophils counted for phagocytosis assays on biofilms.*

| | | **WT PA01** | **PA01 Δ*pel*Δ*psl*** | **SA pJY209** |
|---|---|---|---|---|
| **Biological replicates** | | 4 | 4 | 3 |
| **Total neutrophils counted** | 0% collagen, untreated | 636 | 565 | 375 |
| | 0% collagen, + collagenase | 608 | 649 | 338 |
| | 10% collagen, untreated | 433 | 540 | 392 |
| | 10% collagen, + collagenase | 581 | 582 | 319 |
| | 20% collagen, untreated | 477 | 539 | 365 |
| | 20% collagen, + collagenase | 448 | 502 | 318 |

*Data Analysis*

Statistical significance was determined by Student T-tests and single-factor ANOVA tests in Microsoft Excel. The threshold for significance was set for p-values less than or equal to 0.05. The p-values for each dataset and statistical test are shown in Table 2.

*Table 2. p-values for statistical tests to assess the impact of growth with collagen and treatment with collagenase. The type of test used (ANOVA or Student T-test) is indicated for each row.*

| | **WT PA01** | | | **PA01 Δ*pel*Δ*psl*** | | | **SA pJY209** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Collagen concentration** | 0% | 10% | 20% | 0% | 10% | 20% | 0% | 10% | 20% |
| Effect of growth with collagen (ANOVA) Fig. 1 black * | NS | | | .003 | | | .003 | | |
| Effect of growth with collagen (Student T-test, compared with 0% collagen) Fig. 1 red * | | NS | .04 | | .02 | <.001 | | .008 | .007 |
| Effect of growth with collagen, normalized per day to 0% collagen (ANOVA) Fig. 2 black * | .01 | | | <.001 | | | .006 | | |
| Effect of growth with collagen, normalized per day to 0% collagen (Student T-test, compared with 0% collagen) Fig. 2 red * | | .05 | .007 | | <.001 | <.001 | | <.001 | .001 |
| Effect of collagenase (Student T-test, compared with no collagenase) Fig. 3 | NS | NS | NS | NS | .04 | NS | NS | <.001 | .01 |
| Effect of collagenase, normalized per day to no collagenase (ANOVA) Fig. 4 bracket * | NS | | | NS | | | .04 | | |
| Effect of Collagenase, normalized per-day to no collagenase (Student T-test, compared with unity) Fig. 4 red * | NS | <.001 | <.001 | NS | .002 | .004 | NS | .003 | .03 |
| Effect of collagenase, normalized per-day to no collagenase (Student T-test, compared with 0 % collagen + no collagenase) Fig. 4 black * | | NS | NS | | NS | .04 | | .006 | .02 |

*Preparation of alginate hydrogels*

Alginate gels were prepared by dissolving 4% sodium alginate (Sigma-Aldrich, Cat.# 180947) and 1% Bovine Serum Albumin (HyClone, GE Life Sciences Cat# SH30574.01) in deionized water. This solution was stirred constantly on a hot plate for at least one hour. Then, the solution was transferred to 24-well plates and mixed with fluorescent tracer beads (polystyrene Dragon Green beads, diameters 0.955 mm, Cat# FSDG004; Bangs Laboratories, Fishers, IN, USA) at a concentration of 1 µL tracer per 100 µL of alginate solution. The mixture is stored at 4°C overnight to form hydrogel samples. Information on replicate numbers and statistical outcomes for these experiments are given in Table 3.

*Preparation of Escherichia coli suspension*

We used the DH5α strain of *Escherichia coli*. The frozen stock was streaked onto sterile LB agar plates and grown overnight at 37°C. One colony was selected and cultured in 4 mL of LB liquid medium, shaking overnight at 37°C. The following day, the liquid culture was centrifugated at 10,000 x g, resuspended with 100 µL collagenase solution or PBS, and incubated for 1 hour. Then 500 µL of the pHrodo working solution (see *Staining biofilms with pHrodo*) was added to each sample and incubated at room temperature for 30 minutes. The stained samples were washed 3 times with PBS by centrifuging at 10,000 x g to remove the extra stain, resuspended in PBS, and diluted to an optical density (OD600) of 1.

After the dilution, 100 µL of the *E. coli* suspension was added to each well in a 24-well plate before the *phagocytosis assay* (described above). Information on replicate numbers and statistical outcomes for these experiments are given in Table 3.

*Table 3. Statistics for phagocytosis assays on hydrogels and bacterial suspensions.*

| | Alg. Untreated | Alg. + collagenase | *E. coli* untreated | *E. coli* +collagenase |
|---|---|---|---|---|
| Biological replicates | 3 | 3 | 3 | 3 |
| Technical replicates | 2 for each biological replicate | | | |
| Total neutrophils counted | 847 | 887 | 782 | 733 |
| Student t-test p-value | NS | | NS | |
| ANOVA p-value | NS | | NS | |

**Results and Discussion**

**The incorporation of collagen in biofilms impedes the engulfment of bacteria by neutrophils**

In general, both the bacterial cell membrane and the biofilm surface are typically anionic [43]; this will tend to promote the electrostatic attraction of cationic materials, of which collagen is one example. *P. aeruginosa* and *S. aureus,* specifically, are classic biofilm-forming model organisms that can produce a variety of extracellular polymers to form the matrices of their biofilms.

The major polysaccharide components in *P. aeruginosa* biofilms are negatively charged alginate, cationic Pel, and neutral Psl; each of these can be produced in varying amounts depending on the bacterial strain and incubation conditions [44]. Pel and Psl can play a role in antibiotic tolerance, and alginate protects the bacteria against antibiotics and immune response [14]. In addition to the wild-type (WT) *P. aeruginosa* PAO1 strain, we also used the PA01 Δ*pel*Δ*psl* strain that does not produce either Pel or Psl.

In *S. aureus* biofilms, polysaccharide intercellular adhesin (PIA), negatively charged extracellular DNA (eDNA), and proteins are the major components of the EPS matrix [45, 46], protecting the bacteria from the host immune system and stabilizing the biofilm structure [47].

We grew biofilms from *P. aeruginosa* PA01 wild type (WT), PA01 Δ*pel*Δ*psl*, and *S. aureus* SA pJY209 in the presence of three concentrations of collagen: 0%, 10%, and 20% in LB media. We have recently shown that, for multiple strains of *P. aeruginosa,* including both PA01 WT and PA01 Δ*pel*Δ*psl*, the incorporation of collagen decreases biofilm compliance and increases relative elasticity [14]. This may arise from collagen adhering to bacteria or entangling physically with the EPS matrix [14, 15].

Here, biofilms were stained with a pH-dependent dye and then incubated with freshly-isolated human neutrophils for 1 hour. Imaging with confocal laser fluorescence scanning microscopy allows us to identify bacteria that have been internalized into neutrophils' highly acidic phagosomes because they fluoresce in a different color from external bacteria, including those stuck to the surfaces of neutrophils (Fig. S1). For each sample, imaged fields of view were chosen at random until the number of analyzed neutrophils exceeded 100. Usually, 3-10 fields of view were analyzed for each sample, with each field of view containing 1-120 neutrophils. Phagocytic success is quantified by measuring the percentage of imaged neutrophils that contain bacteria. Representative images for each bacterial strain and collagen concentration are shown in Fig. S2 A-C.

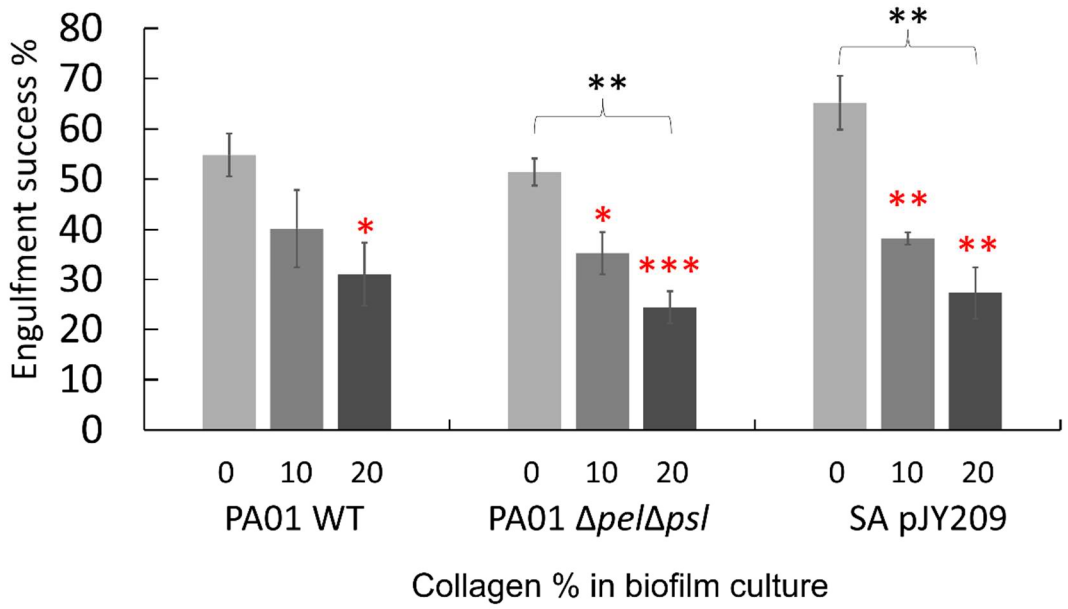


Figure 1. For neutrophils applied to biofilms for one hour, phagocytic success rates are negatively correlated with the concentration of collagen in which the biofilms were grown. This is true for biofilms grown from three different bacterial strains. Error bars represent the standard error of the mean. Statistically significant differences between the indicated groups of data are shown by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. Statistical markers in red indicate a comparison to the corresponding control (0% collagen for each bacterial strain). Statistical markers with brackets indicate overall comparison within a strain, for all three collagen concentrations. The p-value for single bars came from the Student t-test, and the overall p-value for each strain came from the ANOVA test. The replicate numbers and p-values (N) for each strain are shown in Tables 1 and 2. 3-4 biological replicates were measured for each condition.

In the absence of collagen, the average phagocytic success rate of neutrophils applied to biofilms of WT *P. aeruginosa* was 55%. However, this figure decreased to 40% and 31% when the biofilms were grown with 10% and 20% collagen, respectively (Fig. 1, left); this result was statistically significant for biofilms grown with 20% collagen. For neutrophils applied to biofilms of *P. aeruginosa* Δ*pel*Δ*psl* (which produces neither Pel nor Psl), the average phagocytic success rate declined from 51% (when biofilms were grown without collagen) to 35% and 24% when biofilms were grown in the presence of 10% and 20% collagen, respectively (Fig. 1, center); this result was statistically significant. For neutrophils applied to biofilms of *S. aureus*, the phagocytic success rate also declined from 65% (when biofilms were grown without collagen) to 38% and then to 27% with increasing collagen concentration in the biofilm growth medium (Fig. 1, right); this result was also statistically significant.

Our results suggest that in the two species of highly clinically relevant biofilm-forming bacteria, the incorporation of collagen into biofilms inhibits phagocytosis by neutrophils. In our experiments, 3 bacteria strains producing different EPS components, as summarized at the top of this subsection, were

tested [14, 44, 45]. The trend of decreasing phagocytic success with increasing collagen concentration is seen for all three strains tested.

To quantify the effects on phagocytic success upon the addition of 10% and 20% collagen, we report the data as a fold change, comparing to the success rate at 0% collagen (Fig. 2). Here, a fold change of unity represents no change. In contrast, a fold change greater or less than unity represents an increase or decrease, respectively, in phagocytic success. This normalization approach is intended to account for day-to-day variations in both biofilm properties and neutrophil efficacy, and it parallels day-by-day normalization approaches we have used in previous work [27].

In most cases, the sizes of day-to-day fluctuations in engulfment success are comparable to the sizes of average changes in engulfment success. In Fig. 2, each dotted line represents the fold changes measured on one day from one biological replicate, while solid lines represent the average of fold changes measured for different biological replicates on different days

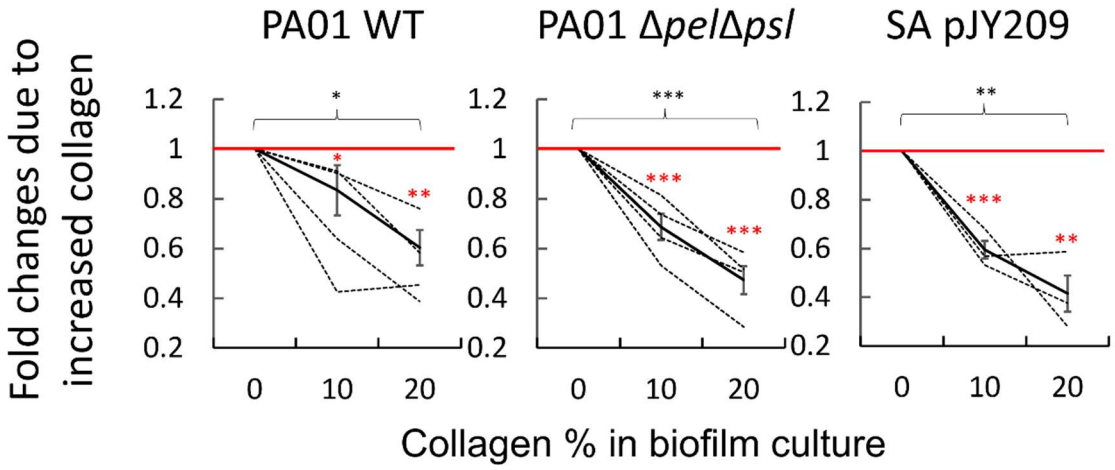


Figure 2. Collagen's effect on engulfment success, shown as fold changes from the control condition (0% collagen for each bacterial strain). Dashed lines represent individual biological replicates. Solid lines represent the average of replicates. The error bars represent the standard error of the mean. Statistical markers in red indicate comparing the fold change to unity (which would mean no change), using Student t-tests. Statistical markers with brackets indicate overall comparison within a strain, for all three collagen concentrations, using the ANOVA test. Statistically significant differences between the indicated groups of data are shown by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. The p-values and replicate numbers (N) for each strain are shown in Tables 1 and 2. 3-4 biological replicates were used for each tested condition.

Adding 10% collagen decreased the engulfment success rate of *P. aeruginosa* WT strain to 0.8 of its original value, that of Δ*pel*Δ*psl* strain to 0.8 of its original value, and that of *S. aureus* pJY209 strain to 0.6 of its original value. With 20% collagen, those fold changes became 0.6, 0.5, and 0.4, respectively. PA01 WT biofilms contain more EPS, in amount and variety, than do Δ*pel*Δ*psl* biofilms. This may be related to why incorporation of collagen, another polymer, has more impact on the engulfment success of Δ*pel*Δ*psl* biofilms than on that of WT. The ability of *S. aureus* to specifically bind to collagen may be related to why the incorporation of collagen has more impact on the engulfment success of *S. aureus* biofilms than on that of either *P. aeruginosa* strain [36].

**Collagenase treatments of biofilms promote neutrophil engulfment**

In our previous research, we demonstrated that enzymes that target the main polymer component of the biofilm matrix can compromise the biofilm's mechanical properties [48]. In light of our previous findings of a potential connection between biofilm mechanics and phagocytic success [26, 27] and that incorporating collagen into biofilms increases their relative elasticity and decreases their creep compliance [14, 15], we expect that enzymatically breaking down collagen within biofilms might enhance the success rate of phagocytosis of biofilm bacteria by neutrophils. To investigate this, we grew biofilms with different concentrations of collagen (0%, 10%, and 20%) and then treated the biofilms with collagenase before incubating them with neutrophils. We then measured phagocytic success as above. Representative images for each bacterial strain and collagen concentration are shown in Fig. S2 A-C. For the control groups, an equal volume of PBS was used instead of collagenase treatment. Each pair of test and control experiments were done on the same day, on the same biological biofilm, using neutrophils isolated on that day in the same batch.

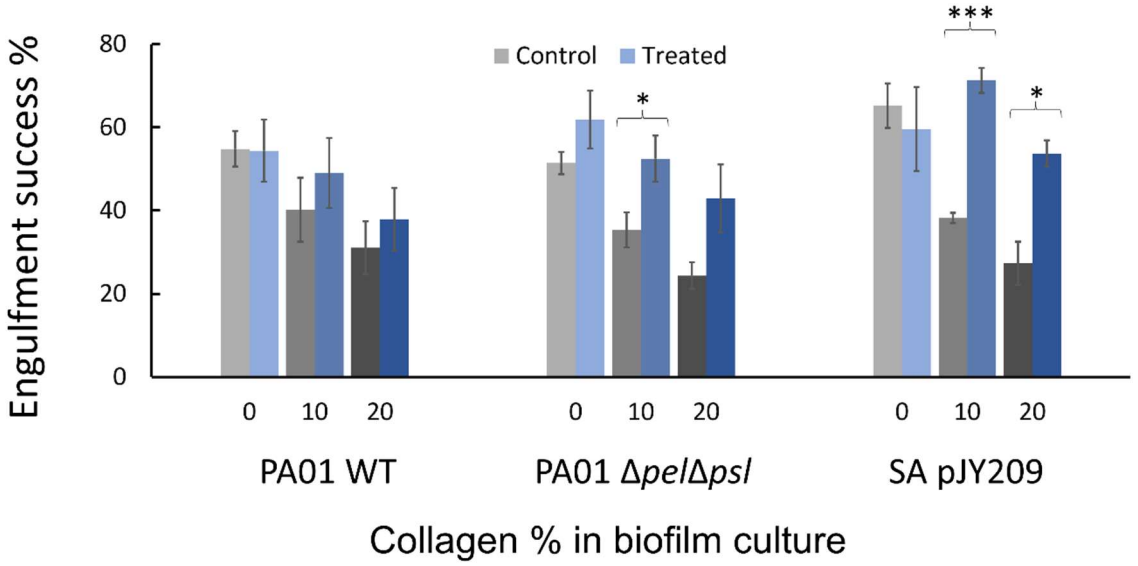


Fig 3. For biofilms grown with collagen, treatment with collagenase increased the phagocytic success rate. Significance markers represent a significant difference between the treated and control data in the indicated group. Statistically-significant differences between the indicated groups of data are shown by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. The p-value came from the student t-test. Error bars represent the standard error of the mean. The p-values and replicate numbers (N) for each strain are shown in Tables 1 and 2. 3-4 biological replicates were used for each condition.

In every case, the phagocytic success rate was increased by collagenase. For PA01 WT, collagenase treatment had no effect on the average phagocytosis success rate for biofilms without collagen. For biofilms with 10% collagen and biofilms with 20% collagen, collagenase treatment increased 9% and 7%, compared to their respective untreated control; however, these increases were not statistically significant (Fig. 3, left). For PA01 Δ*pel*Δ*psl* with 0%, 10%, and 20% collagen, collagenase treatment increased the average phagocytosis success rate by 10%, 17%, and 19%, respectively; however, only the increase for the biofilm with 10% collagen was statistically significant (Fig. 3, center). For *S. aureus* pJY209, treatment with collagenase increased the average phagocytic success rate by 11%, 42%, and

22% for biofilms grown with 0%, 10%, and 20% collagen, respectively; this increase was statistically significant for biofilms grown with 10% and 20% collagen. (Fig. 3, right).

The impact of collagenase treatment was quantified by calculating the fold change of the phagocytic success rate for each biological replicate at each collagen concentration, compared with the same biological replicate and collagen concentration treated only with PBS (no collagenase) (Fig. 4). A fold change of unity indicates no change, while a fold change greater (or less than) unity indicates an increase (or decrease) in phagocytic success. This approach normalizes the data to account for day-to-day fluctuations in the fold change associated with collagenase treatment. In many cases, the sizes of day-to-day fluctuations in the fold change are comparable to the sizes of the average fold changes; this can be seen by comparing dashed lines in Fig. 4, which each represent the fold change measured on one day for one biological replicate, with solid lines in Fig. 4, which represent the average of fold changes measured for different biological replicates on different days.

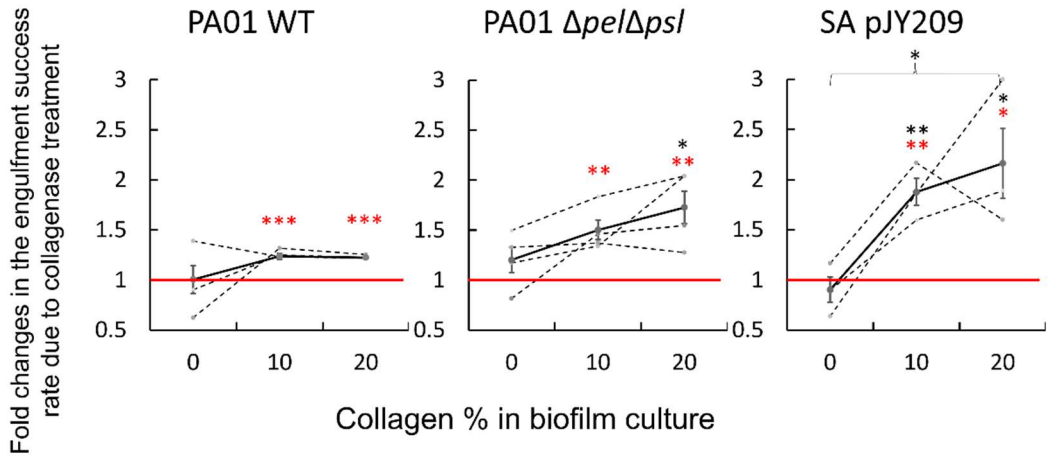


Fig 4. Fold changes in phagocytic success rates arising from treating biofilms with collagenase. Dashed lines represent individual biological replicates. Solid black lines represent the average of replicates. A red line is shown to indicate a fold change of unity, which corresponds to no effect of enzyme treatment. Above the data points, red statistical markers indicate a comparison to unity, and black statistical markers indicate a comparison to the biofilm with 0% collagen and no collagenase treatment; these used the Student t-test). For SA pJY209 (right), the statistical markers with the bracket indicate overall comparison by ANOVA test. Statistically significant differences are shown by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. The p-value between 2 groups came from the student t-test, and the overall p-value for each strain came from the ANOVA test. Error bars represent the standard error of the mean. The p-values and replicate numbers (N) for each strain are shown in Tables 1 and 2. 3-4 biological replicates were used for each condition.

For biofilms grown with 10% collagen, collagenase treatment increased the engulfment success rate of *P. aeruginosa* WT strain to 1.2 of its original value, Δ*pel*Δ*psl* strain to 1.5 of its original value, and *S. aureus* pJY209 strain to 1.9 of its original value. For biofilms grown with 20% collagen, those fold changes due to collagenase treatment became 1.2, 1.7, and 2.2, respectively. It should be noted that although the raw data for PA01 WT (Fig. 3, left) and Δ*pel*Δ*psl* with 20% collagen (Fig. 3, middle) did not show statistical significance, normalization of the day-to-day variation resulted in statistical significance (Fig. 4). For each

bacterial strain tested, collagenase treatment of biofilms grown with collagen resulted in an increased phagocytic success rate, with statistical significance (Fig. 4).

**Outside the biofilm context, collagenase does not impact phagocytic success**

Our result showed that for biofilms lacking collagen there were no statistically significant changes in phagocytic success induced by treatment with collagenase. This result aligns with our expectations, as collagenase does not break down the EPS in *S. aureus* and *P. aeruginosa* biofilms. However, prior studies have shown that collagenase can modify and regulate the bioactivity of chemokines upstream of Interleukin-8, thus possibly involved in the recruitment and activation of neutrophils[49, 50]. We did two sets of experiments to evaluate the impact of collagenase on the neutrophil activity in our experimental conditions, as follows:

To assess the effect of collagenase on neutrophils' extraction of sub-portions of a large target structure, we performed engulfment assays using alginate hydrogels that contained no collagen. Tracer beads that were incorporated into the alginate solution before it gelled were used to measure phagocytic success; this is the same approach we used in our earlier work [26, 27]. The hydrogels were subjected to collagenase treatment of the same dose as in the biofilm experiments and then incubated with fresh human neutrophils for 1 hour. Representative images are shown in Fig. S2 D. In this case, there was no statistically significant effect of collagenase treatment on the engulfment success rate (Fig. S3, left). This parallels our finding that, for biofilms grown without collagen, there is no statistically significant effect on phagocytic success (Fig. 4).

Further, to assess whether collagenase affects the interaction between neutrophils and live bacteria, engulfment experiments were conducted using a planktonic suspension of *E. coli* [51]. Mid-log phase cultures of *E. coli* were washed multiple times by centrifugation to minimize the remaining EPS in the suspension, then treated with collagenase of the same dose as above, then stained with pH-dependent dye, washed again by centrifugation to remove the excess dye, and incubated with neutrophils for one hour. The control groups were treated with equal volumes of PBS instead of collagenase. Representative images are shown in Fig. S2 D. The phagocytic success rate was evaluated as above. The results revealed that the addition of collagenase did not yield statistically significant changes in the engulfment success rate for planktonic bacterial cells (Fig. S3, right).

Thus, in collagen- and EPS-free systems, we do not find a statistically significant enhancement of phagocytic success by collagenase. Therefore, we conclude that collagenase's effect on enhancing the engulfment of biofilm bacteria primarily arises from the enzymatic digestion of collagen incorporated into the biofilm matrix rather than any direct biochemical influence on the neutrophils.

**The greater collagen's impact, the greater the opposing impact of collagenase**

The fold changes in phagocytic success due to incorporated collagen, as well as those due to treatment with collagenase, vary by roughly a factor of two across the three tested bacterial strains. It is reasonable to expect that the more strongly collagen impedes phagocytosis, the more strongly collagenase may increase phagocytosis. To assess the quantitative relationship between the reduction in phagocytic success due to collagen and the increase in phagocytic success due to collagenase, we plot the fold changes due to collagenase treatment versus the fold changes due to increased collagen. We observe an overall negative correlation between these quantities (Fig. 5). This correlation shows that the greater the

reduction in phagocytic success caused by collagen (smaller fold change value on the horizontal axis), the greater the enhancement in phagocytic success caused by collagenase (larger fold change values on the vertical axis). These trends are well-described by individual linear fits, shown as a red dashed line for biofilms grown with 10% collagen and as a purple dotted line for biofilms grown with 20% collagen; these fits have $R^2$ values of 0.96 and 0.97, respectively (Fig. 5, right). The slope of the linear fit for 20% collagen is almost twice that of 10% collagen, which indicates that a higher concentration of collagen not only inhibits phagocytosis more effectively but also makes biofilms consequently more susceptible to collagenase treatment.

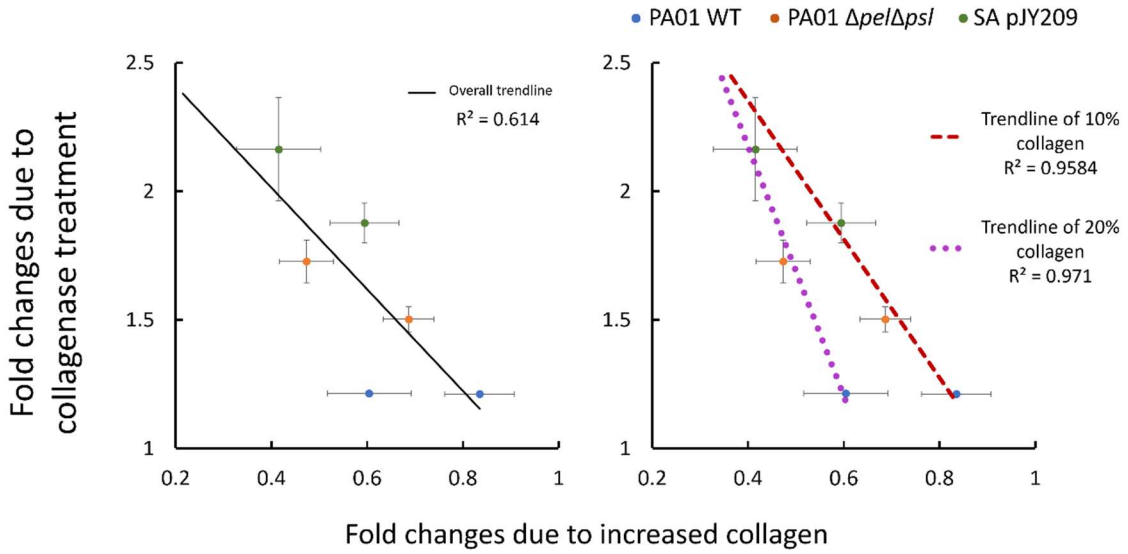


Fig. 5. Collagenase enhances phagocytosis more in cases where the reduction in phagocytic success caused by collagen incorporation is greater. This corresponds to higher fold changes on the vertical axis associated with lower fold changes on the horizontal axes. On the left, for a line fit to all the data overall, the equation of the line is $y = -1.98x + 2.80$. On the right, the data from biofilms grown with different collagen concentrations were fit separately. For 10% collagen, the red dashed fit line has equation $y = -2.69x + 3.43$. For 20% collagen, the purple dotted fit line has equation $y = -4.85x + 4.11$. For the two separately-fit lines in the right panel, $R^2 > 0.95$.

**Conclusion**

Bacterial biofilms are notorious for their ability to evade antibiotics and clearance by the immune system. Biofilms commonly cause persistent infections in many scenarios, including chronic wounds, cystic fibrosis lungs, periodontitis, urinary tract infections (UTIs), and recurrent ear, sinus, and bladder infections [20-25]. One common thing among these infection sites is the abundance of collagen in the connective tissues. Therefore, the ability of biofilms to incorporate host material, such as collagen in the wound bed, may be critically important to researchers developing methods to clear the infection. Here, we present an *in vitro* study of biofilms grown from two highly clinically relevant bacterial species, *P. aeruginosa* and *S. aureus*. We supplement the biofilm growth culture with three concentrations of collagen: 0%, 10%, and 20%. We find that the incorporation of collagen into the biofilm matrix leads to

decreased success of neutrophils at engulfing bacteria. As a corollary, we find that treatment of collagen-supplemented biofilms with collagenase results in increased phagocytic success.

These results suggest that the incorporation of collagen, and perhaps other host materials, into biofilm infections may contribute to biofilms' notorious evasion of immune clearance. These results further suggest that the degradation of incorporated host materials may compromise the biofilms and thereby enhance the efficacy of the innate immune system at clearing such biofilm infections. This could potentially reduce the need for antibiotic treatment and consequently slow the development of antibiotic resistance.

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