

Magnetotactic Phage-based Microrobotic Systems for the Detection of Live Bacteria

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Abstract—The concept of a novel biosensing system for the detection of bacteria and bacterial growth that could potentially offer shorter detection periods, specificities, and higher sensitivities compared with conventional methods is briefly described. The proposed biosensing microsystem uses magnetotactic bacteria that can be controlled by software and a microelectronic circuit embedded in the detection microsystem. These magnetotactic bacteria attach themselves to microbeads previously coated with special virus called phages. By controlling the swimming path of the magnetotactic bacteria, a sweep of the sample that potentially contains the pathogenic bacteria would be performed. Specificity could be assured since binding would normally occur only during a collision between a microbead being pushed by a magnetotactic bacterium and a target bacterium that will attach only to the microbead if it has been previously coated by the appropriate phages. The process would complete by orienting the displacement of the microbeads being pushed by the magnetotactic bacteria towards arrays of microelectrodes for detecting the presence of potential target bacteria.

Index Terms—Magnetotactic bacteria, pathogenic bacteria, phages, biosensors, microsystems, microrobotics, biorobotics

I. INTRODUCTION

A. Motivation

The motivation for research and development in biosensor technologies is quite high. Biosensors can detect, record, and display information regarding a physiological change or the presence of various chemical or biological materials in the environment. More technically, a biosensor is a probe that integrates a biological component, such as a whole bacterium or a biological product with an electronic component to yield a measurable signal.

In particular, market drivers behind the development of biosensors for the detection and quantification of bacterial contaminations are very diverse. It concerns not only national security where the key to protecting a military unit or a community from biowarfare agents is to detect them

before they reach their intended victims, but also in the medical field where new biosensors and lab-on-a-chip offer an especially cost-effective means for the rapid and accurate diagnosis of diseases. For instance, early warning sign of emergence of antibiotic-resistance is a must since antibiotic-resistant infections are an increasingly important health concern for humans. Multi-drug resistant bacteria cause up to 60% of nosocomial (hospital-acquired) infections. Strains of *Staphylococcus aureus* resistant to methicillin and other antibiotics are now endemic to hospitals and nursing homes and a limited number of drugs remain effective against these infections. Exposure of human beings to bacteria especially in particular environments is real since human beings on average, harbour more than 150 types of bacteria inside and outside the body [1].

The detection of bacterial pathogens in raw food materials, food products, processing and assembly lines, hospitals, ports of entry, and drinking water supplies continue to rely on conventional culturing techniques. Conventional methods involve enriching the sample and performing media-based metabolic tests. These tests are elaborate and typically require two to seven days to obtain results, a delay that is often responsible for severe consequences. In contrast, a field-ready biosensor can be ease of use, portable, and provide results in a much shorter time period. Higher sensitivity and faster detection with the desired degree of specificity are the main objectives in designing such field-ready biosensors. For rapid detection, it is often suitable to detect a single bacterium in a volume sample of 1 ml or even 100 ml. Hence, for a biosensor with a relatively high sensitivity, it is necessary to wait for the population of bacteria to grow sufficiently to be detectable. In the best cases, durations between 10 and 12 hours may be necessary for the detection of pathogenic bacteria with typical doubling time of 20-60 min. Detection periods as low as 5 min. have been reported but only on pre-concentrated solutions of approximately 10^8 CFU/ml instead of a natural culture of possibly 10 CFU/ml. Unlike a pure or pre-concentrated culture, in a natural or untreated/non-processed sample solution, a single pathogenic bacterium may share a relatively large sample volume with other types of bacteria, making detection and specificity much more difficult.

B. Actual Methods

Recent optical, piezoelectric and electrochemical biosensors have been developed for the detection of pathogenic bacteria. For instance, a fiber optic evanescent wave biosensor was reported to detect *Salmonella*, *Listeria*, and *Vibrio* species as low as 10^2 CFU/ml in 20 min. [2] and another one for the detection of *E. coli* O157:H7 in 25 min. [3]. An integrated optical interferometer for detecting *S. Typhimurium* has been developed with sensitivity of $10^5 - 10^7$ CFU/ml [4]. A surface plasmon resonance biosensor was also reported for the detection of *E. coli* O157:H7 [5, 6]. Also, a quartz crystal microbalance sensor coated with a thin culture medium was able to detect *Staphylococcus epidermidis* in the range of 10^2 CFU/ml [7].

Electrochemical biosensors, often referred to as amperometric, conductometric or impedimetric, have the advantage of being highly sensitive, rapid, inexpensive, and are highly amenable toward microfabrication, hence they represent very good candidates for field-ready biosensors as opposed to optically-based or other types of sensors [8, 9]. The recent development of an amperometric immunoassay [10] utilizing antibodies bound to a carbon electrode and enzyme amplifiers that was able to detect *S. aureus* concentrations down to 10^3 CFU/ml in 30 min. is one example that reflects the best results achieved by existing electrochemical sensors. Again, the remaining problem is the relatively high concentration, in this case being 10^3 CFU/ml. In reality, early detections require concentrations closer to 10^0 CFU/ml in non-processed samples, and actually the detection time of these biosensors would be extended until the detecting threshold of the biosensor is reached meaning extended by at least several additional hours of bacterial duplication time.

II. PROPOSED SOLUTION

The proposed concept is entirely novel and consists of an array (or matrix) of pyramidal-shape microelectrodes with dimensions (exposed surfaces) adjusted to the size of target bacterial pathogens (typically a few micrometers (μm)) for maximum sensitivity. A very simplified schematic of the proposed detection process is depicted in Fig. 1. The electrically conducting microbeads between the electrodes are coated with a specific type of phages (showed as dot lines in Fig. 1) acting as bioprobes that show specificity to particular pathogenic bacteria.

A solution of highly concentrated non-pathogenic magnetotactic bacteria [11] (MTB) such as *Magnetospirillum gryphiswaldense* [12] would be first injected in reservoir R2 through port P2 at the same time that a volume sample is injected in reservoir R1 through port P1 (Step 1 in Fig. 1). Immediately after injecting the samples, the swimming direction of all the MTB in reservoir R2 would be changed (Step 2 - Fig. 1) through magnetotaxis [13-15] and by the generation of magnetic field lines using

very small electrical current passing through a special conductors network (not shown in Fig. 1 for clarity). The long chain of magnetosomes imparts to the MTB a magnetic moment that generates sufficient torque so that the bacteria can align themselves to magnetic field lines. As the MTB swim in the direction of the reservoir R1, they would pass through a narrow channel between two electrodes such that some MTB could come in contact and self-bind to a conducting microbead or micro-sphere, a few micrometers in diameter (Step 3 in Fig. 1).

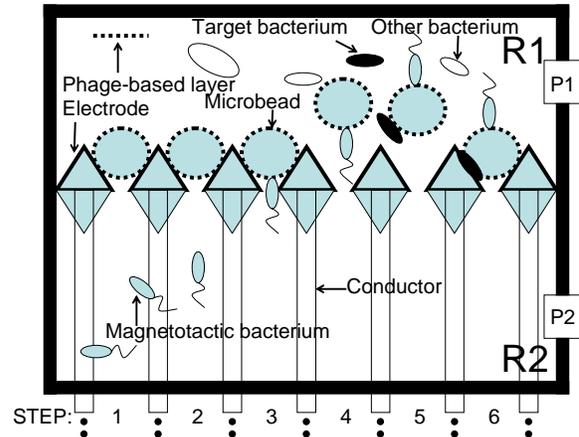


Fig. 1 - Simplified schematic (not to scale) of the detection process based on magnetotactic bacteria pushing microbeads coated with phages chosen specifically for targeting pre-selected types of pathogenic bacteria.

Prior to the detection process, the MTB are forced to generate activation of lipopolysaccharides to allow them to bind to the microbeads through a very simple procedure described later. We have shown in past experimental studies that they are capable of pushing microbeads in an aqueous medium and under software control (see Fig. 2 where a 3-micron sphere is being pushed by one controlled magnetotactic bacterium).

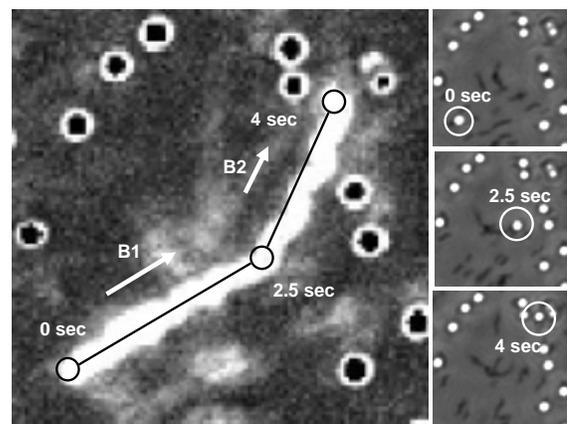


Fig. 2 - Example of a microbead (diameter of $3 \mu\text{m}$) being pushed by a single magnetotactic bacterium acting under computer control with a controlled directional change of approximately 30° anti-clockwise after 2.5 seconds.

Hence, once a MTB is attached to a microbead, a controlled sweep of the surrounding volume sample or area can be achieved by controlling the direction of displacement of each mobile micro-sphere (Step 4 in Fig. 1). Our previous experiments show that a single MTB can move a bead of 3 μm and 10 μm in diameter for instance with an average speed of ~ 16.3 and $4.9 \mu\text{m s}^{-1}$ respectively, corresponding from Stokes' law, to a thrust of ~ 0.5 pN per MTB. This suggests that sweeping the entire surrounding area would only require a few seconds, depending on the size of micro-volumes being scanned (see Fig. 3 for an example of a volume sample being swept by a swarm of magnetotactic bacteria).

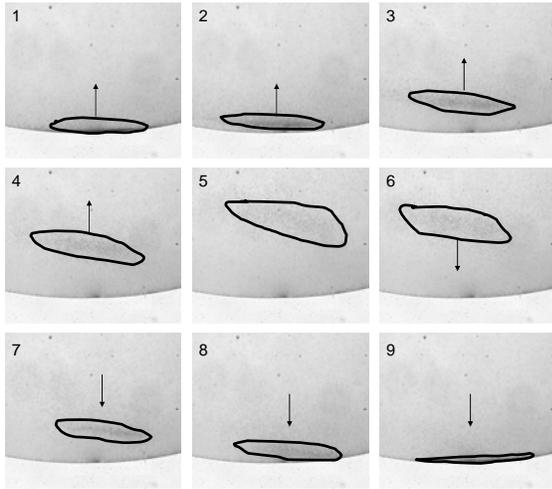


Fig. 3 – Swarm of MC-1 magnetotactic bacteria sweeping an aqueous solution at an average speed of $130 \mu\text{m/s}$ with a total round-trip distance being swept of approximately 1 mm.

Furthermore, we recently showed that another type of MTB known as MC-1 were much faster (about 4 times) and we verified experimentally that they could even swim in human blood, a very promising result that opens huge possibilities in medical-related detections. During the sweeping process, since each micro-sphere is previously coated with phages with a given specificity, only the target pathogenic bacteria will immediately bind to the micro-sphere when a contact occurs and not any other bacteria including MTB. Because of the presence of other types of bacteria in the samples, pumping flow (by mechanical means that require a very large pressure drop which severely limits their performance, electro-osmosis with limited applications because it relies on the electrical properties of the liquid, or other means) towards the electrodes would not only bring the pathogenic bacteria towards the biosensors but also the other types of bacteria and organisms that may be in much larger quantity. These non-pathogenic bacteria could prevent bacterial pathogens to come in contact with the very small electrodes and potentially interfere with the detection of pathogens, hence affecting sensitivity. Other well known techniques and in particular dielectrophoresis

(DEP) [16] could also be used but would have the same disadvantages as pumping since DEP force will also be induced on other organisms present in the samples, hence increasing the risks of interfering at the microelectrodes level and leading to a reduction of the sensitivity and specificity of the system. Unlike other actuation methods, bacteria are very effective for many operations in low Reynolds number hydrodynamics [17] as it is the case in microfluidic systems. Furthermore, unlike other microactuation methods, the proposed controlled bacterial actuation offers specificity by providing a displacement force that acts only on target pathogenic bacteria, a substantial advantage in this context where sensitivity must be maximized allowing only the microbeads with or without a pathogenic bacterium attached to it to be brought to the sensor electrodes (Step 5 in Fig. 1). Microbeads with diameters from 1 μm to 100 μm and coated with different types of material (including copper, a material suited for phage-binding) are available commercially [18] and are relatively low cost. Theoretically, the microbeads could have been eliminated by coating with small conducting nanoparticles the magnetotactic bacteria directly. Although the concept is interesting, it would demand too much on the microfabrication process which has tolerances typically too high to support such approach. Finally, impedance measurements are taken through conductors (see Fig. 1) to determine if a pathogenic bacterium has been trapped (Step 6 on Fig. 1). This detection phase is discussed later in more details.

III. MAIN ISSUES

A. Mobility Studies and Optimization of MTB-actuation

Although we have demonstrated that controlled bacterial actuation is possible at approximately $20\text{-}24^\circ\text{C}$ (see one example in Fig. 2), temperature effects on magnetotactic bacterial motion and efficiency need to be investigated for a higher temperature range of $30\text{-}42^\circ\text{C}$ used for the detection of pathogenic bacterial growth even if quick tests showed that MTB still motile when temperature is increased. The output of the MTB flagellar motor which is a rotary engine embedded in the cell wall and cytoplasm membrane is characterized by its torque and speed. It is known that this motor operates in two dynamic regimes [19]. Fortunately, the transition of low and high rotational speed regimes of the flagella where the relative torque decreases (known as the knee) occurs at higher rotational speeds with higher temperature with a lower rate of decline of torque. But the maximum rotational speed will be limited by the rate of proton transfer in the cell, and as such other parameters such as modifying the viscosity of the medium (with the addition of Ficoll for instance) to improve bacterial actuation, needs to be investigated as well. Hence, although bacterial actuation would most likely work at these higher temperature levels, its efficiency needs to be better

characterized under these conditions while finding ways to exploit it for this particular application.

B. MTB-microbead Binding Process and Optimal Density

Another important aspect is to determine the optimal density or concentration of MTB to be injected in the microsystem to obtain the best results. Several concentrations need to be tested to identify the minimum concentration that needs to be used to ensure that most microbeads become mobiles.

The binding process also needs to be improved. To force the MTB to generate activation of lipopolysaccharides to allow them to stick to the microbeads, the bacteria were remixed in a medium poor in concentration of nutrients for 5 minutes prior to inject a concentration between 1×10^6 and 1×10^8 BMT ml^{-1} in a new medium containing an average concentration of 5×10^6 3- μm diameter microbeads ml^{-1} . It was observed that during the first 5 min. when placed in the new medium in custom-made microfluidic channels, less than 1% of the bacteria attached themselves to the microbeads and after another ~ 5 min. of pushing the microbeads, one side of the bodies of the MTB were entirely glued to the micro-spheres resulting to an uncontrolled rotational motion, or the mobile microbeads suddenly stopped when the flagella were glued to the beads. Since in the new system, the MTB will become in contact through special channels between the electrodes instead of a random distribution of beads, we believe that the success rate will be higher or at least be compensated in part by a higher concentration of MTB. But in all cases, effort needs to be made to further improve the binding process and other self-attachment methods should be investigated as well.

C. Impedimetric Sensing and Electronics

The simple circuit model for the impedance of bacteria immobilized between two electrodes is depicted in the Fig. 4 below.

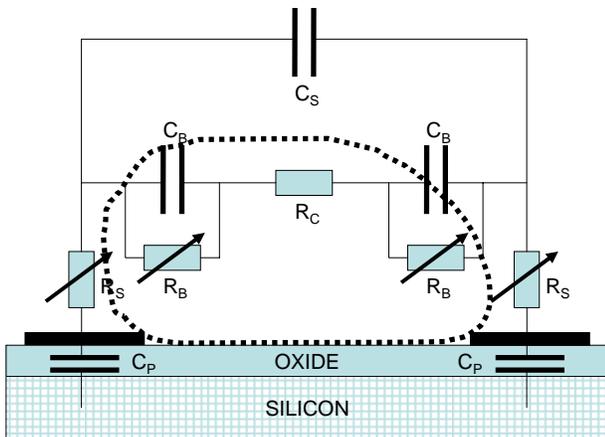


Fig. 4 – Simple circuit model for the impedance of a bacterium immobilized between two electrodes.

The resistivity of the bacteria cytoplasm [20] alone (R_C in Fig. 4) of $10^6 \Omega/\text{cm}^2$ is extremely high and prevents the detection of a single pathogenic bacterium with conventional approaches using electrodes reduced to the size of the target species for maximum sensitivity and detection speed. This is the reason that other biosensors wait for a larger number of pathogenic bacteria to bind to a larger surface electrode in order to have several R_C in parallel and then reducing the effective R_C at the cost of a lost in sensitivity and detection speed. In the proposed approach, the size and particular shape of the biosensors, the impedances of the microbead, cytoplasm, and the medium are combined to offer better performance.

Due to the large number of biosensors, the detection electronic circuit is maintained simple by considering three potential cases namely: 1. a very low impedance (high conductivity) when a microbead is located between 2 microelectrodes (Fig. 1) but has no pathogenic bacteria, 2. a very high impedance (appear like an open circuit or floating connection) due to the cytoplasm of a pathogenic bacterium between one microelectrode and the conducting microbead located between two microelectrodes causing signal saturation on the sensing circuit, and 3. an intermediate impedance ($\sim 70\text{k}$) through the medium when no microbead is located between a given pair of microelectrodes. Notice that we know that the phages with dimensions of approx. 90 nanometers (nm) do not affect impedance significantly. We have conducted some experiments on various mediums such as saline solutions, salt water, blood, etc. and recorded impedances in the few tenths of k-ohms, allowing a huge selection of aqueous solutions and a relatively easy and simple threshold detection scheme due to the larger difference between the 3 potential conditions or impedance ranges. Moreover, the special shape of the microelectrode unlike planar electrodes contributes to lower unwanted characteristics in some cases such as the resistivity and capacity of the solution (R_S and C_S in Fig. 4) for instance. Furthermore, the pyramidal-like shape with the pushing force exerted on the biosensor by the MTB contributes to a better contact and sensitivity with the sensing electrodes. To avoid the complexity and unrealistic packaging issues due to the large number of interconnects involved, one must integrate the switching circuitry on-chip and provide an interface port such as I2C and a few analog connections at the periphery of the integrated circuit (IC) for selecting through a serial protocol the electrode pair, input for the stimulation signals, and to record the impedance outputs.

D. Microfabrication

Because of the potentially large number of microelectrodes for relatively large volume samples, the microelectrodes must be built on the silicon IC itself. The encapsulation of a reservoir to contain the bacterial samples on top of the silicon-based system is a conventional process. The part of the microfabrication which is less conventional

is the implementation of the microelectrodes on the surface of the IC. Several microfabrication approaches are available and include but are not limited to the use of surface micromachining, several etching and deposition techniques, etc.

It should be noted that the shape of the electrodes can vary around a pyramidal, conical, or being as simple as a bump, making its implementation potentially simpler. Two possible approaches among others can be envisioned. First, a high fill factor array of metallic electrode hemispheres using the melting resist technology [21] can be produced. This technology has been developed to fabricate microlenses. It can be applied as well to metals in order to generate high fill factor hemispheres arrays, or half-donut shapes arrays. The advantage of the half-donut shape is that it can provide a receptacle while minimizing the number of electrodes from four to two 180-degrees electrodes, potentially improving the effective detector density further. The size of the microbeads can be enlarged if required to facilitate the microfabrication of electrodes but at the cost of a lower scan speed of the MTB. The second approach is to bond a Si wafer to the CMOS integrated circuit and to polish the Si wafer down to a couple of microns. Anodic bonding has been demonstrated not to damage the CMOS circuitry [22]. The bonded single crystalline Si layer can then be patterned in an array of tips similar to the ones used in scanning force microscopy [23].

E. Integration and Operating Cost Reduction

Efforts are also essential on the integration aspect including the integration of the microbeads. More specifically, the optimal number of microbeads to be included in the microsystem to ensure that there is a bead between each or most electrodes pairs needs to be determined. A method considered to put the beads previously coated with phages is to fill the reservoir with a larger quantity of beads and to “wash” the extra with a lateral flow with velocity adjusted so that the encapsulated beads (between pair of electrodes) remain in place. The excess microbeads could also be used to fill the next round (usage) or system. Operating cost is always an important issue that may prevent the exploitation of a technology and as such, effort dedicated to operating cost reduction is a must. Among several options, the possibility to re-use the same detection system several times is an attractive option. In past attempts by other groups, small electrodes were coated with antigens or the like and were impossible to clean. With the proposed system, only the microbeads are coated with antigens or phages such as to avoid cleaning. By throwing inexpensive microbeads after each usage, the system would be much easier to clean and potentially lead to a lower operating cost through more tests being performed per system.

IV. DISCUSSION AND CONCLUSION

Our preliminary results on controlled bacterial actuation opens the possibility for the development of novel field-ready biosensing microrobotic systems for the detection of bacteria and bacterial growth with shorter detection periods and higher sensitivities compared with conventional methods. The originality of the proposed biosensing microsystems relies on the combination of phage-based biosensors which have proven to be very effective and controlled magnetotactic bacteria-based micro-actuation methods. By combining for the first time these approaches and integrating them in an innovative design concept adapted to these technologies with sensory elements reduced to the scale of the target species, the three main critical specifications of a biosensor namely, specificity, detection time and sensitivity can be improved significantly compared to existing systems. Specificity is guaranteed by biocapture molecules that consist of natural phages that are isolated by screening against a large repertoire of targeted pathogenic bacteria. Although antibodies could also be used in this concept, phages are favoured in a first approach as they can be easily produced, are more stable than antibodies and hence better suited for biosensors in a field environment [24-26]. Additionally phages have higher binding forces compared to antibodies, a particularly important factor in view of the proposed concept. Moreover, it is also possible to genetically engineer phages in order to recognize any type of living organisms including viruses, molds, fungi, etc., and also toxins or other proteins. These characteristics combined with specificity will allow high levels of specific detection of the most important and dangerous pathogenic bacteria in a two step sensing system detecting in few minutes the presence of bacteria and in few hours the presence of live bacteria. These characteristics are significantly different from existing technologies, which measure both living and non-living pathogens without discrimination, have relatively little flexibility to be adapted to a larger selection of pathogens and have a much higher threshold of detection.

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REFERENCES

- [1] M. Madigan, J. Martinko, and J. Parker, *Brock Biology of Microorganisms*, 8th ed. Upper Saddle River, NJ: Viacom, 1997.
- [2] B. Hoyle, "High-tech biosensor speeds bacteria detection," *ASM News*, vol. 67, pp. 434-435, 2001.
- [3] D. DeMarco and D. Lim, "Detection of *Escherichia coli* O157:H7 in 10- and 25-gram ground beef samples with an evanescent-wave biosensor with silica and polystyrene waveguides," *J. Food Protection*, vol. 65, pp. 596-602, 2002.
- [4] K. Seo, R. Brackett, N. Hartman, and D. Campbell, "Development of a rapid response biosensor for detection of *Salmonella* Typhimurium," *J. Food Protection*, vol. 62, pp. 431-437, 1999.
- [5] F. Fratamico, T. Strobaugh, M. Medina, and A. Gehring, "Detection of *Escherichia coli* O157:H7 using a surface plasmon resonance biosensor," *Biotechnol. Tech.*, vol. 12, pp. 571-576, 1998.
- [6] C. Meeysen, E.C. Alcilija, and W. Osburn, "Detection of *E. coli* O157:H7 using a surface plasmon resonance biosensor," *Trans. ASAE*.
- [7] L. Bao, L. Deng, L. Nie, S. Yao, and W. Wei, "Determination of microorganisms with a quartz crystal microbalance sensor," *Anal. Chim. Acta.*, vol. 319, pp. 97-101, 1996.
- [8] A. Ghindilis, P. Atanasov, M. Wilkins, and E. Wilkins, "Immunosensors: electrochemical sensing and other engineering approaches," *Biosens. Bioelectron.*, vol. 13, pp. 113-131, 1998.
- [9] J. Gau, E.Lan, B. Dunn, C. Ho, and J. Woo, "A MEMS based amperometric detector for *E. coli* bacteria using self-assembled monolayers," *Biosens. Bioelectron.*, vol. 16, pp. 745-755, 2001.
- [10] J. Rishpon and D. Ivnitcki, "An amperometric enzyme-channeling immunosensor," *Biosens. Bioelectron.*, vol. 12, pp. 195-204, 1997.
- [11] R.P. Blackmore, "Magnetotactic bacteria," *Science*, vol. 190, pp. 377-379, 1975.
- [12] K.H. Schleifer, D. Schueler, S. Spring, M. Weizenegger, R. Amann, W. Ludwig, and M. Kohler, "The genus *Magnetospirillum*, gen. nov., description of *Magnetospirillum gryphiswaldense*," *Syst. Appl. Microbiol.*, vol. 14, pp. 379-385, 1991.
- [13] R.B. Frankel and R.P. Blakemore, "Navigational compass in magnetic bacteria," *J. of Magn. and Magn. Materials*, vol. 15-18(Part 3), pp. 1562-1564, 1980.
- [14] C. Denham, R. Blakemore, and R. Frankel, "Bulk magnetic properties of magnetotactic bacteria," *IEEE Trans. on Magnetics*, vol. 16(5), pp. 1006-1007, 1980.
- [15] H. Debarros, D.M.S. Esquivel, and M. Farina, "Magnetotaxis," *Sci. Progr.*, **74**, pp. 347-359, 1990.
- [16] H.A. Pohl, "Dielectrophoresis," Cambridge, UK: Cambridge University Press, 1978.
- [17] J. Happel and H. Brenner, "Low Reynolds number hydrodynamics," *Martinus Nijhoff, The Hague*, the Netherlands, 1983.
- [18] <http://66.33.116.242/glass.php/metaspheres.html>
- [19] X. Chen and H.C. Berg, "Torque-speed relationship of the flagellar rotary motor of *Escherichia coli*," *Biophysical Journal*, vol. 78, pp. 1036-1041, 2000.
- [20] H. Tien and A. Ottova-Leitmannova, "Membrane biophysics as viewed from experimental bilayer lipid membranes," *Amsterdam, The Netherlands: Elsevier*, 2000.
- [21] Ph. Nussbaum, R. Völkel, H. P. Herzig, M. Eisner, and S. Haselbeck, Design, fabrication and testing of microlens arrays for sensors and microsystems," *Pure & Applied Optics*, vol. 6, pp. 617-636, 1997.
- [22] S. van der Groen, M. Rosmeulen, K. Baert, P. Jansen and L. Deferm, "Substrate bonding techniques for CMOS processed wafers", *J. Micromech. Microeng.* Vol. 7, 108-110, 1997.
- [23] O. Wolter, Th. Bayer and J. Greschner, *J. Vac. Sci. Technol. B* vol. **9**, 1353-1357 (1991).
- [24] V. Petrenko *et al.*, "A library of organic landscapes on filamentous phage," *Protein Eng.*, **9**(9), 797-801, 1996.
- [25] G. Smith and V.A. Petrenko, "Phage display- Review," *Chemical Reviews*, **97**(2), pp. 391-410, 1997.
- [26] V.A. Petrenko and G.P. Smith, "Phages from landscape libraries to substitute antibodies," *Protein Engineering*, **13**(8), pp. 589-592, 2000.