Chemotactic Behavior of Bacteria Propelled Microbeads in Linear Microfluidic Gradients

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Abstract:

Flagellated bacteria have been embraced by the micro-robotics community as a highly efficient microscale actuation method, capable of converting chemical energy into mechanical actuation for microsystems that require a low payload and high rate of actuation. Along with being highly motile, *Serratia marcescens* (*S. marcescens*), our bacterium species of interest, is a highly agile biomotor capable of being steered via chemotaxis. In this paper, we used a biohybrid system of *S. marcescens* bacteria attached to polystyrene beads to create microrobots that can propel themselves towards a chemoattractant source. Using a three-channel linear chemical gradient generator, we compared the behaviors of bacteria-propelled beads in the presence and absence of a chemoattractant, L-aspartate. Additionally, we tested and compared the behavior of three different bacteria-attached bead sizes, and noted their behavioral differences. By using a particle tracking algorithm, we analyzed the behavior of each bead: the results indicate that in the presence of a chemoattractant, the *S. marcescens*-attached polystyrene beads exhibit a clear indication of directionality and steering control through the coordination of the bacteria present on each bead. Moreover, this directionality exists in all bead size cases.

Keywords:

*S. marcescens, micro-robotics, chemotaxis, bacterial propulsion*
1. Introduction

The recent prominence of miniature mobile robots has been the result of a burgeoning number of developments in the field of micro-robotics. Many envision micro-robots being able to carry out tasks in small spaces that large robots would otherwise be unable to execute successfully. However, the miniaturization of existing systems can exhibit bottlenecks when it comes to creating viable actuation and power sources necessary for effective and efficient mobility at the microscale (Sitti 2009).

A number of actuation techniques have been proposed to enable the development of non-tethered micro-robots. The first of two methodologies utilizes external sources for actuation and power. For example, one group developed an electrostatically actuated robot utilizing a patterned substrate to move on (Donald et al. 2006), while another two groups created an untethered power source with an external magnetic field that can be modulated to produce actuation (Pawashe et al. 2009; Yesin et al. 2006). However, these micro-robots can only operate within a limited workspace and require bulky and expensive external instrumentation. The second, more promising methodology, utilizes biomotors for on-board actuation. With simple nutrients such as glucose, biomotors are capable of converting chemical energy to mechanical energy with high efficiency. More importantly, scientists can exploit the integrated sensors already present within the cell to control cell movement over a large area.

*Serratia marcescens* (*S. marcescens*) is a flagellated bacterium species that is highly agile and a power efficient swimmer at very low Reynolds numbers. Moreover, the sensors built within the bacterium allow for steering via chemotaxis or phototaxis (Steager et al. 2007). The use of *S. marcescens* for micro-scale manipulation and actuation has been well documented in a variety of applications. For example, *S. marcescens* was used to accomplish active mixing by adsorbing swarmer cells onto PDMS (polydimethylsiloxane) surfaces, and also to achieve propulsion of microscale objects by attaching them to the microobject surface (Darnton et al. 2004). With an ultraviolet (UV) light source, *S. marcescens* was also able to accomplish active mixing in microfluidic channels, as well as propulsion of PDMS barges by the same bacteria (Steager et al. 2007; Kim and Breuer 2007) via phototaxis. Behkam and Sitti explored the behavior of bacteria-propelled objects of various geometries in a static fluid, in addition to investigating a chemical switching method to establish on/off control of the bacteria-propelled objects (Behkam and Sitti 2007; Behkam and Sitti 2008). Moreover, in order to reduce opposing bacteria forces attached to each micro-object, they proposed chemical and physical patterning techniques to allow for controlled bacteria adhesion in order to achieve higher speeds and more directional motion trajectories (Behkam and Sitti 2008; Behkam and Sitti 2009).

This paper investigates the chemotactic steering behavior and the increased directionality of *S. marcescens* propelled micro-beads when placed in a microfluidic channel enclosure that produces a uniformly linear chemical gradient. Samples of three different bead sizes were taken to compare differences in bacteria-attached bead behavior, both with and without the presence of the chemoattractant, L-aspartate. Using a tracking algorithm, the displacement of each bead was recorded and analyzed to determine displacement, speed, and directionality results.
2. Methods and Materials

2.1 Bacteria Cultivation

The bacteria, *S. marcescens* (ATCC 274, American Type Culture Collection, Manassas, VA), were first grown in nutrient broth (25 g Difco LB Miller Broth and 1 L deionized (DI) water, pH 7.0) for 4 hours at 37 °C. A 2 µL aliquot was placed on one end of a nutrient agar plate (25 g Difco LB Miller Broth, 6 g Bacto Agar, 5 g glucose, and 1 L DI water, pH 7.0) and incubated at 30 °C for 14 to 18 hours (Kim et al. 2010). After bacteria swarming behavior developed, the inoculation site generally contained a pink pigmentation, exclusive to *S. marcescens*. The swarming colony spanned across the plate with the most motile bacteria faintly pigmented and located along the leading edge of the swarm (Fig. 1).

2.2 Polystyrene Bead Preparation

An aliquot of Polystyrene (PS) beads (Fluka, Sigma-Aldrich, Germany) was diluted in DI water 5 times. Because these beads are pre-coated with a surfactant, we performed a cleaning procedure to remove the surfactant and allow the bacteria to bind to the bead surface. The suspension was centrifuged and resuspended in 1:1 DI water/Isopropyl alcohol (50% IPA). The wash procedure was repeated five times to ensure removal of the adsorbed surfactant (Behkam and Sitti 2008). After the cleaning sequence, the beads were placed into 1 ml of motility medium (0.1 M potassium phosphate tribasic, 10^{-4} M ethylenediaminetetraacetic acid (EDTA), 0.067 M sodium chloride, 0.01 M glucose, and 0.002% Tween-20, pH 7.0) (Adler and Templeton 1967). The beads were then concentrated five-fold. This preparation method was performed for all 5, 10, and 20 µm diameter beads.

A 10 µl aliquot of the final suspension was pipetted into the leading edge of the swarm plate. The sample was incubated at room temperature for 5 minutes. During this time, bacteria randomly interact with the beads and some adhere to the beads through hydrophobic interactions at an approximate density of 1 bacterium/10 µm^{2} (Behkam and Sitti 2009). After 5 minutes, the aliquot was pipetted back into 1 ml of motility medium (Fig. 2), and the resulting solution is ready for observation.

2.3 Materials for Bacterial Chemotaxis

Various microfluidic gradient generators have been proposed in the past, each falling under one of two categories, flow-based and diffusion-based. Flow-based chemical gradient generators can create steady gradients of arbitrary shape. However, flow can also affect the trajectory of free-swimming cells, not only due to shear stresses that can affect motility, but also due to temporally variable gradients as they are moved by flow. Consequently, flow-based gradient generators are only appropriate for use in studying surface-attached cells. On the other hand, diffusion-based microdevices can generate a chemical gradient without flow, making them appropriate for use on free-swimming microobjects. Three approaches have been proposed to create diffusion-based gradient generators, the most promising approach utilizing source and sink microchannels to maintain a gradient through hydrogel (agarose) layers (Ahmed et al. 2010). A test channel is
located between the source and sink to place our bacteria-propelled microbeads to examine their behavior in the presence of the chemical gradient (Fig. 3).

To create this microfluidic channel setup, a polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) positive mold of the channels was first fabricated. To do this, the three-channel pattern was created on Solidworks, and etched onto a 150 µm thick acrylic film using a laser cutter, which was then mounted to the base of an empty petri dish. An 8 mL PDMS solution was poured into the petri dish and cured at 30 °C for 24 hours. The PDMS around the mounted acrylic film was cut out to produce a positive mold.

A step-by-step procedure to create the experimental platform with the agarose-based channels is illustrated in Figure 4. (1) The PDMS positive mold was placed on a rough, non-stick surface, and a PDMS spacer around 300 µm thick was placed on top of the mold; (2) an aqueous solution of agarose (40 g Eiken Agar and 1 L DI water) was heated on a hot plate and poured in the area enclosed by the spacer. The agarose was promptly covered by a 1 mm thick Plexiglas manifold containing holes at the inlets and outlets of each of the 3 channels. Care was taken to make sure that the inlets and outlets were aligned with those on the PDMS positive mold; (3) after allowing 10 minutes for the agarose to gel sufficiently, the platform was flipped, and the PDMS positive mold was removed, exposing the channels. A 1.8 mm diameter capillary tube (Kimble Glass, KIMAX-51, Vineland, NJ) was used to puncture a hole at the inlets and outlets of each channel through the agarose to the holes on the Plexiglas manifold. This is done to permit the subsequent insertion of solutions from the holes on the acrylic piece into the channels; (4) lastly, the exposed channels are covered by a glass slide, and the platform is flipped back upright. In order to ensure proper adherence between layers and to prevent leakage between channels, the platform was placed into an aluminum mounting bracket to sandwich the layers together with sufficient force (Cheng et al. 2006).

Upon mounting the platform, 10 µl motility medium was placed at the test channel via the inlet hole in the top Plexiglas layer and left for a few minutes to ensure no leakage into other channels. Upon confirmation, 10 µl of a 10⁻³ M L-aspartate (chemoattractant) solution was placed at the source channel, and 10 µl motility medium was placed in the sink channel: L-aspartate is one of four ideal chemoattractants (L-aspartate, L-glutamate, L-threonine, and glycine) for attracting flagellated bacteria (Park et al. 2003). The diffusion of 10⁻⁴ M fluorescein at such a concentration through agarose has been characterized by Cheng et al., and was shown to effectively diffuse linearly, given a sufficiently low concentration under 10⁻³ M; therefore, we utilized a 1 mM L-aspartate concentration for our application (Cheng et al. 2007). Given the dimensions of the channels, the chemoattractant was given approximately 90 minutes to diffuse through the test channel and generate a uniformly linear chemical gradient.

Following the chemical gradient generation, a small aliquot of bacteria-attached beads was placed in the test channel. Upon placement into the channel, the apparatus was left for 10 minutes to allow the test channel flow to settle, and for the chemoattractant gradient to restore itself through the test channel. In order to maintain high flow through the sink and source channels to maintain the chemoattractant gradient, 10-20 µl chemoattractant solution or motility medium was placed in their corresponding channels approximately every 20 minutes (Cheng et al. 2007).
2.4 Behavior Tracking of \textit{S. marcescens} attached PS Beads

For non-chemotactic experiments, the source and sink channels were both filled with motility medium, while the PS beads were placed in the test channel. For chemotactic experiments, the source and sink channels were filled with chemoattractant and DI water, respectively. The motion of the bacteria-attached PS beads was observed via using an inverted optical microscope (Axiovert 100, Zeiss, Germany) under 20x and 32x objective lenses, as well as a 40x oil immersion objective lens (Zeiss, Germany). Videos were recorded at 23 FPS for each bead sample using a CCD camera (WV-CD110-A, Panasonic, Japan). These videos were processed in Image J, using a particle tracking plugin (free software and plugin available from NIH), and the trajectory data output from the plugin was placed into MATLAB to determine and plot mean square distance.

3 Results

Upon placement in the test channel, the PS beads exhibited different behavior based on two scenarios: near the glass surface at the bottom of the apparatus, and away from the glass surface. When near the surface, the beads exhibit a wall effect and are consequently slower in speed; when the beads are around 5 times a bead’s diameter away from the surface, the beads tend to be faster, as they are less influenced by a wall effect (Goldman et al. 1967). When the beads were first placed in the test channel, the liquid within the channel exhibited a high flow rate, and the bead trajectories could not be accurately measured. After approximately 10 minutes, the flow stopped and the chemical gradient was restored throughout the channel. Moreover, by the end of this waiting period, the beads tend to settle near the glass. Therefore, for both non-chemotactic and chemotactic experiments, we observed the beads near the surface only.

To ensure that free-swimming background bacteria within the bacteria-attached PS bead solution do not engage in collective behavior and consequently affect the movement of the beads themselves, we diluted the bead solution with motility medium to reduce the concentration of background bacteria. In a study on the concentration dependency of dynamic collective behavior, it was shown that for density numbers (volume fraction) under 0.14, no collective behavior is observed (Sokolov et al. 2007). In our experiments, we diluted the various bead solutions accordingly to ensure a background bacteria density under 0.14.

For our chemotactic bead behavior study, we examined the bead behavior for 3 different bead diameter sizes: 5 µm, 10 µm, and 20 µm. For both non-chemotactic and chemotactic cases, these beads were fully coated with bacteria, observed under an optical microscope for a duration lasting 90 seconds (1.5 minutes), and subsequently tracked to determine the mean square displacement of each bead as a function of time. The specific duration was chosen to ensure accuracy in tracking, while maintaining an observation window well under the randomization time ($\tau_R$) (Behkam and Sitti 2009). The two-dimensional displacement of bacteria propelled beads follows the following general equation (Behkam and Sitti 2008, Howse et al. 2007):

$$\Delta l^2 = 4D \Delta t + \frac{\nu_{\text{mean}} \tau_R^2}{2} \left[ e^{-\frac{2\Delta t}{\tau_R}} + e^{-\frac{2\Delta t}{\tau_R}} - 1 \right] \quad (1)$$
where \( t \) is the time, \( V \) and \( D \) are, respectively, the velocity and the diffusion coefficient of the mobile micro-bead, and \( \tau_R \) is the randomization time. When \( t \) is much smaller than \( \tau_R \), (1) can be approximated to the following equation:

\[
\Delta L^2 \approx 4D\Delta t \quad (2)
\]

By fitting (2) to a plot of the mean squared displacement of each bead, we determined the speed of each bead.

### 3.1 Non-Chemotactic Bead Behavior

To accurately analyze the bead behavior and establish a baseline for chemotactic experiments, we first tracked the motion of bacteria-attached beads in the absence of a chemoattractant for all 3 bead sizes. Because there is no chemoattractant, the bead exhibited no preferential direction of movement; this bead behavior is consistent with the free swimming bacteria’s “random walk” motion. Additionally, as mentioned above, these beads were tracked while near the surface, resulting in reduced motility due to a wall effect between the bacteria-attached bead and the glass surface at the bottom of the experimental apparatus.

The trajectories of every bead were recorded for 90 seconds and placed, corresponding to their bead size. The trajectories of the beads for a 30-second duration were placed onto 3 separate graphs, as illustrated in Figure 5(a)-(c). Each bead is represented by a different color on the graph, and the starting points of each are denoted by a double concentric circle. Through plotting the trajectories, one can observe that in the absence of a chemoattractant, the beads exhibited no preferential direction of travel. This lack of directionality is characteristic of a “random walk” behavior. The square displacement plots of the non-chemotactic cases for each bead size were plotted over 10, 30, and 60 seconds.

Determining a best fit line, and fitting this to the form of (1), we determined the mean speed, \( V_{\text{mean}} \). The mean speeds of each bead throughout different durations, calculated by the total distance travelled of the bead over time, are organized by bead size and shown in Table 1.

Through the results, it was also observed that as bead size increases, the mean bead speed decreases. There are several hypotheses to explain this behavior. Firstly, as the size of the bead increases, the surface area of bead increases as well. However, because our experiments were performed near the glass surface at the bottom of our channels, an increase in contact surface area translates to a greater magnitude of the wall effect. Additionally, while surface area increases by an order of 2 as radius increases, and more bacteria are able to attach to the bead surface, propulsive force only increases by an order of 1. As a result, propulsive force does not scale proportionally with an increase in bead size (surface area).

### 3.2 Chemotactic Bead Behavior

In the presence of a chemoattractant, the *S. marcescens* attached to the PS beads are expected to propel the beads up the chemical gradient, towards the source of the chemoattractant. As described previously, a diffusion-based three-channel agarose chemoattractant gradient generator was fabricated to generate a uniformly linear gradient throughout the width of the test channel (Cheng et al. 2007). By maintaining a flow in the side channels (source and sink channels), the
chemical continues to diffuse through the agar and test channel, maintaining the gradient. Each bead sample was tracked for 90 seconds, and their trajectories for 30 seconds were plotted in Figure 5 (d)-(f), with respect to their own starting positions within the capture frame, and superimposed upon each other.

The mean speeds for each bead size throughout various durations for the chemotactic case are shown in Table 2. From the trajectories illustrate a clear indication of directionality; that is, the bacteria-attached beads have the tendency to propel themselves towards the source of the chemoattractant. When the bacteria attached to the bead detect a chemoattractant gradient, the run to tumble ratio greatly increases, minimizing the “random walk” behavior as it seeks out the source of the chemoattractant gradient.

3.3 Comparison of Non-chemotactic and Chemotactic Cases

As shown in Tables 1 and 2, we calculated the mean speed for each bead size over 3 durations. Between non-chemotactic and chemotactic cases, the mean speed of each bead size fall within the standard deviation of each other, and can therefore be assumed to be roughly equal. For each non-chemotactic and chemotactic case, all sample data within one bead size was taken within a single bacterial cultivation. As a result, the standard deviation is relatively minimal. If we took samples of each bead size throughout different bacterial cultivations, we expect to see a greater standard deviation for mean speed.

The chemotactic nature of the bacteria-attached beads under the influence of a chemical gradient is most clearly illustrated in Figure 6, which illustrates angle histograms based on 30-second trajectory data from all bead samples, based on bead size. For each frame in the capture video, the bead exhibits a displacement, which can be quantified by a vector. The angles of these vectors are calculated with respect to the chemoattractant, located at -90°, and plotted onto the histogram. Because the bead trajectory is calculated based on the order of 1 pixel per frame, and our bead tracking algorithm tracked around 23 frames per second, this can result in pixel discretization errors. Therefore, we determined our displacement vectors by taking the difference between the 1st and 4th frames, 2nd and 5th frames, etc., and graphed the trajectories based on these vector calculations.

In the non-chemotactic case, we expect to see a uniformly random distribution throughout all directions (-270° to 90°). In Figure 6 (a)-(c), we can see that this is primarily the case with all the non-chemotactic bead angle histogram results, due to the fact that there are no pronounced modes in any specific direction. On the other hand, given the presence of a chemical gradient, whose source lies at -90°, we expect the corresponding angle histograms to exhibit a unimodal distribution centered at or around -90°. As seen in Figure 6 (d)-(f), each of the three bead sizes show a peak at or around -90°. Comparing the histograms between the non-chemotactic cases (Fig, 6(a)-(c)) and chemotactic cases (Fig. 6 (d)-(f)), it is clear that in the presence of a chemoattractant, the bacteria attached to the bead achieve a sense of steering control, helping to propel the bead towards the chemoattractant source.

Lastly, the orientation by which S. marcescens can attach to the PS bead surface can affect its overall displacement. As observed in a small percentage of beads, the beads not only exhibit a two-dimensional displacement, but also engage in a rolling movement. If the cell body of the
bacterium attaches by its side to the bead, then its “run” mode can potentially contribute to a rolling motion of the bead. For our calculations and models, we only observed the translational bead motion.

4 Conclusion

This paper demonstrated the behavior of S. marcescens-attached 5 µm, 10 µm, and 20 µm PS beads in the absence and presence of a chemoattractant, L-aspartate. When no chemoattractant is present, the bacteria-attached beads engage in a random-walk motion, in accordance with the behavior documented by previous studies. However, when the beads are placed in the presence of a chemoattractant, L-aspartate, the bead behaviors change. By using a diffusion-based microfluidic chemical gradient generator and observing their behaviors, the following conclusions could be made: through the trajectory maps, their run-to-tumble ratio greatly increases, resulting in a straighter trajectory; and through the angle histograms, the bacteria-attached beads have a tendency to move towards the direction of the chemoattractant source. Additionally, in both non-chemotactic and chemotactic cases, as bead size increases, the mean speed decreases. This is due to an increased wall effect, as well as a decreasing proportion of propulsive force over surface area as the bead diameter increases.

This study has demonstrated the potential for utilizing bacteria-attached micro-robots controlled by bacterial chemotaxis as a passive steering control method for various applications. Moreover, through testing various PS bead sizes, it can be concluded that there exists an optimal robot body size that allows for a balance between propulsive force and wall effects. By improving bead patterning and bacteria attachment techniques, these may further improve the motility of the bacteria-propelled micro-robot.

Acknowledgements

The authors of this paper would like to thank the members of the NanoRobotics Lab at Carnegie Mellon University for their help and discussions. We would also like to thank Joseph Suhan of Carnegie Mellon University for help in SEM imaging. This work was supported by the NSF CPS-Medium (CNS-1135850).
Figures and Tables

**Fig. 1.** Photo of swarming *S. marcescens* on a nutrient agar plate. The most pigmented area on the right marks the inoculation site (denoted by the orange circle), while the leading edge is seen slightly over halfway across the plate. The PS beads are placed near the leading edge, marked by the yellow line.

**Fig. 2.** Images of *S. marcescens*-attached PS Beads. (a) Scanning Electron Microscope (SEM) image of 30 µm beads. The individual bacteria are denoted as small dots on the smooth surface of the PS bead. (b) Inverted optical microscopic image (using a 40x oil immersion objective lens) of 20 µm beads suspended in motility medium. Attached bacteria can be seen roughly as black spots on the bead surfaces.
Fig. 3. Schematic of the imaging enclosure. (a) In order from bottom to top, 1: glass slide, 2: thin PDMS layer, 3: agarose shaped with three channels, and 4: acrylic manifold with six holes for access to channel inlets and outlets. (b) Channel layout of diffusion-based gradient generator, from top to bottom: chemoattractant (as source), test channel (for placement of microbeads), and DI water (as sink).

Fig. 4. Step-by-step preparation of three-channel chemical gradient generator, similar to design by Cheng et al. (a) A PDMS spacer is placed on top of a PDMS positive mold to create a reservoir for hot agarose. (b) Upon placement of hot agarose, the setup is covered by an acrylic manifold, ensuring that the inlet and outlet holes line up with those on the positive mold. (c) After the agarose is given sufficient time to gel (~10 min), the PDMS positive mold is removed. Holes are then created for solution insertion, and the exposed side is covered by a glass slide. (d) The final enclosure is then sandwiched together and mounted between two metal frames for secure insertion of solutions.
Fig. 5. Trajectory Maps of *S. marcescens*-attached microbeads through a duration of 30 seconds: Non-chemotactic behavior of (a) 5 µm, (b) 10 µm, and (c) 20 µm diameter microbeads; chemotactic behavior of (d) 5 µm, (e) 10 µm, and (f) 20 µm diameter microbeads. For chemotactic behavior, the chemoattractant source is located at the bottom of each map. The starting point of each bead trajectory is marked by a double concentric circle.
Fig. 6. Angle Histograms of *S. marcescens*-attached microbead directionality, through a duration of 30 seconds: Non-chemotactic behavior of (a) 5 µm, (b) 10 µm, and (c) 20 µm diameter microbeads; chemotactic behavior of (d) 5 µm, (e) 10 µm, and (f) 20 µm diameter microbeads. In the absence of a chemoattractant, the beads should exhibit no preferential direction, and therefore the corresponding angle histograms show a fairly uniform distribution among all angles. However, in the presence of a chemoattractant, the beads have a tendency to move towards the chemoattractant source, located at -90°. As seen on the chemotactic angle histograms, there is a peak, in each case, at or near -90°, indicating an increased directionality.

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<th>60 sec</th>
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Table 1. Mean speeds of 5, 10, and 20 µm non-chemotactic bacteria-attached bead propulsion (in µm/s) over three durations.
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Table 2. Mean speeds of 5, 10, and 20 µm chemotactic bacteria-attached bead propulsion (in µm/s) over three durations.

REFERENCES