

Phagocytosis of *Escherichia coli* biofilm cells with different aspect ratios: a role of substratum material stiffness

Yanrui Zhao^{1,2} · Fangchao Song¹ · Hao Wang¹ · Junlin Zhou² · Dacheng Ren^{1,3,4,5} 

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Abstract Bacterial biofilms play an important role in chronic infections due to high-level tolerance to antibiotics. Thus, it is important to eradicate bacterial cells that are attached to implanted medical devices of different materials. Phagocytosis is a key process of the innate immunity to eliminate invading pathogens. Previous research demonstrated that the efficiency of phagocytosis is affected by the aspect ratio of polymer beads. Recently, we reported that the stiffness of polydimethylsiloxane (PDMS) influences *Escherichia coli* biofilm formation and the biofilm cells on stiff (5:1) PDMS are 46.2% shorter than those on soft (40:1) PDMS. Based on these findings, we hypothesized that *E. coli* cells attached on stiff PDMS can be more effectively removed via phagocytosis. This hypothesis was tested in the present study using viability

assays, flow cytometry, and cell tracking. The results revealed that shorter *E. coli* cells detached from stiff PDMS were easier to be phagocytized than the longer cells from soft PDMS surfaces. Furthermore, macrophage cells were found to be more motile on stiff PDMS surfaces and more effective at phagocytosis of *E. coli* cells attached on these surfaces. These results may help the design of better biomaterials to reduce fouling and associated infections.

Keywords Stiffness · Polydimethylsiloxane · Phagocytosis · Bacteria · Biofilm

Introduction

A total of 60–70% of hospital-acquired infections are associated with medical devices of different materials (Bryers 2008). These infections are normally persistent when bacteria produce an extracellular matrix and establish multicellular structures known as biofilms (Vergidis and Patel 2012). Due to the protection of the extracellular matrix and slow growth, biofilm cells can tolerate up to 1000 times higher concentrations of antibiotics than the planktonic cells of the same strain (Anwar et al. 1990; Costerton et al. 1987; Khoury et al. 1992; Olsen 2015; Dubois-Brissonnet et al. 2016; Martens and Demain 2017).

As a major mechanism of innate immunity, phagocytosis is a receptor-mediated immune response of macrophage cells against invading pathogens (Aderem and Underhill 1999). It plays an important role in stopping bacterial pathogens before a long-term infection can be established. During phagocytosis, macrophage cells recognize pathogens using surface receptors, engulf the pathogens into plasma membrane-derived phagosomes, and eventually eliminate the pathogens using multiple strategies (Tauber 2003; Underhill and Goodridge 2012). While phagocytosis of free-swimming bacteria is

Yanrui Zhao and Fangchao Song contributed equally.

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✉ Junlin Zhou
doctorzyr@163.com

✉ Dacheng Ren
dren@syr.edu

¹ Department of Biomedical and Chemical Engineering, Syracuse University, Syracuse, NY 13244, USA

² Department of Orthopedics, Beijing Chao Yang Hospital, Capital Medical University, Chaoyang District, Beijing 100020, China

³ Department of Civil and Environmental Engineering, Syracuse University, Syracuse, NY 13244, USA

⁴ Department of Biology, Syracuse University, Syracuse, NY 13244, USA

⁵ Syracuse Biomaterials Institute, Syracuse University, Syracuse, NY 13244, USA

well-documented, such defense against biofilm cells is not well-understood.

Bacteria adhesion and biofilm formation are influenced by material properties, such as surface charge, hydrophobicity, topography, stiffness, and surface chemistry (Song et al. 2015). Recently, we reported that *Escherichia coli* and *Pseudomonas aeruginosa* attach more on soft (40:1) polydimethylsiloxane (PDMS) surfaces than stiff (5:1) PDMS; we also demonstrated that cells attached to soft surfaces are longer (2.6 ± 0.07 vs 1.4 ± 0.03 μm) and more susceptible to antibiotics (Song and Ren 2014).

Previously, phagocytosis has been shown to be affected by the size and shape of the target particles. Champion and Mitragotri (2006, 2009) used polymeric particles with 2–3- μm diameters to represent bacterial cells with different sizes and shapes. The results showed that longer cells are less susceptible to phagocytosis. Doshi and Mitragotri (2010) also reported that the geometry of particles plays an important role in the recognition and clearance by macrophages, and membrane ruffles of macrophages are important to target recognition. Furthermore, Blakney et al. (2012) reported that stiff poly(ethylene glycol)-based hydrogels promote macrophages to spread and secrete a number of molecules involved in inflammation. These findings led to our hypothesis that material stiffness affects phagocytosis of attached bacterial cells. To test this hypothesis, *E. coli* was used as a model organism to study phagocytosis of attached cells during early-stage biofilm formation by the human macrophage cell line U937.

Materials and methods

Macrophage

The human monocyte macrophage cell line U937 (ATCC CRL1593.2, Manassas, VA, USA) was cultured in RPMI medium (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) and 5% CO_2 at 37 °C. To activate the macrophages, 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Cayman Chemical, Ann Arbor, Michigan, USA) was added to the cultures at least 15 h before experiments. The activated macrophages were released from the surface of T-flasks (Falcon, Corning, NY, USA) with ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Waltham, MA, USA) before being tested on biofilm cells.

Bacterial strains and growth conditions

E. coli RP437 (CGSC No. 12122) (Parkinson and Houts 1982), *E. coli* RP437/pBAD-mPlum, and *E. coli* RP437/pGLO (Hou et al. 2007) were used in this study. The plasmids of pBAD-mPlum (Addgene, plasmid no. 54565) and pGLO

(Bio-rad) allow the cells to produce purple and green fluorescences, respectively, induced by arabinose. Ampicillin and arabinose were supplemented at a concentration of 100 $\mu\text{g}/\text{mL}$ and 2 mg/L, respectively, to keep plasmids and induce the fluorescence genes. All strains were routinely grown at 37 °C in lysogeny broth (henceforth LB medium) (Hou et al. 2011) which contains 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl.

Preparation of PDMS surfaces

PDMS surfaces were prepared using SYLGARD184 silicone elastomer kit (Dow Corning Corporation, Midland, MI). The stiffness was adjusted by varying the mass ratio of base to curing agent as we described previously (Evans et al. 2009; Fuard et al. 2008; Wang 2011). Specifically, the base to curing agent ratios (*w/w*) of 5:1, 20:1, and 40:1 were tested in this study. To prepare PDMS, the elastomer base and curing agent were thoroughly mixed and degassed under vacuum for 40 min. Then, the mixture was added into 24-well plates (0.2 mL each) or 35 mm \times 10 mm petri dishes (0.5 mL each) (Thermo Fisher Scientific, Waltham, MA, USA), cured at 50 °C for 24 h, and incubated at room temperature for another 24 h to fully polymerize. Finally, the PDMS surfaces were sterilized by soaking in 200 proof ethanol for 20 min and dried at 50 °C for 1 h. All of the sterilized PDMS substrates were stored at room temperature until use.

Bacterial attachment and biofilm formation

E. coli cells from overnight cultures were harvested by centrifugation at 8000 rpm for 3 min at 4 °C, washed with fresh phosphate-buffered saline (PBS, pH 7.3) three times, and then resuspended in fresh PBS to desired cell density (Thermo Fisher Scientific, Waltham, MA, USA). Cell suspensions were added to 24-well plates or 35 mm \times 10 mm petri dishes containing sterilized 5:1, 20:1, or 40:1 PDMS surfaces, after a serial dilution ($\times 2$, $\times 4$, $\times 8$, $\times 16$, and $\times 32$) from a suspension with an optical density at 600 nm (OD_{600}) of 0.01. The biofilm samples were incubated at 37 °C for 2 h without shaking, then the PDMS surfaces with attached cells were gently washed three times with fresh PBS (changed to clean PBS for each step). After washing, the medium was changed from PBS to LB to allow the attached cells to grow for 5 h at 37 °C. Using different inoculum cell density allowed us to obtain similar numbers of attached cells on soft (40:1), medium (20:1), and stiff (5:1) PDMS surfaces to start biofilm growth, which was determined using the drop plate assay as described previously (Chen et al. 2003).

Effects of *E. coli* cell size on phagocytosis

Phagocytosis of *E. coli* cells was studied with two complementary approaches. In the first work flow, macrophages were

cultured in petri dishes in RPMI medium supplemented with 10% FBS and 5% CO₂ at 37 °C and the attached macrophages were activated by 100 ng/mL PMA for 15 h. *E. coli* cells after 5-h biofilm growth on PDMS with varying stiffness were released from surfaces by pipetting for 30 s and resuspending in 2 mL PBS. The size of these cells was examined using an inverted fluorescence microscope (AX10, Carl Zeiss, Berlin, Germany). Then, the cells were concentrated using a filter plate (Pall Corporation, Ann Arbor, MI, USA) and resuspended in 300 µL PBS. The *E. coli* cells were added to activate macrophages and incubated at 37 °C for 90 min in 1 mL RPMI medium supplemented with 10% FBS and 5% CO₂. After incubation, the cells were harvested by pipetting for 30 s and the numbers of viable *E. coli* cells before and after incubation were determined by counting CFU to evaluate the efficiency of phagocytosis. *E. coli* cells incubated in 1 mL RPMI medium supplemented with 10% FBS but without macrophages were used as control. In the second workflow, each PDMS with 5-h biofilm cells was washed three times with PBS. Then, the biofilm cells were challenged with 10⁴ cells/mL macrophages in 3 mL RPMI medium supplemented with 10% FBS and 5% CO₂ at 37 °C for 90 min to evaluate phagocytosis of surface-attached cells. The bacterial cells incubated in 3 mL RPMI medium supplemented with 10% FBS but without macrophages were used as control.

Effects of PDMS stiffness on phagocytosis

Macrophages were cultured on PDMS surfaces with different stiffness in RPMI medium supplemented with 10% FBS and 5% CO₂ at 37 °C, and the attached macrophages were activated by 100 ng/mL PMA for 15 h. *E. coli* cells from an overnight culture were added to activated macrophages at 10⁵ cells/mL and incubated in 1 mL RPMI medium supplemented with 10% FBS and 5% CO₂ at 37 °C for 90 min. After incubation, the cells were harvested by pipetting for 30 s. The numbers of viable *E. coli* cells before and after incubation were determined by counting CFU to evaluate the efficiency of phagocytosis. *E. coli* cells incubated in 1 mL RPMI medium supplemented with 10% FBS on different stiffness of PDMS surfaces but without macrophages were used as controls.

Tracking macrophage movement on different PDMS

After activating macrophage for 15 h with PMA, *E. coli* cells were added to study phagocytosis as described above but the cells were incubated in a custom-made flow cell chamber to allow direct monitoring of phagocytosis and the movement of macrophages. The chamber has two compartments: one (7 cm × 4 cm × 1 cm) is filled with water and another (7 cm × 7 cm × 1 cm) is for holding samples under a glass cover. The water temperature was controlled at 37 °C using a

heating coil; and 5% CO₂ was purged into the water before entering the culture compartment. An inverted fluorescence microscope (AX10, Carl Zeiss, Berlin, Germany) was used to monitor phagocytosis and the movement of macrophages in the chamber (Fig. S1). The cell tracks were generated from the acquired images using ImageJ.

Effects of PDMS stiffness on the activity of macrophages

To study the effects on macrophages specifically, U937 cells were cultured in RPMI medium supplemented with 10% FBS and 5% CO₂ at 37 °C on different PDMS surfaces (5:1, 20:1, and 40:1) for 1 day first. Then, the macrophage cells were activated with 100 ng/mL PMA for 15 h; *E. coli* cells from an overnight culture were dispersed into 1 mL PBS by pipetting for 30 s and added to the cultures containing activated macrophages. After incubation for 90 min, the macrophages and bacterial cells on PDMS surfaces were released by pipetting for 30 s. The number of viable *E. coli* cells after incubation was determined by counting CFU. The macrophages in the co-culture were harvested by centrifugation at 125×g for 5 min and resuspended in PBS. The number of macrophages that had uptaken bacteria was determined using flow cytometry (FACSaria II SORP, BD Biosciences). To corroborate the results, phagocytosis on different PDMS surfaces was also directly monitored using an inverted fluorescence microscope (AX10, Carl Zeiss, Berlin, Germany). At least five images were randomly taken from each sample to count the total number of macrophages and the number of macrophages that were associated with *E. coli* cells.

Statistical analysis

All data are presented as mean ± standard deviation. Statistical significance was assessed with one-way ANOVA followed by Tukey test or Pearson correlation analysis. Statistical significance was set as $p < 0.05$. All the analyses were performed using SAS 9.4 software (SAS Institute, Cary, NC, USA).

Results

E. coli cells attached on stiff PDMS are more susceptible to phagocytosis

This experiment was conducted by adding U937 cells to *E. coli* RP437/pGLO biofilms on different PDMS surfaces. The results show that 94.0 ± 2.5% *E. coli* biofilm cells on stiff (5:1) PDMS were killed by macrophages based on CFU data, while only 59.0 ± 1.3% *E. coli* biofilm cells on soft (40:1) PDMS were killed ($p = 0.007$, one-way ANOVA; Fig. S1). Although the initial cell density was the same on soft and stiff surfaces, the number of 5-h biofilm cells on soft surfaces was

much higher than that on stiff surfaces (10^7 vs 10^5 cells/mL), likely due to faster growth on soft PDMS, as we reported previously (Song and Ren 2014). Thus, the ratio of biofilm cells to macrophages on stiff surfaces was lower than that on soft surfaces. To further evaluate the effects of PDMS stiffness without the confounding factor of *E. coli* cell density, the cell densities of 5-h biofilm cells on soft and stiff PDMS surfaces were controlled to be the same by adjusting the initial cell density in the inocula. We found that inoculum densities of 1.0×10^7 , 1.3×10^6 , and 3.1×10^5 cells/mL *E. coli* RP437/pGLO led to similar biofilm cell density after 5 h of incubation on 5:1, 20:1, and 40:1 PDMS surfaces (all around 1.0×10^4 cells/cm²). As shown in Fig. 1, the number of viable *E. coli* RP437/pGLO cells decreased due to phagocytosis and the killing effects increased when PDMS got stiffer; e.g., $83.8 \pm 3.4\%$ on 5:1 PDMS, $71.4 \pm 3.1\%$ on 20:1 PDMS, and $63.3 \pm 4.4\%$ on 40:1 PDMS ($r = 0.94$, $p < 0.001$, Pearson correlation analysis).

Corroborating the CFU results with biofilm imaging

As shown in Fig. 2, the percentages of positive macrophage cells (associated with *E. coli* cells) based on microscopy were 10.4 ± 1.5 , 7.2 ± 0.8 , and $5.4 \pm 0.6\%$ on 5:1, 20:1, and 40:1 PDMS, respectively, indicating that phagocytosis increased with PDMS stiffness ($r = 0.92$, $p < 0.0001$, Pearson correlation analysis). This is consistent with the CFU results and supports our hypothesis that phagocytosis is more effective on stiff PDMS under our experimental condition.

Similar results were observed with *E. coli* RP437/pBAD-mPlum cells

Flow cytometry was used to quantify the number of macrophages involved in phagocytosis. However, U937

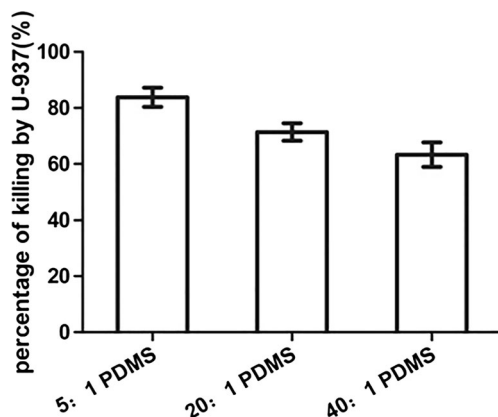


Fig. 1 Phagocytosis of 5-h *E. coli* RP437/pGLO biofilm cells on 5:1, 20:1, or 40:1 PDMS surfaces. The graph shows the percentage of killing after incubating with U937 for 90 min in RPMI medium supplemented with 10% FBS at 37 °C and 5% CO₂ ($r = 0.94$, $p < 0.001$, Pearson correlation analysis)

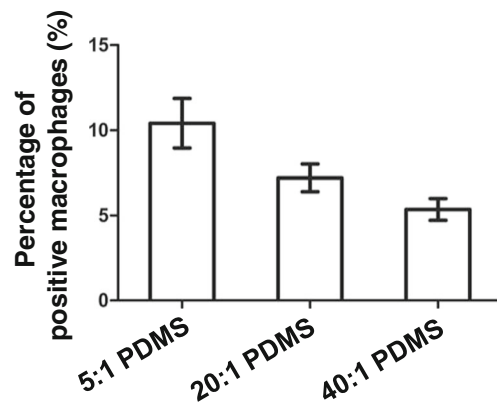


Fig. 2 Percentage of positive macrophage cells (associated with *E. coli* cells). The graph shows the percentage of positive U937 cells under microscopy after incubating with 5-h *E. coli* RP437/pGLO biofilm cells for 90 min in RPMI medium supplemented with 10% FBS at 37 °C and 5% CO₂ ($r = 0.92$, $p < 0.0001$, Pearson correlation analysis)

macrophage cells were found to have green autofluorescence which interferes with the fluorescence signal from *E. coli* RP437/pGLO. To address this challenge, we constructed another strain, *E. coli* RP437/pBAD-mPlum, to detect *E. coli* cells based on the mPlum, which does not overlap with the GFP spectrum. As shown in Fig. S2, the number of viable *E. coli* RP437/pBAD-mPlum cells also decreased due to phagocytosis and the killing effects increased as PDMS got stiffer, e.g., $81.7 \pm 9.2\%$ on 5:1 PDMS, $70.1 \pm 5.1\%$ on 20:1 PDMS, and $61.4 \pm 18.8\%$ on 40:1 PDMS ($r = 0.63$, $p = 0.0019$, Pearson correlation analysis). The results of positive macrophages (associated with *E. coli* cells) were 10.9 ± 3.0 , 7.0 ± 0.8 , and $4.8 \pm 1.2\%$ on 5:1, 20:1, and 40:1 PDMS, respectively ($r = 0.83$, $p < 0.001$, Pearson correlation analysis; Fig. S3). It was also found that macrophages were more spread on stiff PDMS, and membrane extensions of pseudopods were visible. The *E. coli* cells on stiff PDMS (5:1) surfaces were smaller than those on soft PDMS (40:1) surfaces, consistent with our previous report (Song and Ren 2014).

Phagocytosis analyzed with flow cytometry

Flow cytometry was used to corroborate the results of CFU and microscopy. In principle, three populations were obtained: *E. coli*, U937, and U937 associated with *E. coli* cells. The cells of *E. coli* RP437/pBAD-mPlum have far-red fluorescence that does not overlap with the autofluorescence of U937 cells. Thus, the three populations can be differentiated based on size, morphology, and fluorescence (plum from *E. coli* cells and background green autofluorescence from U937). As shown in Fig. 4, the phagocytosis rates (the amount of macrophages associated with *E. coli* cells) were 12.4 ± 4.1 ,

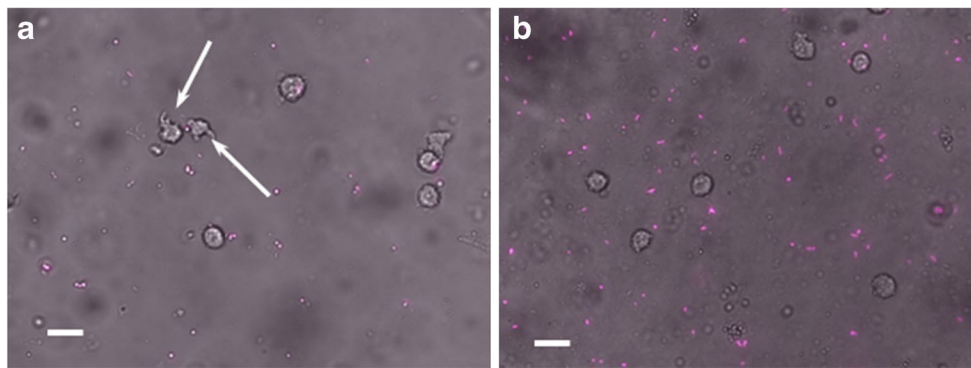


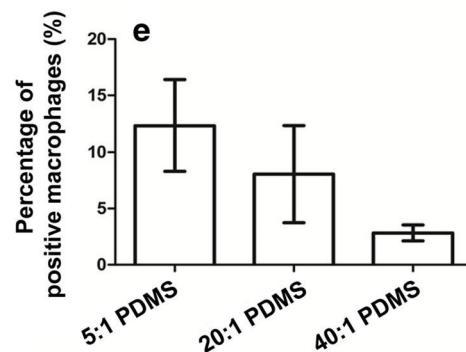
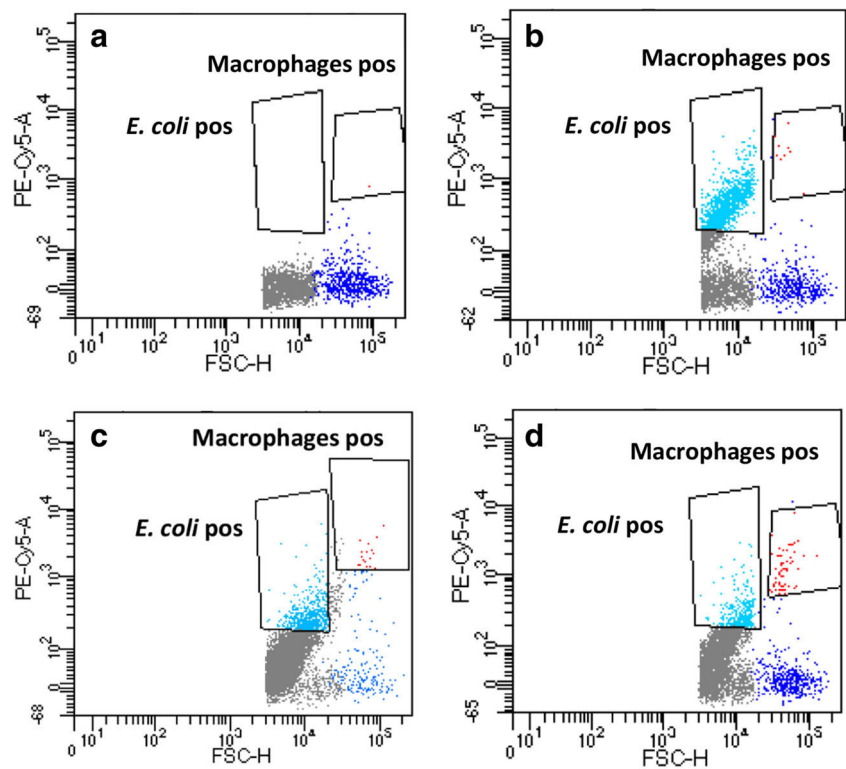
Fig. 3 Microscope images of *E. coli* RP437/pBAD-mPlum cells and U937 cells on 5:1 (a) and 40:1 (b) PDMS surfaces. U937 cells were added to 5-h *E. coli* RP437/pBAD-mPlum biofilm cells on 5:1 or 40:1

PDMS surfaces after 90 min of incubation in RPMI medium supplemented with 10% FBS at 37 °C and 5% CO₂. Arrows indicate macrophage cell membrane extensions. Bar = 20 μm

8.1 ± 4.3, and 2.8 ± 0.7% on 5:1, 20:1, and 40:1 PDMS surfaces, respectively ($r = 0.64$, $p < 0.001$, Pearson

correlation analysis). This is consistent with the results of microscopy.

Fig. 4 Flow cytometry analysis of phagocytosis of 5-h biofilm cells. The plots show the macrophage control (a) and the cells of *E. coli* and U937 on 40:1 PDMS (b), 20:1 PDMS (c), and 5:1 PDMS (d). e The percentage of positive macrophage cells (associated with *E. coli* cells) after incubating with *E. coli* RP437/pBAD-mPlum biofilms for 90 min in RPMI medium supplemented with 10% FBS at 37 °C and 5% CO₂ on 5:1, 20:1, or 40:1 PDMS surfaces ($r = 0.64$, $p < 0.001$, Pearson correlation analysis)



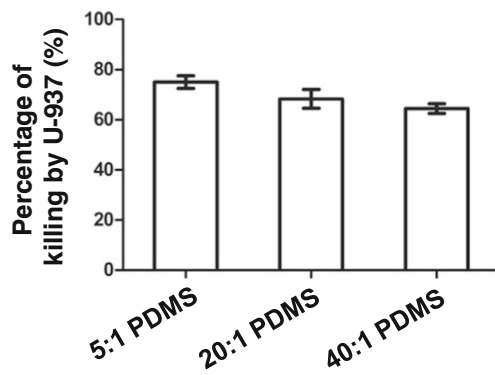


Fig. 5 Phagocytosis of detached 5-h *E. coli* RP437/pBAD-mPlum biofilm cells. The graph shows the percentage of killing after incubating with macrophage U937 (attached on 5:1, 20:1, or 40:1 PDMS surfaces first) for 90 min in RPMI medium supplemented with 10% FBS at 37 °C and 5% CO₂ ($r = 0.62$, $p = 0.07$, Pearson correlation analysis)

Effects of *E. coli* cell size on phagocytosis

To understand if the biofilm matrix has effect on the observed difference in phagocytosis, we also conducted a test with macrophages attached on different PDMS surfaces first. We then added *E. coli* cells detached from biofilms on PDMS surfaces with varying stiffness. This allowed us to study the effects of *E. coli* cell size without the influence of the biofilm matrix. As shown in Fig. 5, the number of viable *E. coli* RP437/pBAD-mPlum cells decreased due to phagocytosis and the killing effects increased with PDMS stiffness, e.g., 75.0 ± 2.5% on 5:1 PDMS, 68.3 ± 3.8% on 20:1 PDMS, and 64.4 ± 1.9% on 40:1 PDMS ($r = 0.62$, $p = 0.07$, Pearson correlation analysis). As shown in Fig. 6, the percentages of positive macrophages were 13.5 ± 1.0% on 5:1 PDMS, 8.9 ± 1.9% on 20:1 PDMS, and 7.5 ± 0.8% on 40:1 PDMS ($r = 0.92$, $p < 0.0001$, Pearson correlation analysis). This is consistent with the above results

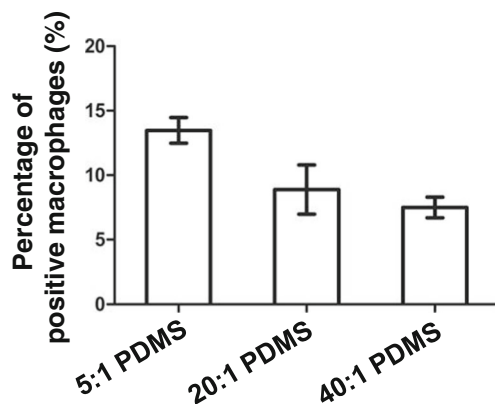


Fig. 6 Percentage of positive macrophage cells (associated with *E. coli* cells) after incubating with detached 5-h *E. coli* RP437/pBAD-mPlum cells. U937 cells were attached on 5:1, 20:1, or 40:1 PDMS surfaces for 90 min in RPMI medium supplemented with 10% FBS at 37 °C and 5% CO₂ on 5:1, 20:1, or 40:1 PDMS surfaces ($r = 0.92$, $p < 0.0001$, Pearson correlation analysis)

of direct phagocytosis of attached *E. coli* cells in biofilms, supporting the finding that biofilm cells on stiff PDMS are more susceptible to phagocytosis, both with and without the biofilm matrix.

To further corroborate the results, we also performed a study where macrophages were attached on the same surfaces of petri dish, followed by the addition of *E. coli* cells detached from 5-h biofilms on PDMS surfaces with varying stiffness. As shown in Fig. S4, the number of viable *E. coli* RP437/pBAD-mPlum cells decreased due to phagocytosis and the killing effects increased when the size of *E. coli* decreased, e.g., 88.8 ± 1.3, 80.0 ± 4.0, and 77.8 ± 5.6% of *E. coli* RP437/pBAD-mPlum cells from 5:1, 20:1, and 40:1 PDMS, respectively ($r = 0.80$, $p = 0.0095$, Pearson correlation analysis). Although the differences are rather moderate, this result shows that the *E. coli* cell size does affect phagocytosis and cells attached on stiff surfaces are more susceptible.

Effect of PDMS stiffness on the activity and movement of macrophages

To evaluate the effects of PDMS stiffness on macrophages more specifically, we allowed macrophages to attach on the PDMS surfaces with different levels of stiffness and then added *E. coli* cells from an overnight culture (to have the same cell size). As shown in Fig. 7, the number of viable RP437/pBAD-mPlum cells decreased due to phagocytosis and the killing effects increased when the PDMS surfaces got stiffer, e.g., 79.5 ± 5.0% on 5:1 PDMS, 69.7 ± 3.0% on 20:1 PDMS, and 63.1 ± 4.7% on 40:1 PDMS ($r = 0.89$, $p = 0.0014$, Pearson correlation analysis). From the images, we also observed that macrophage cell membrane extension spread longer on stiff PDMS (5:1) surfaces than on soft PDMS (40:1) surfaces (Fig. 3a). Using a custom-built chamber, we also monitored *E. coli* and

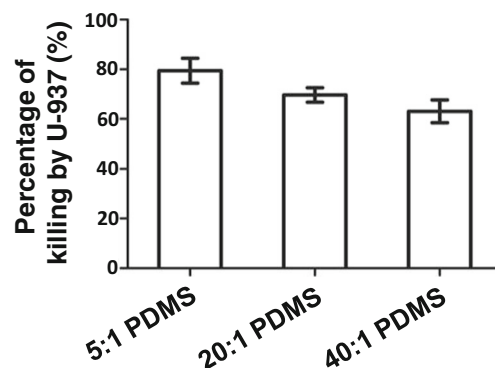


Fig. 7 Effects of PDMS stiffness on the activity of macrophage U937 cells. The graph shows the killing of *E. coli* RP437/pBAD-mPlum from overnight cultures. U937 cells were attached on 5:1, 20:1, or 40:1 PDMS surfaces in RPMI medium supplemented with 10% FBS at 37 °C and 5% CO₂ ($r = 0.89$, $p = 0.0014$, Pearson correlation analysis)

U937 cells in real time during phagocytosis. By taking images every 3 min for 3 h, the movement of macrophages was tracked. As shown in Fig. 8, it was found that macrophages move faster on the stiff (5:1) PDMS, compared to the soft (40:1) PDMS (0.6 vs 1.3 $\mu\text{m}/\text{min}$, $p = 0.005$, one-way ANOVA adjusted by Tukey test).

Discussion

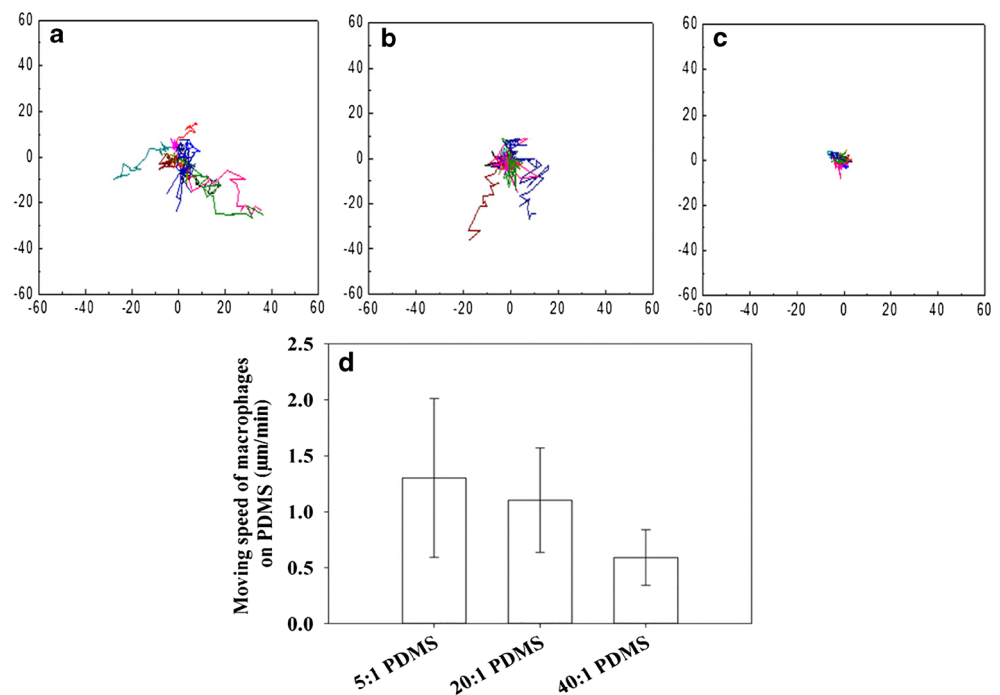
Recently, we reported that the early-stage biofilm cells on stiff PDMS (5:1) surfaces have lower aspect ratio, grow slower, and are less susceptible to antibiotics compared to those on soft (40:1) PDMS surfaces (Song and Ren 2014). This finding shows that material stiffness has profound impacts on the physiology of attached bacterial cells. To understand if such effects play a role in the interaction between bacteria and host cells, we conducted this study to investigate phagocytosis of early-stage biofilm cells on soft and stiff surfaces. Previously, Champion and Mitragotri (2009) showed that the IgG-opsinized poly(lactide-co-glycolide) (PLGA) particles with low aspect ratio (AR) are more easily internalized by macrophages. Because early-stage *E. coli* biofilm cells on stiff PDMS exhibited low AR, we hypothesized that early-stage *E. coli* biofilm cells on stiff PDMS will be uptaken more than those on soft PDMS. The obtained results support this hypothesis since more *E. coli* biofilm cells on stiff PDMS were killed by macrophages than those on soft surfaces. Further experiments revealed that *E. coli* cell size does affect phagocytosis. In addition to size and shape, the type and abundance of

surface appendages and proteins may also differ between cells on stiff and soft surfaces. These may also play a role in phagocytosis and thus deserve further study. This is part of our ongoing work.

In addition to biofilm cells, we found that macrophages on stiff PDMS surfaces were more active than on soft PDMS surfaces. The macrophages on stiff PDMS surfaces moved faster and showed longer movement tracks, and the cells were more spread than those on soft PDMS surfaces. These results are consistent with the report of Blakney et al. (2012) that the macrophages on stiff PEG hydrogel (840 kPa) are more spread than those on soft PEG hydrogel (130 kPa) and the report of Adlerz et al. (2016), which showed that the human monocyte-derived macrophages move faster on stiff polyacrylamide hydrogel (280 kPa) than soft polyacrylamide hydrogel (3 kPa), although the materials and the ranges of stiffness are different from our study. While previous works on the effects of surface stiffness on macrophages focus on hydrogels, our results show that macrophages are also more active in the presence of *E. coli* biofilm cells on hydrophobic PDMS surfaces.

By testing phagocytosis in complementary experiments, we found that the higher activity of phagocytosis of biofilm cells on stiff PDMS is likely attributed to two factors: small cells of *E. coli* and higher motility of macrophages. As shown in Fig. S4, macrophages in petri dishes internalized the relatively short *E. coli* cells detached from stiff (5:1) PDMS faster than the longer ones detached from soft (40:1) PDMS. The results in Figs. 5, 6, and 8 showed that macrophages are also more active on stiff PDMS than soft ones. Based on these findings, we speculate that bacterial infections associated with

Fig. 8 Tracking U937 cells during phagocytosis of *E. coli* RP437/pBAD-mPlum on PDMS surfaces with different levels of stiffness. **a–c** The moving tracks of U937 cells on stiff (5:1; **a**), medium (20:1; **b**), and soft (40:1; **c**) PDMS surfaces. All cell tracks are shown with the same starting point for the convenience in comparison. **d** The moving speeds of U937 cells (5:1 PDMS vs 40:1 PDMS, $p = 0.005$, one-way ANOVA adjusted by Tukey test). The cells were tracked for 3 h.



implanted biomaterials can be better controlled by optimizing material stiffness. Since PDMS is commonly used in medical devices, it is plausible to test this hypothesis.

Beyond the different physiology of biofilm cells on soft and stiff PDMS surfaces, why and how the macrophages respond to them differently are also interesting. Phagocytosis is highly dependent on surface receptors. For example, the IgG-opsionized polymeric particles used in previous studies are recognized by Fc receptors of macrophage (Champion and Mitragotri 2006, 2009) but the recognition of bacterial pathogens by macrophages is based on complement receptors. What receptors are used by macrophage cells to internalize biofilm cells and how material stiffness affects the receptor binding and internalization rate are important questions for further investigation. In addition, because macrophages produce secretory mediators after binding to bacterial pathogens, it will be interesting to investigate the amount of secretory mediators, such as IL-1 and TNF- α , involved in the phagocytosis of biofilm cells on soft and stiff PDMS surfaces and the contribution of secretory mediators to the higher phagocytosis rate of biofilm cells on stiff surfaces.

In summary, we demonstrated in this study that the stiffness of substrate material can affect the phagocytosis of attached bacterial cells. Specifically, increase in the stiffness of PDMS (40:1, 20:1, and 5:1 PDMS surfaces were studied) led to higher efficiency of phagocytosis of *E. coli* biofilm cells. The effects were attributed to higher motility of macrophages and smaller size of bacterial cells on stiff surfaces compared to soft ones. Collectively, the results from this study indicate that material stiffness is an important factor that affects the interaction between bacteria and host cells and thus, it may be possible to develop better biomaterials by tailing the mechanical properties to reduce fouling and/or to promote the antimicrobial activities of host immune cells.

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Compliance with ethical standards This article does not contain any study with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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