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Chemotaxis for enhanced immobilization of *Escherichia coli* and *Legionella pneumophila* on biofunctionalized surfaces of GaAs

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The authors have investigated the effect of chemotaxis on immobilization of bacteria on the surface of biofunctionalized GaAs (001) samples. *Escherichia coli* K12 bacteria were employed to provide a proof-of-concept of chemotaxis-enhanced bacterial immobilization, and then, these results were confirmed using *Legionella pneumophila*. The recognition layer was based on a self-assembled monolayer of thiol functionalized with specific antibodies directed toward *E. coli* or *L. pneumophila*, together with the enzyme beta-galactosidase (β-gal). The authors hypothesized that this enzyme together with its substrate lactose would produce a gradient of glucose which would attract bacteria toward the biochip surface. The chemotaxis effect was monitored by comparing the number of bacteria bound to the biochip surface with and without attractant. The authors have observed that β-gal plus lactose enhanced the immobilization of bacteria on our biochips with a higher effect at low bacterial concentrations. At 100 and 10 bacteria/ml, respectively, for *E. coli* and *L. pneumophila*, the authors observed up to 11 and 8 times more bacteria bound to biochip surfaces assisted with the chemotaxis effect in comparison to biochips without chemotaxis. At 10^4 bacteria/ml, the immobilization enhancement rate did not exceed two times. © *2016 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4947048]

I. INTRODUCTION

Rapid detection of pathogenic organisms in complex media is critical to ensure water quality, food safety, management of infectious disease, reliable agricultural production, and as a counter measure against biological weapons. While many sophisticated biosensors and assay methods have been developed for detecting pathogenic microorganisms, much of the effort has focused only on detection as a stand-alone task while the importance of, and challenges in, sample preparation prior to detection have been largely overlooked. Most detection methods use a very small sample volume, which limits the power to detect small quantities of pathogens dispersed in large quantities of material such as food or water.[1] Thus, being able to concentrate bacteria in small volumes of sample designed for analysis is of paramount importance to the development of advanced biosensing methods. For instance, a submicron superparamagnetic anion exchanger (SiMAG-DEAE, Chemicell, Berlin, Germany) was used to target dilute aqueous solutions as a method to improve sensitivity for subsequent polymerase chain reaction detection.[2] Collection and concentration of bacteria have also been achieved via ion-exchange, immunoaffinity, lectins, and metal hydroxides, as well as centrifugation, filtration, aqueous two-phase partitioning, and magnetic separation.[3–5]

Gallium arsenide (GaAs) is a popular photonic material found in fabrication of semiconductor lasers and light emitting devices. We have recently demonstrated rapid detection of *Escherichia coli* at 10^3 CFU/ml,[6,7] and *Legionella pneumophila* at 10^4 CFU/ml,[8] using photoluminescence (PL) emitting GaAs/AlGaAs microstructures. The detection sensitivity of such photonic biosensors depends on the efficiency of bacteria immobilization on their surfaces. This also applies to other biosensing platforms, such as that represented by a quartz crystal microbalance biosensor designed for specific capture of bacteria on gold surfaces.[9] Thus, concentrating the bacteria near, or at the surface of such biosensors could potentially result in increased sensitivity of these devices. We postulated that chemotaxis could be used as an efficient and simple way to enhance detection of bacteria. Chemotaxis (or more accurately, chemokinesis) is the process by which a cell alters its speed or frequency of turning in response to an extracellular chemical signal and permits drift toward spatial regions optimum for growth and survival (e.g., with high nutrient concentration).[10] This has been shown for *E. coli* or *Salmonella* to involve a motile system consisting of several helical flagella, each with a rotary

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motor at their base, embedded in the cell wall.[10] If the motor turns counter-clockwise, the flagella come together to form a spinning bundle and the cell swims forward (a run) for 1.0 s. If one or more of the motors turn clockwise, the bundle becomes unstable, and the cell turns at random (a tumble) for 0.1 s, with no net displacement. The purpose of a tumble is to reorient the cell to a new (random) direction. In the absence of an attractant gradient, this results in a diffusive random walk with a diffusion constant of 4×10^{-6} cm^2/s.[10–12] The *E. coli* chemotaxis pathway is so sensitive that it is able to sense a change in a few molecules of attractants.[13] In the presence of a gradient of attractant (or repellent), the bacteria use temporal comparisons of the attractant concentration over the preceding ≈3–4 s to determine if conditions are improving or deteriorating.[14,15] If the tumbles are suppressed, cells move in a favorable direction. Conversely, when conditions deteriorate, the cell increases its chances of finding a favorable new direction by tumbling. This combination of run-and-tumble swimming and bias control leads to a drift velocity (in steep gradient of attractant) of around ≈7 μm/s.[16]

In this manuscript, we investigate the use of chemotaxis to enhance *E. coli* K12 and *L. pneumophila* immobilization on immune-recognition layer biofunctionalized GaAs (001) biochips by attaching β-galactosidase to the biochip surface to create a glucose/galactose gradient by lactose hydrolysis[17] inducing chemotaxis of flagellated bacteria toward the surface where they could be captured by specific antibodies also bound to the biochip surface.[18]

II. EXPERIMENT

A. Materials

Polyclonal antibodies against *L. pneumophila* coupled with biotin and unconjugated anti-*E. coli* antibodies were obtained from ViroStat, Inc. (Portland, Marine). *E. coli* K12 were provided from the bacterial collection of the Biology Department of the Université de Sherbrooke Faculty of Sciences. *E. coli* carry peritrichous flagella.[19] *L. pneumophila* ssp1 were provided by Magnus (Boucherville, Canada). These bacteria carry a single monopolar flagellum.[20] BCYE agar medium was provided by Becton, Dickinson and Company (Sparks, MD, USA). Neutravidin was obtained from Molecular Probes Invitrogen (Burlington, Canada). HS-CH_{11}-EG_3-biotin (Biotinylated polyethylene glycol (PEG) thiol) was obtained from ProChimia Surfaces (Gdansk, Poland). 16-mercaptohexadecanoic acid (MHDA), 11-mercaptoundecanoic acid (MUDA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), *N*-hydroxysuccinimide (NHS), β-galactosidase, Luria Bertani broth, lactose, and phosphate buffered saline (PBS) solution were purchased from Sigma-Aldrich, Oakville, Canada. OptiClear, acetone, anhydrous ethanol, and isopropanol (2-propanol) were obtained from National Diagnostics (Mississauga, Canada), ACP (Montreal, Canada), Commercial Alcohols, Inc. (Brampton, Canada), and Fisher Scientific (Ottawa, Canada), respectively. Ammonium hydroxide (28%) was obtained from Anachemia (Richmond, Canada). Water was deionized to 18.2 MΩ resistance (DI water) with a Millipore purification custom system built by Culligan (Quebec, Canada). Wafers of GaAs (001), series VW 10680–53 and 54, were bought from Wafer Technology, Ltd. (Milton Keynes, UK).

B. Biochip functionalization

1. GaAs surface cleaning

Chips of 2 × 2 mm, obtained by cleaving GaAs (001) wafers, were cleaned sequentially for 5 min in an ultrasonic bath of undiluted OptiClear, acetone, and isopropanol. The chips were then dried using a flow of high-purity (99.9995%) compressed nitrogen and etched with a solution of concentrated ammonium hydroxide (28%) for 2 min at room temperature to remove surface native oxides, such as Ga_2O_3, As_2O_5, and As_2O_3.

2. Binding architectures

Two recognition layer architectures were investigated: MUDA thiol and PEG-biotin thiol, for the immobilization of *E. coli* and *L. pneumophila*, respectively.

a. MUDA thiol based architecture. Reference MUDA GaAs biochips: The cleaned GaAs chips were immersed for 20 h in MUDA thiol solution (0.5 mM) in degassed anhydrous ethanol. Consequently, the thiol modified surface was activated using a mixture of EDC (0.1 mM) and NHS (0.4 mM) prepared in 18.2 MΩ resistance DI water. The activated chips were incubated in a solution of unconjugated *E. coli* antibody in PBS (100 μg/ml) for 1 h. Finally, the antibody functionalized chips were incubated for 1 h in bovine serum albumin (BSA) (2%) in PBS to saturate the remaining active sites. We note that other thiol-based architectures have also been investigated for biofunctionalization of GaAs,[21–24] and noble metal substrates.[25–27]

MUDA GaAs biochips for chemotaxis: In order to induce chemotaxis, a supplementary functionalization step was integrated into the procedure: following the unconjugated *E. coli* antibody attachment step, the biochips were incubated for 1 h in 50 IU (1 IU corresponds to the quantity of enzyme that cleaves 1 μmol of lactose substrate into glucose and galactose per min at 25 °C) of β-galactosidase in PBS solution. Figure 1(a) presents a schematic representation of the MUDA based recognition layer architecture.

b. PEG-biotin/MHDA thiol based architecture. Reference PEG-biotin/MHDA GaAs biochips: The cleaned GaAs chips were immersed for 20 h in a mixture of PEG-biotin and MHDA thiol [0.5 mM 1:15 (M/M)] in 5 ml of degassed anhydrous ethanol. Subsequently, the thiol-modified chips were incubated for 2 h with 200 μl of neutravidin (200 μg/ml) in PBS in a 0.5 ml microfuge tube in order to fix the neutravidin to the thiol layer by its association with the biotinylated thiol. The extra biotin binding groups in the neutravidin layer allowed immobilization of biotinylated *L. pneumophila* antibody after the incubation of the modified chips in 200 μl

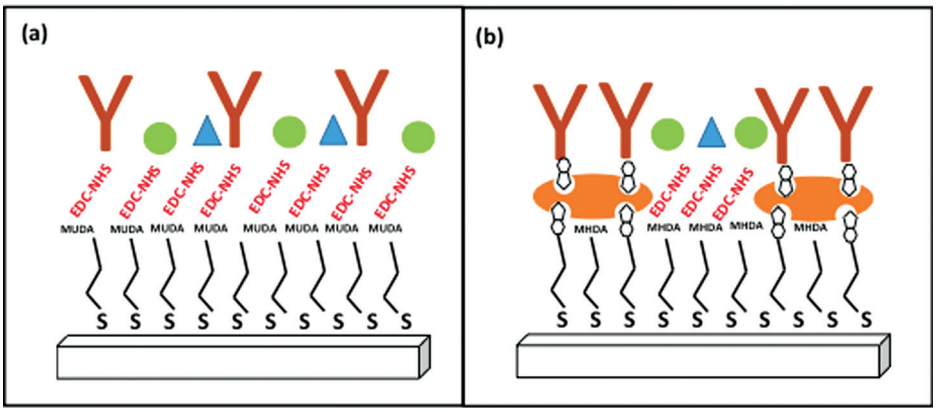

FIG. 1. (a) MUDA thiol-EDC/NHS architecture of the biochip for chemotaxis of *E. coli*. Antibody is represented as a brown Y, β-galactosidase (green ●), BSA (blue ▲). Reference MUDA GaAs biochip (not shown) was identical but without β-galactosidase. (b) PEG-biotin/MHDA architecture of the biochip for chemotaxis of *L. pneumophila*. Neutravidin is represented as an orange oval with four biotin binding sites, biotinylated *L. pneumophila* antibody represented as a brown Y with black biotin tag, β-galactosidase (green ●), BSA (blue ▲). Reference PEG-biotin GaAs biochip (not shown) was identical but without β-galactosidase.

of antibody solution prepared in PBS (100 μg/ml) in a 0.5 ml tube. This step was carried out for 1 h at room temperature. We employed MHDA thiols rather than hexadecanethiol used previously,[6] to allow β-galactosidase immobilization as described in the below paragragh.

PEG-biotin/MHDA GaAs biochips for chemotaxis: The initial functionalization procedure was similar to that of the PEG-biotin functionalization of a reference biochip, but the procedure was modified to immobilize the β-galactosidase enzyme. Prior to neutravidin addition, the thiolated surface was activated for 30 min with EDC-NHS (0.4/0.1 M) prepared in DI water. The biochips were then incubated for 1 h in 50 UI β-galactosidase in PBS solution. The remaining active sites were blocked with a solution of 2% BSA. Neutravidin was added, and subsequent steps were followed as for the reference PEG-biotin biochips. Figure 1(b) presents a schematic representation of the PEG-biotin based recognition layer architecture.

C. Reaction of functionalized surfaces with bacteria

E. coli was grown overnight in Luria-Bertani broth. Colonies of *L. pneumophila* ssp1 on BCYE agar medium were suspended in PBS. After a centrifugation step (25 min at 3000 rpm) to remove culture medium and suspension in PBS, the concentration of the bacteria was quantified with a spectrophotometer (Thermo-Fisher, Model 40). By diluting, plating on petri dishes and counting the bacterial colonies, we determined that an optical density at 600 nm of 0.1 corresponded to 10^8 *E. coli* CFU/ml or 8×10^7 *L. pneumophila* CFU/ml. Dilutions were carried out in PBS or in PBS plus lactose to reach final concentrations of bacteria at 10, 10^2, 10^3, and 10^4 CFU/ml. Bacteria would not be expected to multiply in PBS with only lactose as they require at least a source of nitrogen.

The *E. coli* K12 biofunctionalized chips were exposed to bacterial solutions at concentrations ranging from 0.5×10^2 to 10^4 CFU/ml and incubated for 1 h in 0.5 ml microcentrifuge tubes. Exposure of *L. pneumophila* to biofunctionalized chips was carried out for 1 h in a 150 μl microfluidic chamber with concentrations ranging from 10 to 10^4 CFU/ml. No agitation or fluid flow was applied when attempting to bind either *E. coli* or *L. pneumophila*. After PBS washing, *E. coli* was quantified using an optical microscope (Zeiss, Axiotech) operating in a differential interference contrast mode, whereas *L. pneumophila* was quantified by incubating the biochips for 1 h in 50 μg/ml of fluorescein tagged anti-*Legionella* antibodies. These biochips were subsequently exposed to isopropanol for 1 h to inactivate *L. pneumophila*. The number of bound *L. pneumophila* was quantified by fluorescence microscopy using a fluorescence microscope (Olympus IX71). Numbers of bacteria were counted manually.

The PEG-biotin based architecture showed a high nonspecific interaction with *E. coli* K12, even at 2.5×10^5 CFU/ml, leading to coverage of 55 ± 8 bacteria per mm^2 in the absence of antibody. In the case of the MUDA based architecture (without antibody), only 1 ± 0.5 bacteria per mm^2 were immobilized at a bacterial concentration of 2.5×10^5 CFU/ml. On the other hand, *L. pneumophila* did not show a high affinity to neutravidin with a coverage not exceeding 0.25 bacteria/mm^2 for a bacterial concentration of 10^5 CFU/ml. Consequently, MUDA based architectures were used for *E. coli* K12 and PEG-biotin for *L. pneumophila*.

III. RESULTS AND DISCUSSION

A. Immobilization of *E. coli* K12

The *E. coli* functionalized reference and chemotaxis GaAs biochips were exposed separately to *E. coli*. As can be seen in Fig. 2, for the reference biochip, 1 ± 0.25, 4 ± 1, and 13 ± 2.5 bacteria were immobilized per mm^2, respectively, for concentrations of 10^2, 10^3, and 10^4 CFU/ml. At the 0.5×10^2 CFU/ml bacterial concentration, we did not observe any bacteria. The chemotaxis effect was investigated for the functionalized GaAs biochips exposed to solutions of *E. coli* K12 at 10^2, 10^3, and 10^4 CFU/ml in samples mixed with 50 mg/ml lactose. The number of *E. coli* immobilized from these solutions was, respectively, 11 ± 1, 19 ± 1, and

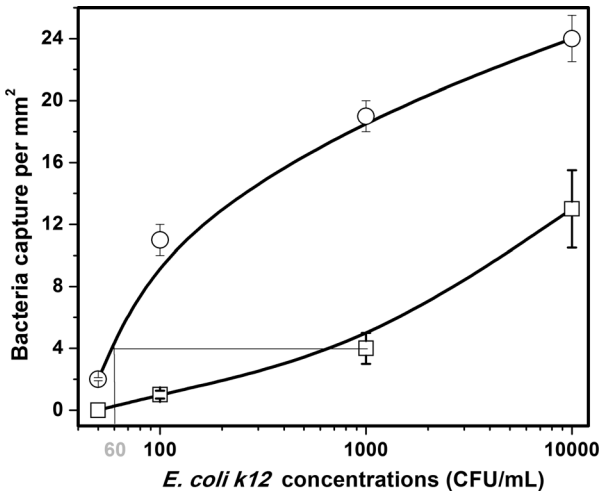

FIG. 2. *E. coli* K12 surface capture as a function of the concentration of bacteria achieved with (○) and without chemotaxis (□).

24 ± 1.5 bacteria per mm^2. It can be seen that 4 ± 1 of *E. coli* per mm^2 were immobilized under unassisted conditions from 10^3 CFU/ml. We note that detection of *E. coli* at 10^3 CFU/ml is the limit achieved with photocorroding GaAs/AlGaAs biochip microstructures functionalized with PEG-biotin/HDT (hexadecanethiol)-based architectures,[7] which could be associated with the presence of bacteria immobilized at such surface density. Thus, if assisted with chemotaxis, this same number of bacteria could be counted from a concentration of ~60 CFU/ml, indicating a potential 16-fold sensitivity improvement of a photocorrosion based biosensor.

The enhancement of the number of *E. coli* K12 captured on the biochip that was induced by chemotaxis is presented as a function of the concentration of bacteria in Fig. 3. It can be seen that the enhancement associated with chemotaxis is stronger at lower bacterial concentrations. Chemotaxis increased bacterial capture ~1.8 times at 10^4 CFU/ml, but 11 times more bacteria were captured from a bacterial solution at 10^2 CFU/ml.

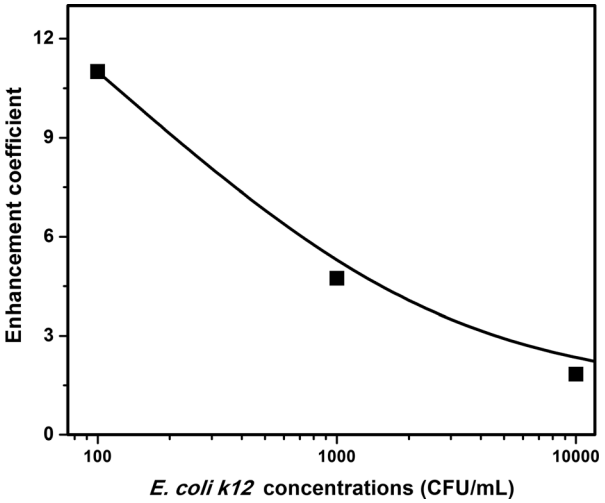

FIG. 3. Enhancement of *E. coli* K12 surface capture induced by chemotaxis as a function of the concentration of bacteria.

B. Immobilization of *L. pneumophila*

L. pneumophila functionalized reference and chemotaxis GaAs biochips were exposed separately to 10, 10^2, 10^3, and 10^4 *L. pneumophila* CFU/ml. Without chemotaxis, ~0.75, 12 ± 1.5, 19 ± 1.5, and 23 ± 2 bacteria, respectively, were immobilized per mm^2 whereas with chemotaxis, immobilization of 6 ± 0.25, 28 ± 1, 41 ± 3, and 42 ± 2 bacteria per mm^2, respectively, were observed. These results are summarized in Fig. 4. We have reported that the lower limit of detection by PL for *L. pneumophila* was 10^4 CFU/ml.[8] It can be seen that ~23 *L. pneumophila* per mm^2 were immobilized under unassisted conditions at the concentration of 10^4 CFU/ml. In contrast, this same number of bacteria could be immobilized by a chemotaxis-assisted process from a suspension at ~70 CFU/ml, which is 140 times less.

The enhancement of the surface capture of *L. pneumophila* that was induced by chemotaxis is presented as a function of the concentration of bacteria in Fig. 5. It illustrates that chemotaxis again plays a more important role at lower concentrations of bacteria, with more than eight times enhanced bacterial immobilization observed for 10 CFU/ml. The enhancement factor falls below 2 for 10^4 CFU/ml.

We have reported that with similar MUDA-antibody or PEG-biotin-neutravidin-biotinylated antibody architectures we were able to detect *E. coli* at 10^3 CFU/ml,[7] and *L. pneumophila* at 10^4 CFU/ml.[8] In fact, to ensure PL detection, a critical minimum number of bacteria should be immobilized on the surface. Other detection methodologies also require critical minimum numbers of bacteria immobilized on the surface to yield a positive signal. For *E. coli* and *L. pneumophila*, immobilization of bacteria at concentrations under the detection limits can be visualized microscopically,[6–8] but the achieved coverage was not enough to induce PL signal variation of photocorroding GaAs/AlGaAs biochips. We have thus employed microscopic visualization and counting of bacteria to quantify the effect of chemotaxis. We propose that chemotaxis could improve the sensitivity of PL detection of *E. coli* and *L. pneumophila* by 16 to 140-fold. The

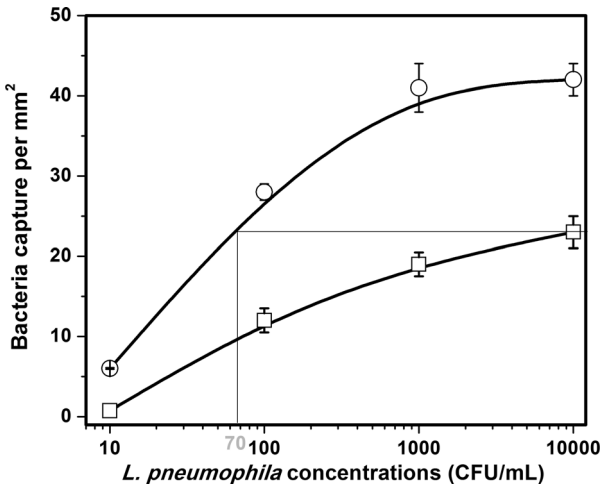

FIG. 4. *L. pneumophila* surface capture as a function of the concentration of bacteria with (○) and without chemotaxis (□).

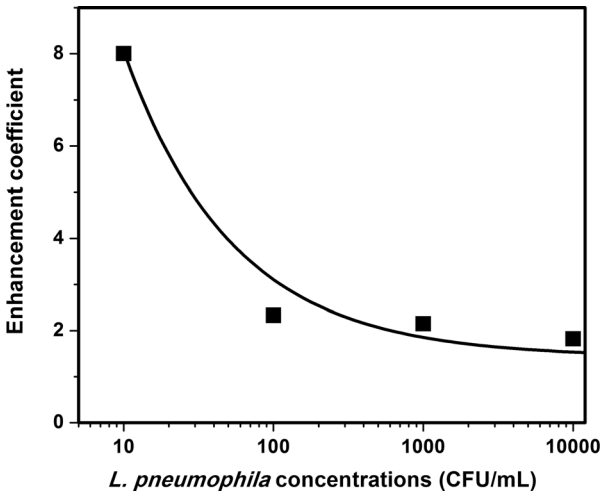

FIG. 5. Enhancement of *L. pneumophila* surface capture induced by chemotaxis as a function of the concentration of bacteria.

sensitivity of detection of other detection strategies could also, no doubt, be improved by chemotaxis.

We propose that the mechanism of action of chemotaxis depends on the generation of glucose at the surface of the biochip. Glucose is the preferred energy and carbon source for most, if not all, microorganisms. In the presence of glucose and lactose, lactose use is repressed until all of the glucose is consumed giving rise to diauxic growth.[28] Chemotaxis, thus, attracts bacteria toward sources of glucose. In the absence of an attractant gradient, bacteria move in a diffusive random walk, whereas the presence of an attractant leads to a direction and increase in velocity. This directed motility of the bacteria together with an increased speed of movement explains why, in the case of chemotaxis, increased numbers of bacteria are immobilized on the biochip surface. Normally, negatively charged bacteria are repulsed from the negatively charged biochip surfaces. We propose that chemotaxis partially overcomes these repulsive forces by attracting bacteria toward the surface where they are captured by antibodies. Increasing concentrations of bacteria would increase the probability of random interactions with the surface and so chemotaxis would have less influence. Chemotaxis lured bacteria with food toward the surface where they were captured, analogous to the Sirens of Greek mythology, whose song lured sailors toward their islands where their ships were wrecked and thus immobilized on the rocks.

IV. CONCLUSIONS

We have investigated the influence of chemotaxis on the immobilization of *E. coli* K12 and *L. pneumophila* on antibody biofunctionalized GaAs (001) biochips. Chemotaxis was induced with the enzyme beta-galactosidase (β-gal) that together with its substrate lactose produced a gradient of glucose acting as a bacterial attractant toward the biochip surface. A comparison between bacteria immobilized on the reference (no β-gal) and chemotaxis assisted biochips revealed 11 and 8 times enhancement of the surface immobilized *E. coli* and *L. pneumophila*, respectively, for 10^2 and 10^4 CFU/ml concentrations of these bacteria. The enhancement factor decreased with increasing concentration of bacteria, and for 10^4 CFU/ml, it was reduced to less than 2. The results suggest that with chemotaxis, the GaAs/AlGaAs photonic biosensor has the potential to detect *E. coli* at 60 CFU/ml or *L. pneumophila* at 70 CFU/ml. The sensitivity of detection of other detection strategies could also, no doubt, be improved by chemotaxis.

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