Engineering a Bacterial Flagella Forest for Sensing and Actuation – A Progress Report

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ABSTRACT

Flagella can be used to make magnetically-controlled microfluidic and nanoscale devices for biomedical applications in both vitro and vivo environments. They are capable of operating with high precision on the cellular and subcellular level. So far, scientists and engineers have successfully used monolithic inorganic materials or photoactive polymers [1] to mimic the helical bacterial flagella whose rotary-propulsion mechanism effectively overcomes the dominant viscous forces that prevail in a low Reynolds-number environment. Here, we focus on bacterial flagella and their rotary motion. The bacterial flagellum is an ideal biomaterial for constructing self-propelling nanoswimmers because it can reversibly change its geometry in response to different environmental stimuli such as pH, the local concentration of certain organic solvents, and mechanical force on the flagella. The bacterial flagellum is very easy to manipulate because it is composed of flagellin which can be mechanically isolated through vortexing and centrifugation, which enables flagella to be used as nanoscale sensors and mechanical transducers. Our project focuses on fabricating a bacterial flagella forest which consists of an ordered array of flagella on a glass substrate. Flagella are attached to magnetic nanobeads via biotin-avidin bonding for actuation by oscillating magnetic field.

1. INTRODUCTION

Nanoscale robots or nanoswimmers have emerged as a new class of active matter over the past decade because of their potential of being operated at the cellular and subcellular level. The viscous force and energy source are the major challenges we have to overcome when giving the nanorobots mobility in the low Reynolds-number environment. In the low Reynolds-number (Re << 1) environment, the nanoswimmers experience a viscous force that is many orders of magnitude stronger than inertial forces. According to the Stokes equation, flows in the low Reynolds-number regime are instantaneous and time-reversible. As a consequence, in the incompressible and Newtonian fluid, the low Reynolds-number swimmer executes a sequence of identical shape changes when reversed, and the net displacement is zero, that is reciprocal motion [37].

The large part of reported small-scale robots takes the approach of imitating locomotion strategy of microorganisms. Some microorganisms such as bacteria Escherichia coli and Salmonella typhimurium propel themselves by beating and rotating their flagella in the microfluidic environment. The nonreciprocal motion [14] they employed breaks the time-reversal symmetry of Stokes flow and generating viscous drag. Some reported micro-swimmers use flexible metallic nanowires [15,16], magnetic particles linked by DNAs [17] to mimic the method of propulsion of bacteria. Depending on the properties of micro/nano-motor, the swimmers can be actuated by [18, 19] oscillating field [20,21], chemical gradient, light [34] and acoustic signals [19] and so on. While those products are effective swimmers, the fabrication process may be very complicated that involves the using of top-down fabrication techniques such as 3D laser writing [22], 3D template-assisted electrodeposition [23], physical vapor deposition [24] and shadow growth. Also, the toxicity of swimmer materials has to be cautiously considered when implementing those swimmers into human bodies.

In comparison to the artificial flagella using traditional metallic materials, natural bacterial flagella are easier to be fabricated and own better structural and mechanical abilities. The flagellum is a thread-like appendage protrudes from the bacterial basal body. Its primary function is locomotion, but it also often functions as a sensor which is sensitive to the environmental factors such as temperature and light [36]. Bacterial flagella are self-assembled helical structures with rotary motors at their bases, which control the flagella rotate clockwise or counterclockwise. Each flagellum seems like a hollow tube with an inside diameter of 2 nm and outside diameter of 20 nm. It is entirely composed of flagellin subunits, a 55 kDa globular protein [25]. The subunits non-covalently bond to each other in a repeated helical pattern (usually left-handed). They are surprisingly durable for a protein, surviving pHs from a range of 3 to 11 and can resist denaturation up to 60°C [26].

Bacterial flagella experience polymorphic transformations [24], interconvert between different helical forms in response to external stimuli such as temperature [27], pH [28], electrostatic field [32], mechanical forcing [29] and possibly the concentration of specific ligands. Our

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project is utilizing and manipulating those properties to construct a bacterial flagellar forest on the glass substrate. The forest is composed of arrays of bacterial flagella attached on magnetic microbeads. Then placing the forest in microfluidic systems [12] vary in the fluid types and flow directions to test and characterize the dynamics of individual flagella within the forest in response to different external stimuli. Through examining their responses to changes of the environmental condition, we can manipulate the physical properties of the flagella and construct them with the purpose of gathering them into densely packed ordered arrays on a substrate for a bacterial flagellar forest. The resulting biomaterial, the bacterial flagella forest whose properties are well determined, can be harnessed to autonomously and actively sense environmental changes and to generate macroscopic pumping flow.
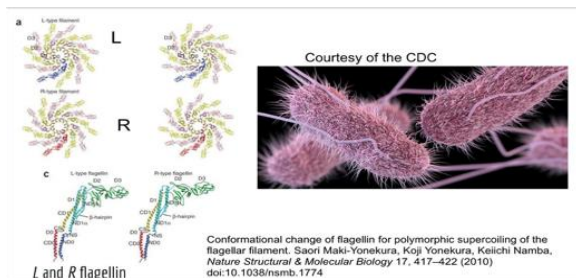

Figure 1. Conformational Change of Flagellin. Flagella can form both left-handed and right-handed helices, depending on local conditions. Reproduced from ref. 3. Copyright 2010 Nature Structural & Molecular Biology

From term Fall 2017 to Spring 2018, we used the flagella of bacteria Salmonella as raw materials to synthesize flagella greater than 10 nm and attached them to ferromagnetic nanobeads via biotin-avidin bonding. Now, we are working on testing and characterizing the dynamics of individual flagella within the forest in response to different external stimuli.

2.   RESEARCH DESIGN

The AD5933 impedance analyzer was selected for this project because of the capabilities of the chip and the features of the evaluation board and its software.
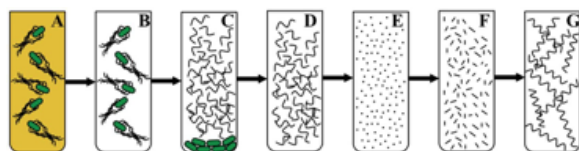
Polymerization

Figure 2. Diagram of flagella polymerization process. Reproduced from ref.1 Copyright 2017 Sci. Rep.

We followed the protocol provided by Dr. Kim [1, 11] to polymerize the bacteria. We first cultured the Salmonella typhimurium (SJW 1103) in a modified LB broth (1% tryptone, 1% yeast extract, 0.1% glucose, in 10 mM potassium phosphate buffer) (A) for approximately 14-

16 hours in order to yield as much bacteria as possible. Next, we centrifuged the bacteria and resuspended in a phosphate buffer (B). Flagella were isolated from bacterial basal bodies by sharing via vortexing (C). Flagella were then separated from the cell bodies by centrifugation at 10,000 x g and resuspended in phosphate buffer (D). The isolated flagella were depolymerized into flagellin monomers by heating at 65°C for 15 minutes (E). Next, we added an equal volume of 2M Mg2SO4 to the solution of flagellin monomers and allowed them to polymerize for 2 hours at room temperature to generate flagellar fragments (seeds) (F). The seeds were added to the solution of depolymerized flagella to facilitate repolymerization.

Flagellin self-assembles into flagella-like polymers. In vivo polymerization is quite complicated (involving, among other things, the newly synthesized subunits traveling through the center of the hollow flagella in unfolded form). In vitro polymerization seems to behave reasonably like any other association/dissociation reaction [4]. Flagella can be separated into flagellin monomers by heating (at 60°C, 10 minutes) or by acid treatment (pH < 2.5). This project makes use of heat for separation. Flagellin monomer must be incorporated into the filament right below the HAP2 cap at the filament tip [5], and the presence of CAP protein in the solution will limit the elongation of flagella to our desired length. The HAP2 complex has the highest stability at around pH 5.0 and tends to dissociate when the pH rises above 9.0, but flagellin is still functional in the pH range of 9.0 to 11.0. Therefore, we can utilize this property to filter out the CAP protein from flagellin and allow for longer flagella.

There are a variety of different ways we can check the purification of our sample. Isoelectric focusing is a desirable method of purification because it allows us to separate proteins based on their isoelectric points and also their mass, allowing us to see if there are containment proteins.

Additionally, we need a sufficient concentration of the subunits in solution in order to polymerize long flagella. We can check the concentration of our samples with spectrophotometry. As the aromatic amino acids absorb well at 280 nm, we can use spectrophotometry to get an idea of the total protein concentration.
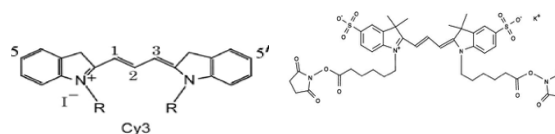
Fluorescent Dying with Cy3

Figure 3. Molecular structure of unbound cy3.

Because flagella are much smaller than the wavelength of light (flagella are 2 nm; visible light is > 300 nm), we cannot directly see the flagella with a light microscope. While there are ways to use electron microscopes to see structures that small, electron microscopes are expensive, typically require freezing the sample, which would prevent us from studying the motion in real time. Instead, we will be using fluorescent imaging

and Cy3 dye to visualize the flagella.

Cy3 (Cyanine) is a synthetic dye belonging to the polymethine group. Cy3 conjugates can be excited to 550 nm maximally and induce a light emission at 570 nm in maximum. Compared to other orange-red fluorescing dye conjugates, it is brighter, more photostable, and gives less background. Cy3 is a derivative non-sulfonated cyanine which has low solubility. Thus, it has to be dissolved in organic solvent such as DMSO before being added to the solution of the biomolecule in aqueous buffer. As Cy3 forming conjugation with flagellin, it increases the range of wavelengths which will form an image of the dyed sample on the film.
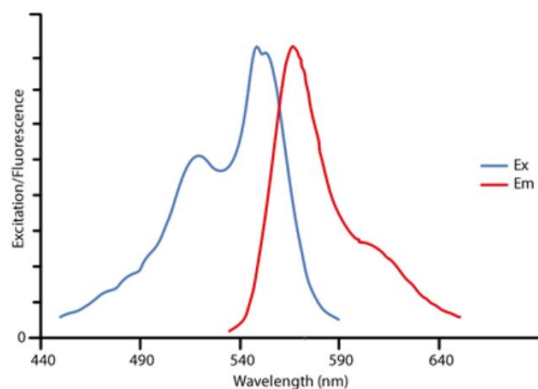

Figure 4. Excitation and emission spectra of Cy3-conjugated affinity-purified secondary antibodies, streptavidin, and purified proteins. Reproduced from ref. 35. Copyright jacksonimmuno.com

After repolymerization, we first centrifuge the flagella at 80,000 x g and use 10 nM potassium phosphate buffer at pH 7.5 to resuspend the pellet. Then reconstitute the Cy3 dye in the solution of 0.1 M sodium bicarbonate and add it to the suspension of flagella, incubate the solution for three hours at room temperature.

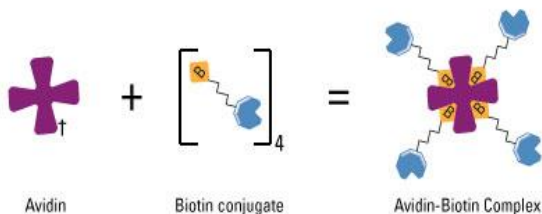*Biotinylation and Attaching the Magnetic Microparticles*

Figure 5. Schematic of avidin-biotin interactions. Reproduced from ref. 6. Copyright Thermo Fisher Scientific
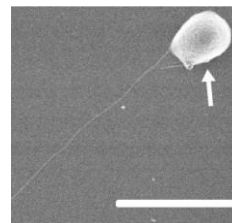

Figure 6. SEM image: a flagellum attached to a ferromagnetic particle (1-micrometer scale bar). Reproduced from ref. 1. Copyright 2017 Sci. Rep.

We use a common biology lab protocol to attach the flagella to the microbeads: biotin-avidin. The biotin-avidin bond is one of the strongest noncovalent bond known in biology (KD ≈ 10−15 M) [33]. Because of the small size of biotin (244.31 kD), biotinylation does not disrupt the function and natural properties of the molecules. With an exception to high specificity and high reaction rate, the interaction between biotin and streptavidin and avidin is not affected by heat, pH, and proteolysis. Thus, making biotinylation possible in extreme environments. Both primary amines and the -SH bond on a cysteine are reasonable targets for biotinylation, and there are a fair number of lysines on the flagellin protein as seen in the figure below.
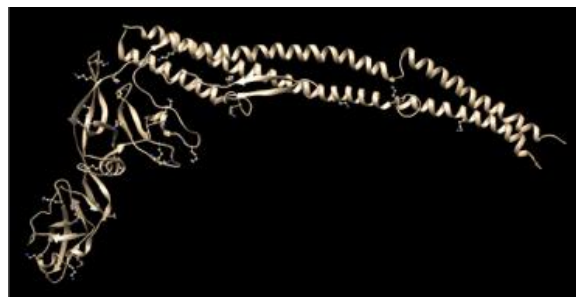

Figure 7. Flagellin with biotinylation sites marked. Structure data from RCSB PDB (3A5X) [3]; image generated with Chimera. There are plenty of possible biotinylation sites. Blue is the primary amine of lysine.

The NHS-biotin is added before the final step of repolymerization. After the NHS-biotin successfully binds to the short repolymerized flagella, we centrifuge the flagella solution to depolymerize them into single subunit. Along with the seeds, we added the biotin-attached flagellins to the solution of short, fragmented flagella to form long repolymerized flagella with biotin at one end. The resultant flagella solution is attached directly to the solution of streptavidin-coated ferromagnetic nanobeads.
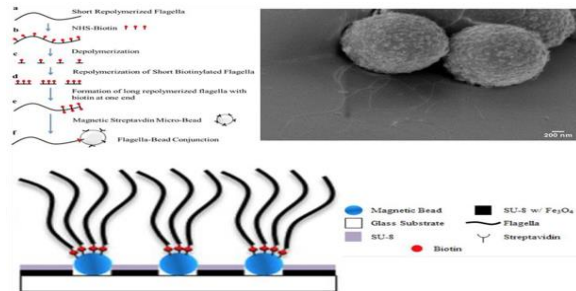
Figure 8. Method of attaching nanoswimmers to a glass substrate. Reproduced from ref. 10. Copyright 2017 Physical Review.

As for attaching the avidin to the magnetic microparticles: magnetic microparticles are composed of iron embedded in a polystyrene shell in order to prevent the iron from interfering with biological processes, and in order to facilitate experiments, most magnetic microparticles are functionalized with either primary amines or carboxylic acid groups so we can attach the avidin enzymatically.

Anchoring Simmers to a Substrate

The difficulty here is that we must anchor the swimmers down in a way that does not impede their spinning while still controlling their location. There are many different ways to place beads precisely on a substrate (including a number of methods that quite resemble inkjet printing [7]) However, it is easiest for us to use lithography to etch little wells into our substrate and use a magnetic force to hold down the magnetic microbeads.
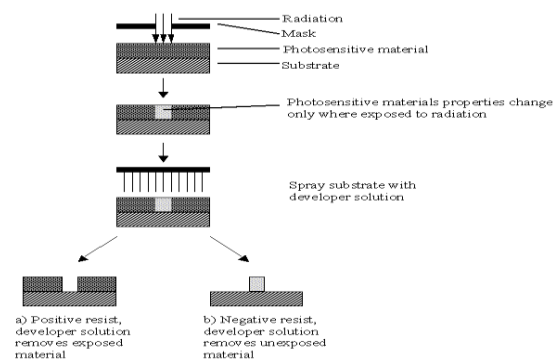

Figure 9. A simple illustration of lithography process

Lithography and masking allow us to efficiently design patterns well below 1 µm feature size, and because we do not need to repeat any masking steps, alignment is not an issue. For this process, we apply a layer of photoresist (SU-8) and use a mask to define areas to etch away in the substrate by exposing some areas of photoresist with UV light, which causes those areas to soften. Next, we wash away the excess photoresist and etch. Finally, we wash away the remaining photoresist.

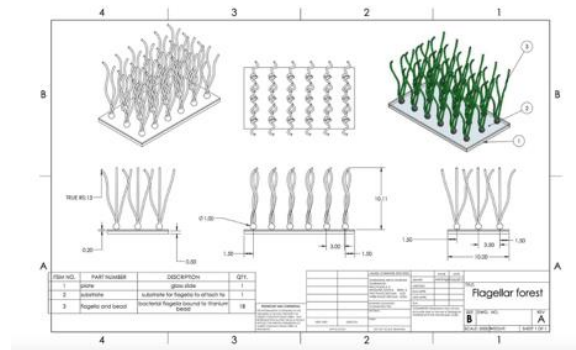For now, we will assume a grid-like pattern; this may also need to be optimized in the future.

Figure 10. Initial design of bacterial flagellar forest (drew by Lauren Metzinger)

Figure 9 is the CAD drawing of our design idea. The final flagellar forest model should have up to three 10 µm-long flagella attached to each magnetic microbead. The separation distance between each row of flagella-magnetic particles we assumed in this picture is 3.0 µm. According to James D. Martindale and Henry C. Fu's research results [10], the stable tilt angle of flagella decreases as the separation distance between arrays increased in the neutral environment. Their findings provided us a design implication to predict the separation distance, length of flagella and the rotational frequency of magnetic field that will cause transformations between straight and curly forms.

Based on their analysis, we will find out the most appropriate distance as we thoroughly examine the interaction between individual flagella, the responses to stimuli, and the effect of spinning microbeads controlled by external magnetic field.

Actuating the Microbeads

To three-dimensionally actuate the magnetic beads, we place the flagellar forest in a Helmholtz coil system, which is composed of three pairs of orthogonally positioned solenoid electromagnets (along x, y, and z-axis). The coil system produces a region of the nearly uniform magnetic field. As a magnetic field rotates the beads, a torque is imparted on each flagellum which in turn generate pumping flow.

We use an epi-fluorescent inverted microscope to visualize the behavior of flagellar forest on glass substrate within the Helmholtz coil system. Previous experiments were done by Jamel Ali and etc. show that under 30 Hz, the rotational response of a magnetic particle experiences a linear increase along with the magnetic field [30,31]. Therefore, to obtain synchronous movement of nanoparticles as well as flagellar forest, we need to set the rotational frequency below 30 Hz.

Figure 11 (left). A sample of three-axis Helmholtz coil system. Reproduced from ref. 8. Copyright DirectVaccum.com
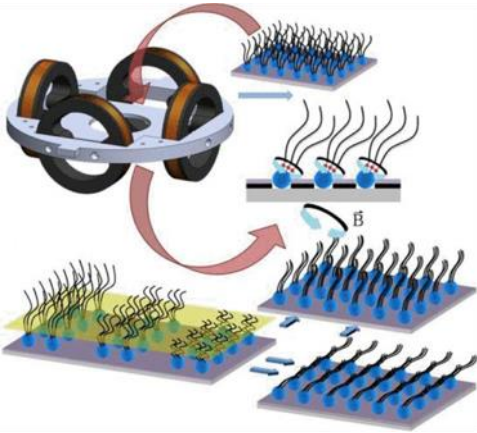

Figure 12 (right). Martindale *et al.*'s electromagnetic setup, which is similar to what we will use. Reproduced from ref. 10. Copyright 2017 *Physical Review.*

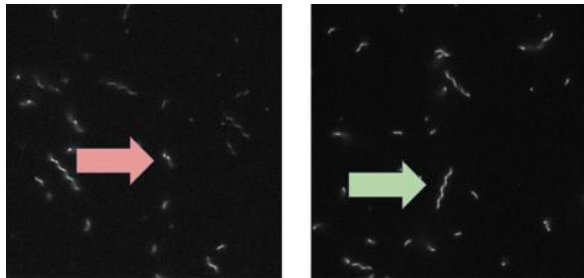## 3.   Outcomes and Current Status

Figure 13. Failed (right) and successful (left) repolymerization outcomes from Louis Rogowski
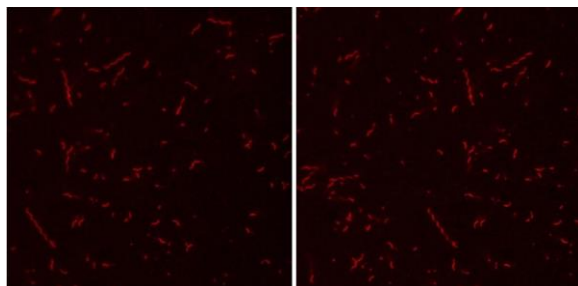

Figure 14. Successful repolymerized flagella (by our team) visualized under inverted epifluorescence microscope

In the Fall 2017 semester, our team generated short fragments of flagella (as shown in Figure 13, left) and viewed them under high-resolution epi-fluorescence microscopy. However, the length was not sufficient to produce collective pumping and mixing motion. The ideal polymerized flagellum should have a length of above 10 μm long, with biotin attached at one end. After making some modification on the concentration of reagents, we finally repolymerized flagella to the desired length at the end of the fall semester. At the start of the Spring 2018 semester, our team experienced some personnel changes, and only Xihe Liu continued working on this project. That caused somewhat delay in our research schedule. In February, we finished the biotinylation and procedures of attaching flagella to microbeads. At the end of March, we started to attach these flagella onto a substrate and put the model within a microfluidic device to test its responses in different environments. From James D. Martindale and Henry C. Fu's research, we learned that in a microfluidic device as shown in Figure 15, the tilt angle of a flagellar forest is a significant factor we need to manipulate to control the direction and magnitude of resultant pumping flow [10]. The non-dimensionalized volumetric flow rate generated by a single flagellum on a no-slip plane depends on tilt angle, polymorphic form (curl intensity) and helical forms (left-handed or right-handed). The volumetric flow rate quadratically increases with the tilt angle when the other two influencing factors are controlled and achieves the maximum value at approximately 45 degrees.
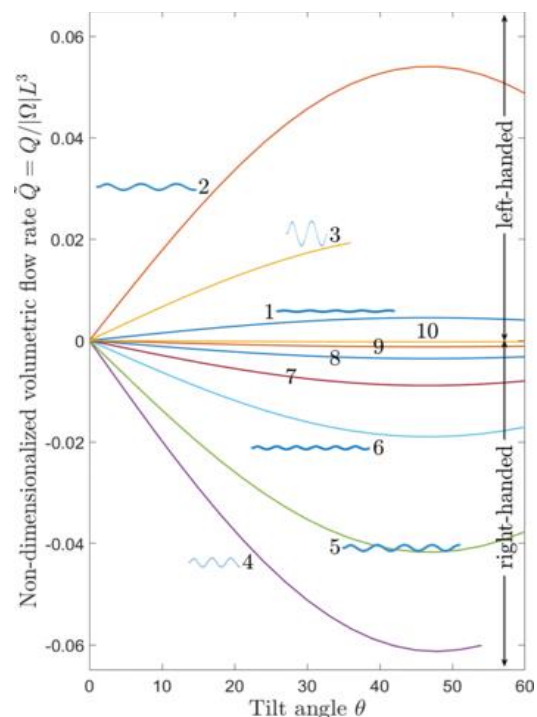

Figure 15. Nondimensionalized volumetric flow rate over one rotational period for the 10 helical bacterial polymorphic forms as a function of tilt angle. Rotational direction is clockwise about the helical axis (Martindale and Fu 3)

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Similarly, the flow rate also goes up with the curl intensity until helical form 4 in Figure 15. Based upon their research, we would more specifically examine the rotation, elastic stretching and polymorphic transformations induced by viscous shear flow applied to flagellar forest within a microchannel. The final collective, parallel response of flagella forest can be harnessed for engineering applications. The final flagellar forest model should have up to three 10 μm-long flagella attached to each magnetic microbead. The separation distance between each row of flagella-magnetic particles we assumed in this picture is 3.0 μm. According to James D. Martindale and Henry C. Fu's research results [10],

4. RESEARCH DESIGN

In the Fall 2017 semester, our team started to work with Ph.D. students Daehee Kim and Louis Rogowski to generate short fragments of flagella and viewed them under high-resolution epi-fluorescence microscopy. We were stuck with the polymerization step for about three months. Under the microscope, some of the flagella we got were popcorn-like particles rather than threadlike helical fragments as we expected. Also, the length of some repolymerized flagella was less than 10 μm, which was not sufficient to produce collective pumping and mixing motion.

For the popcorn-like particles, we guess contamination occurred. It was tough to figure out at which step contamination happened accurately. It might occur at any point during the experiment, such as the steps of transferring supernatant, adding polymerization buffer and culturing bacteria in LB broth. After several attempts, we found that the vital point to avoid contamination was pipetting flagella containing fluid to a new tube after centrifuging the vortexed solution to remove bacterial basal bodies. Touching the bacteria palette at the bottom of the tube would transfer the unnecessary components, for example, basal bodies, CAP proteins, and others that limit elongation of flagella. Thus, we added a checkpoint after centrifuging the resultant flagella containing the solution. We dumped the supernatant and checked if the palette at the bottom of the tube was transparent. If not, then contamination happened.

About the unsatisfactory length of repolymerized flagella, we guessed the cause of it was the temperature. The depolymerized flagella with buffer were supposed to be placed at room temperature to allow growth. However, our lab had issues with temperature control. Instead, we put it in the incubator for 30 minutes to form the short flagella seeds.

We finally repolymerized flagella to the desired length at the end of the semester, as shown in Figure 13. Then we started to attach these flagella onto a substrate and put the model within a microfluidic device to test its responses in different environments. We will examine the rotation, elastic stretching and polymorphic transformations induced by different kinds of viscous shear flow applied to flagellar forest within a microchannel [2]. The final collective, parallel response of flagella forest can be harnessed for engineering applications.

5. ASSESSMENT OF CONTEXT
Global Impact

Since the last decade, a number of research groups have demonstrated the potential of nano/microscale robots to be used in the field of medicine: invasive surgery, targeted drug delivery and attacking cancer cells. In the following text, we will use one potential application of bacterial flagellar forest: lab-on-a-chip device, to illustrate how this microfluidic device can beneficially influence medical treatment and surgery.

The lab-on-a-chip device allows doctors to quickly analyze tiny amounts of biofluids such as blood, sweat, urine, etc. Currently, when most people go to a physical check-up, their doctor draws blood, takes a urine sample, and sends both to an outside lab, which may take days to get a result. If there are any abnormalities, the patient often has to come in for another appointment, which is both costly and inconvenient. Instead, lab-on-a-chip systems allow faster turnaround times and can allow these tests to be performed during an appointment.

Additionally, with organ-on-a-chip systems, we can look into how someone's cardiac, liver, or other cells react to medications using an in vitro test instead of testing within someone's body.

Because microfluidic devices are associated with very low Reynolds numbers, it is difficult to mix two fluids at that scale. Flagella have evolved to work at this scale, however, and can help us mix fluids in microfluidic systems and also pump fluids.

Economic Impact

Lab-on-a-chip devices allow for faster turnaround less utilization of our very expensive medical system, possibly allowing the country to save money. Additionally, using targeted drug delivery will likely reduce side effects, which both saves money and will enable people to be healthier. Our device is not yet at the point of being a commercial product as it is still in the development phase so we do not have a good idea of how much money it can make or save; it is dependent on a lot of other factors.

Environmental Impact

In general, our project should not have any sizable environmental impacts. Bacterial flagella themselves should not cause many ecological impacts because of the number of flagella in our flagellar forests is very small compared to the number of flagella that already exists in the world due to bacteria. Like most proteins, flagellin will degrade quickly if left out in the open. However, the polystyrene magnetic microbeads are problematic. If they escape into the ocean, they may add to the problem of plastics in the sea. (Similar plastic microbeads were banned from cosmetic products recently.) However, our microbeads are much easier to gather up than non-magnetic microbeads and additionally we will not be using many of them.

Effects on Society

Bacterial flagellar forests would allow us to control liquid flow in organ-on-a-chip systems. Organ-on-a-chip systems would let us test specific chemo medications against both cancerous and noncancerous cells in a particular

patient, allowing us to find medicines that are effective against the patient's tumor while reducing side effects. We can screen for adverse reactions to chemo medications and also assess the efficiency of the medication for that particular patient without having to administer the medication to the patient and risk unpleasant side effects. Additionally, we could use bacterial flagella to help navigate magnetic microbeads to specific locations in a patient's body. (We can also use flagella to push the magnetic microbeads through the cell membrane of cancerous cells). Third, because flagella alter their shape in response to environmental factors, we can theoretically create dipsticks with logic, allowing more sophisticated dipstick urine tests. Finally, understanding flagella can help us understand how Salmonella is transmitted from food to person.

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