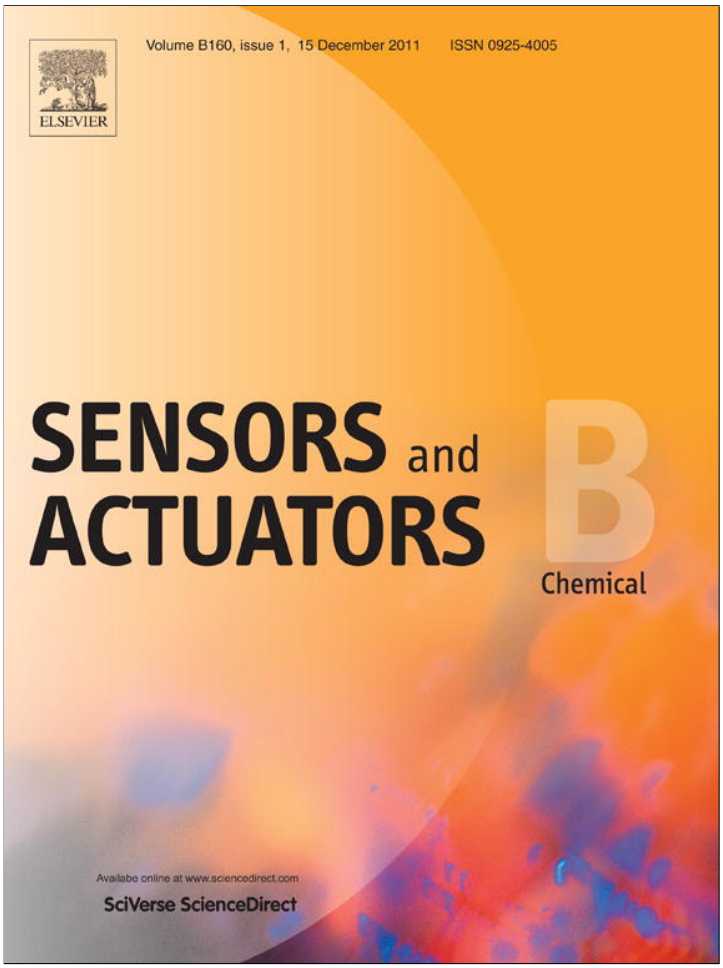

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A photoluminescence-based quantum semiconductor biosensor for rapid *in situ* detection of *Escherichia coli*

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ABSTRACT

This work describes a novel method of detecting *Escherichia coli* using photoluminescence (PL) emission from III–V quantum semiconductor (QS) devices functionalized with two different antibody-based architectures. The first approach employed self-assembled monolayers of biotinylated polyethylene glycol thiols to immobilize biotinylated antibody via neutravidin. In the second approach, we used QS microstructures coated with a thin layer of Si_3N_4 allowing direct functionalization with *E. coli* antibodies through hydrofluoric acid etching and glutaraldehyde-based reticulation. Atomic force, optical and fluorescence microscopy measurements were used to assess the immobilization process. Depending on the biosensing architecture, density of the immobilized bacteria was observed in the range of 0.5–0.7 bacteria/100 μm^2. The detection of *E. coli* at 10^4 CFU/ml was achieved within less than 120 min of the bacteria exposure. It is expected that an even better sensitivity threshold could be achieved following further optimization of the method.

1. Introduction

Traditional methods of pathogenic bacteria detection require the use of sophisticated analytical laboratories, often in centralized facilities, which require considerable capital and a highly skilled workforce [1–4]. However, there are many cases where this approach is inadequate, mainly due to the long time-to-result period. For example, some standard methods, such as, the ISO 11731:1998 and ISO 6222:1999 for the detection of *Legionella pneumophila* require up to 10 days to yield results, as they rely on the ability of micro-organisms to multiply to visible colonies [5]. Molecular detection methods such as polymerase chain reaction (PCR), although more rapid than culture based approaches, again require highly qualified personnel in central laboratory facilities. New, easy to use, technologies capable of rapid, selective and sensitive detection are needed for the detection of various pathogens [6–8]. Examples of methods studied for detection of food-borne and water-borne micro-organisms include surface plasmon resonance (SPR), electrochemical, impedimetric and piezoelectric [6,9–11]. Due to the potentially rapid response of optical effects to surface located biochemical reactions, this approach has increasingly been investigated for detection of biological molecules such as DNA, bacteria, and other proteins or pathogens [12–15]. The use of antibody- or DNA-functionalized nanoparticles has also been investigated for single bacterial cell quantitation [16]. However, to deliver a portable and economically attractive device for rapid detection of *Escherichia coli* (and other bacteria) has remained an elusive problem. We have proposed that bright photoluminescence (PL) of epitaxial quantum dots (QD) could be used to study biochemical reactions on surfaces of semiconductors made from elements of the third and fifth column of the periodic table (III–V semiconductors) [17]. Recently, we have demonstrated that optically confined GaAs/AlGaAs epitaxial microstructures could be used to monitor surface effects related to the formation of self-assembled monolayers (SAM) of alkanethiol [18] and decomposition of thimerosal [19]. The attractive feature of such quantum semiconductor (QS) devices is that their optical response could be monitored with photonic detectors of relatively small dimensions, which makes them suitable for the development of portable biosensing instruments. Generally, the functioning of a QS biosensor depends not only on its sensitivity, but also on the ability to maintain a stable response over an extended period of time. Indeed, the exposure of bio-functionalized, but unprotected III–V surfaces to oxygen and air atmosphere could degrade their electrical and optical properties [20,21]. Two different bio-architectures have been investigated in our laboratory to address this problem and produce a biosensing device. Firstly, the sensor surface (GaAs) was functionalized with biotinylated polyclonal antibodies using polyethylene-glycol (PEG) hexadecanethiol (HDT) and neutravidin. The use of PEG-HDT

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thiols helps to address the surface stability issue as it has been demonstrated that sulphuric inorganic compounds allow passivation of GaAs surfaces [20,22–25]. A PEG thiol-based architecture has recently been reported by us for the successful immobilization of influenza A virus [26]. Secondly, the sensor surface was coated with silicon nitride (Si_3N_4) prior to functionalization using glutaraldehyde. The GaAs surface covered with Si_3N_4 is protected from environmental exposure, while maintaining the functionalization ability [27,28].

Here we report on the development of a photonic biosensor based on PL emitted by a (0 0 1) GaAs/AlGaAs epitaxial microstructure biofunctionalized for *in situ* detection of *E. coli*.

2. Experimental procedure

2.1. Materials

QS wafers (V0729) used in this study comprised a 300 nm thick epilayer of GaAs separated from the surface by 3 $Al_{0.33}Ga_{0.67}As$ barriers (50, 90 and 25 nm thick) and 2 GaAs wells (3.0 and 5.5 nm thick). The microstructure was capped with a GaAs layer (5 nm thick) that was functionalized for specific immobilization of investigated biomolecules. Polyclonal antibodies against *E. coli* coupled with biotin were obtained from ViroStat, Inc (Portland, ME). D-PBS 1X (Dulbecco's phosphate buffered saline, pH 7.4) was bought from Wisent Bioproducts Inc. (Quebec, Canada). Neutravidin was obtained from molecular probes (Invitrogen, Burlington, Canada). Biotinylated PEG thiols were obtained from Prochimia Surfaces (Gdansk, Poland). An HDT solution was purchased from Sigma–Aldrich (Ontario, Canada). Samples of live *E. coli* K12 and *Lactococcus lactis* bacteria were obtained from the Department of Biology of the Université de Sherbrooke (Quebec, Canada). They were grown in Lysogeny broth (LB) or brain heart infusion (BHI) media, respectively. Bacterial growth was performed in liquid medium and followed by regular seeding on a solid-type LB or BHI agar. The bacteria were aliquoted and stored at −26 °C. Nominally anhydrous ethanol (98% v/v) was bought from Commercial Alcohols, Inc. (Brampton, Canada). To remove residual oxygen, a 250 ml flask filled with ethanol was purged for 4 h with a 0.084 Nm^3/h high purity nitrogen stream (>99% pure nitrogen, Praxair Canada Inc.). OptiClear, a solvent designed to remove impurities present at the surface of optical or electric compounds, was obtained from National Diagnostics (Mississauga, Canada). Acetone was bought at ACP (Montréal, Canada); isopropanol (2-propanol) was obtained from Fisher Scientific (Ottawa, Canada), acetic acid (CH_3COOH) was obtained from Fisher Scientific and ammonium hydroxide 28% (NH_4OH) was bought from Anachemia (Richmond, Canada). A solution of hydrofluoric acid and gluteraldehyde were purchased from Sigma–Aldrich. All the solvents were Lab (suitable general laboratory applications) and A.C.S. (high quality) grade and all the products have been used without additional purification.

2.2. Biofunctionalization of the GaAs (0 0 1) surface

In the first approach, the QS samples of 4 mm × 4 mm × 0.63 mm dimensions were cleaned, etched and thiolated according to the procedure described in Supporting Information (Section 1.1). After thiolation, the samples were thoroughly rinsed with IPA and dried with N_2. PEG-based thiols were incorporated in the functionalization procedure as they are known to offer a decreased nonspecific association of the antibodies to certain molecules [29] and, in addition to preventing steric hindrance [2], they possess the ability to sustain stretching which allows the recognition with several antibodies of a large size antigen, such as a bacterium. The samples with biotin terminated SAMs were immersed for 2 h in a degassed PBS solution containing 3.33 μM neutravidin, at room temperature. This step was followed by rinsing the samples with PBS and then with deionised (DI) water. The biotin/neutravidin system was incorporated into the procedure due to the known strong affinity of these molecules and, thus, the increased specificity of the functionalized architecture as compared to other approaches, e.g., including N-hydroxysuccinimide (NHS) treatment [30,31]. The neutravidin-coated samples were exposed to 1 μM biotinylated polyclonal antibodies against *E. coli* diluted in degassed PBS solution. This step was carried out at 4 °C overnight. The samples were then rinsed, sequentially, in PBS and DI water, similar to a previously described procedure [26].

The second biofunctionalization approach involved QS wafers coated with a thin film of Si_3N_4. The potential advantage of Si_3N_4 compared to SAMs of PEG thiols is in a more efficient protection of the GaAs surface against degradation in aqueous solutions. It is known that even most densely packed thiols interact only with a fraction (up to 50%) of the GaAs surface atoms [32], while amorphous Si_3N_4 layer could provide the full surface coverage [33]. However, it was not clear if a 10–20 nm thick Si_3N_4, which is about 5–10 times greater than the thickness of a thiol SAM, would lead to a decreased efficiency of an electric charge transfer from a bacterium to the GaAs surface and, consequently, to a reduced sensitivity and/or dynamics of the sensing process. The samples of dimension 5 mm × 5 mm × 0.63 mm were cleaned as previously stated and etched in concentrated (49%) hydrofluoric acid (HF) for 15 s to remove all native oxides and other surface contaminants [34]. This step was followed by rinsing with DI water and drying with an N_2 gun. A 40 nm thick Si_3N_4 layer was deposited on such prepared surface using the PECVD (plasma enhanced chemical vapour deposition) technique. The resulting Si_3N_4 surfaces were cleaned with OptiClear, acetone and IPA as previously stated. Chemical modification of the Si_3N_4 surface and fabrication of the aldehydized surface was carried out according to the procedure described in Supporting Information Material (Section 1.2). The aldehydized surfaces were incubated for 1 h in 1 μM of polyclonal antibodies against *E. coli* in PBS solution and then rinsed with PBS followed by rinsing with DI water. To prevent non-specific adsorption, the functionalized substrates were incubated for 30 min at room temperature in a 1% bovine serum albumin (BSA) solution.

The SAM- and glutaraldehyde-based detection architectures are schematically illustrated in Fig. 1. The exposure of antibody-functionalized samples to varying concentrations of bacteria was monitored by *in situ* measurements of the PL signal, as discussed further in this paper. Negative control tests were carried out for both detection architectures with *E. coli* antibody-functionalized samples exposed to *L. lactis* at 10^6 colony forming units (CFU) per millilitre.

2.3. Transducer effect and detection of bacteria using PL emission

The presence of high-density surface states on semiconductor surfaces is known to lead to band bending of the semiconductor bulk bands approaching the surface. This results in the formation of a space charge depletion layer that for a lightly n-doped GaAs with a density of electrons of 10^{16} cm^{-3} extends 130 nm into the bulk [35]. The depletion layer related electric field separates electrons (e) and holes (h) generated in the space charge region and, thus, it reduces the radiative rate of the e–h recombination, contributing to the reduced intensity of PL emission from such a material. We have recently demonstrated that formation of the interfacial dipole layer (IDL) takes place near the surface of GaAs upon chemisorption of thiols [36]. It is the electrostatic field of IDL that interact with the space charge depletion layer and leads to a large reduction of carrier surface recombination velocity (SRV) and decrease in the hole/electron ratio of surface carrier capture cross-section.

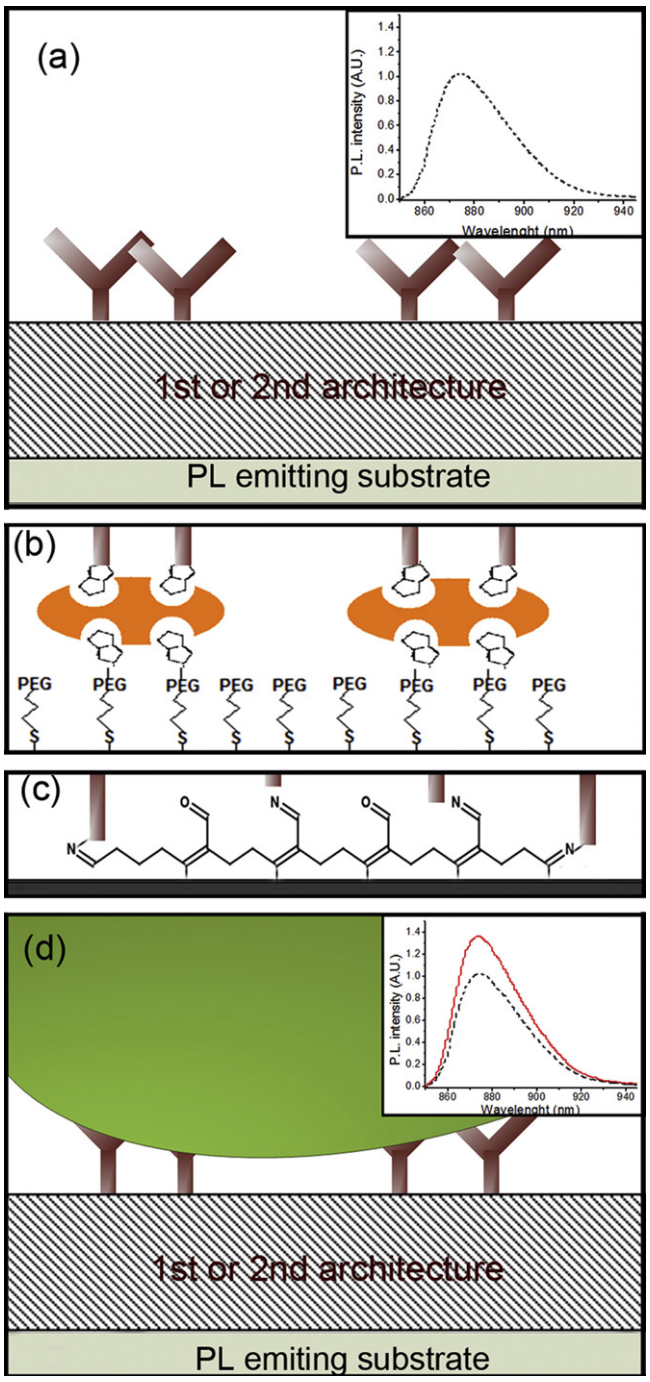

Fig. 1. Schematic illustration of the detection process of a sample (a) functionalized with either (b) SAM and biotin conjugated antibodies, or (c) Si_3N_4 and reticulation of unconjugated antibodies to achieve (d) immobilization of bacteria. The inset schematically depicts the concept of PL-based detection.

The argument has been made that in contrast to the so-called dead-layer model [37], it is the reduction of SRV that is responsible for the increased PL intensity [36]. The presence of negatively charged molecules contacting the thiol SAM is expected to increase the potential of IDL, resulting in further reduction of SRV. Thus, negatively charged molecules trapped on thiolated, or biofunctionalized surface of GaAs coud lead to the additionally increased PL intensity from such semiconductor. Both Gram-negative bacteria, such as *E. coli*, and Gram-positive bacteria exhibit significant negative surface charge formed as a result of the dissociation of related chemical groups present on the bacterial surface. Depending on the pH of the bacterial solution, this negative surface charge can be counterbalanced, partially or entirely, by ions of the opposite charge (counter-ions) present in the liquid [38]. However, for a net negative charge present on the bacterial surface of *E. coli* and other negatively charged bacteria, the increased PL emission is expected following the attachment of such bacteria to the biofunctionalized surface of an intrinsic or lightly n-doped GaAs.

2.4. Interface and surface characterization

The validation of the PEG-thiol SAM architecture for biofunctionalization of GaAs was carried out previously using Fourier transform infrared transmission measurements [26]. To evaluate the effectiveness of surface modification and monitor the reproducibility of the biofunctionalization process applied to Si_3N_4 surfaces, we employed contact angle measurements using a custom made contact angle goniometer (Department of Electrical Engineering, Université de Sherbrooke, Quebec, Canada). A 2 μl droplet of deionized water was gently placed onto the surface. The contact angle measurements were made within 15 s after placing the drop of water. The inner angle between the edge of the droplet and the surface was photographed and analysed using the QCapture Pro software (QImaging, Surrey, Canada). The measurements were repeated in 3 different regions on the surface, giving an accuracy of ±1°. All the measurements were performed in ambient atmosphere at room temperature.

2.5. Optical microscopy

Optical microscopy was used to estimate the density of bacteria immobilized on samples functionalized with 160 μg/ml (1 μM) of antibodies against *E. coli*. Images were obtained using an Eclipse TI microscope (Nikon Instruments, Inc). With a 1000× magnification, it was feasible to resolve individual bacteria required for these measurements. Each sample was imaged at three different regions to provide more reliable statistics.

2.6. Atomic force microscopy (AFM)

Surface morphology of processed samples was investigated using a Nanoscope IIIa microscope (Digital Instruments, Inc.) operating in contact mode. A MLCT-B type tip (Veeco Metrology, Inc.) was used with a cantilever spring constant of 0.03 N/m. The bacteria exposed samples were rinsed, dried and fixed with a 3.7% formaldehyde solution in water. All the AFM measurements were carried out in an air environment. The AFM images were analysed with WSxM 3.0 software [39]. The root-mean square roughness (RMS) values of the investigated surfaces refer to an area of 2.5 μm × 2.5 μm.

2.7. PL signal detection

The collection of PL signal was carried out using a custom designed (Photon Etc., Montreal) Hyperspectral imaging PL mapper (HI-PLM) described elsewhere [18]. The presence of bacteria was monitored by collecting PL maps over a period of up to 5 h. The PL intensity was averaged over the sample area, normalized by subtracting the value for PBS without bacteria, and plotted to demonstrate the dynamics of detection. Each run was repeated 3 times in order to produce error bars as indicated on the respective plots. Additional details concerning the PL data collection procedure are provided in Section 1.3 of the Supporting Information. For static measurements, the functionalized samples were placed in a sealed Teflon chamber. We investigated biofunctionalized samples that were exposed to live *E. coli* concentrations ranging from 10^4 to 10^8 CFU/ml.

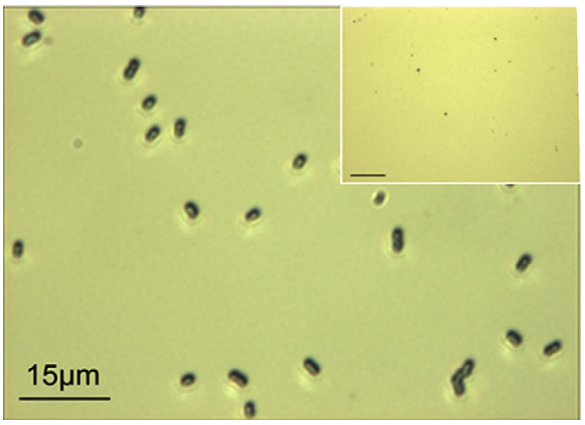

Fig. 2. Example of a bright field optical microscopy image obtained after exposure of the antibody functionalized GaAs samples to 10^6 CFU/ml *E. coli* (inset: a negative control showing an image of a sample exposed to 10^6 CFU/ml *L. lactis*).

Additional experiments were carried out to verify the intensity of the measured PL signal after the bacteria exposed samples were washed *in situ* with PBS solvent. This served to eliminate a possible contribution to the PL signal due to sedimentation and nonspecific physisorption of bacteria from the solution. A 200-μl volume custom made micro-fluidic chamber was employed for these experiments. Only the samples with alkane thiol SAM based architectures were investigated, but it is reasonable to expect that qualitatively similar results would be obtained with the Si_3N_4 coated samples as well. Each sample was, first, exposed to a fixed concentration of bacteria for 30 min. This exposure time was assumed to be sufficient to allow the antibody–antigen reaction at the liquid/solid interface [40]. Subsequently, the samples were washed *in situ* with PBS.

3. Experimental results and discussion

3.1. Density of immobilized bacteria

Optical microscopy images were taken to estimate the density of bacteria immobilized on samples functionalized by both methods and exposed to varying concentrations of *E. coli* bacteria (10^2, 10^4 and 10^6 CFU/ml) and 10^6 CFU/ml of *L. lactis* as a negative control. The images show the presence of bacteria measuring approximately 2–3 μm in length and between 0.8 and 1 μm in diameter. An example of the bright field image obtained for the thiol SAM based architecture and 10^6 CFU/ml of *E. coli* is shown in Fig. 2. The inset in this figure confirms the negligible efficiency of the applied architecture in the immobilization of *L. lactis*. Both architectures gave qualitatively similar results, although for the 10^6 CFU/ml *E. coli* solution, a slightly higher surface coverage was achieved with the Si_3N_4-based functionalization method (0.7 bacteria/100 μm^2) in comparison to the SAM-based method (0.55 bacteria/100 μm^2).

3.2. Surface morphology

The AFM measurements carried out at various stages of sample functionalization revealed details of surface morphology consistent with applied etching steps and constructed bioarchitectures (see Supporting Information, Section 2.2). Fig. 3 shows an AFM picture of a selected area GaAs/Si_3N_4 sample functionalized with *E. coli* antibodies that was exposed to 10^6 CFU/ml of *E. coli* solution. A micro-object of approximately 2 μm × 0.8 μm × 0.35 μm can clearly be seen in this picture. The dimensions of the mirco-object correspond to the dimensions of an *E. coli* bacterium that is known to be a cylindrical object, measuring approximately 2 μm in length and 0.8 μm in diameter [41,42]. The slightly reduced height of the bacterial cell observed in this experiment could be explained by the possible flattening occurring due to the pressure exercised by the AFM cantilever tip as well as shrinking of bacteria due to the drying and fixing procedure [43].

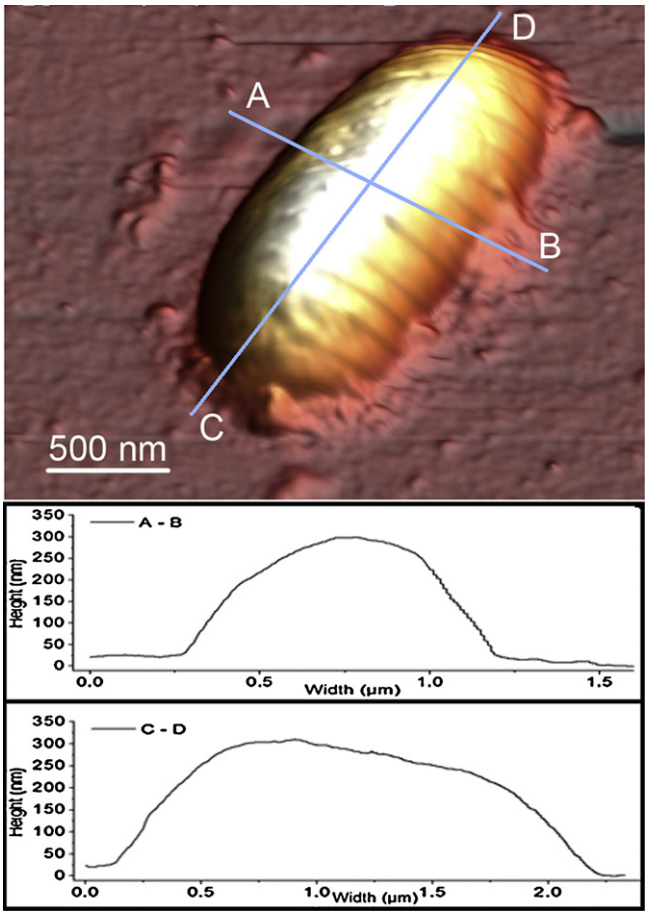

Fig. 3. Selected area of the antibody functionalized Si_3N_4–GaAs/AlGaAs sample that, after the exposure to 10^6 CFU/ml of *E. coli* solution, shows a bacterium immobilized on the surface.

3.3. Dynamics of bacteria detection observed with PL

Fig. 4 shows time-dependent PL data obtained with SAM- and Si_3N_4-based biosensor architectures exposed to different concentrations of bacteria. The net PL signal increases with time, consistent with the expected increase of the concentration of negative electric charge delivered by the bacteria immobilized on the biosensing surface. For the SAM-based architectures exposed to *E. coli* concentrations of 10^5 CFU/ml or greater (Fig. 4a), the process of bacteria sedimentation has not been completed within the investigated time frame of 300 min. However, for the Si_3N_4-based architectures (Fig. 4b), the saturation of the PL signal for 10^4 and 10^6 CFU/ml has been observed within 60 and 300 min, respectively. This suggests that the Si_3N_4-based architecture provides a better capture efficiency of the bacteria, although the PL signal for 10^6 CFU/ml has increased in this case by only 30% in comparison to the increase exceeding 45% observed for the SAM-based architecture. The negative control tests carried out with *L. lactis* at 10^6 CFU/ml for both architectures suggest that the detection of *E. coli* at concentrations exceeding 10^4 CFU/ml was specific. However, this approach required verification as the *in situ* data collection in a stagnant bacterial environment could also include a PL component related to bacteria sedimentation (physisorption) on the biosensor surface.

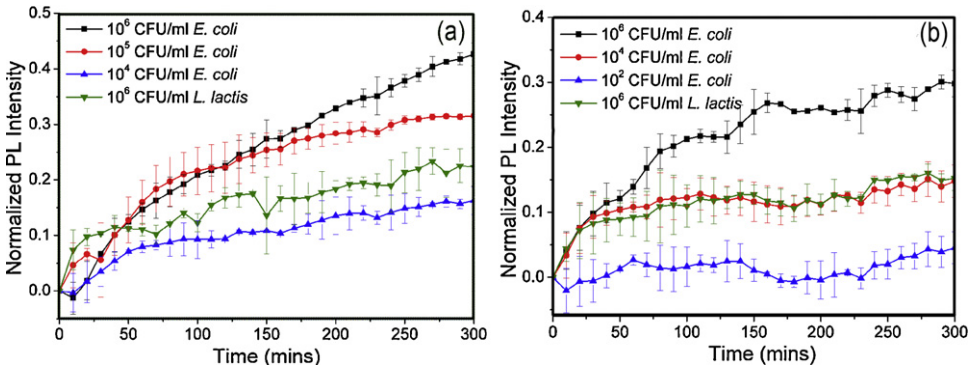

Fig. 4. Normalized PL intensity dependence observed for different concentrations of bacterial solutions as a function of time for (a) thiol SAM based architectures, and (b) of Si_3N_4 with glutaraldehyde crosslinking architectures.

To address this problem, we carried out a series of experiments involving PL measurements with *in situ* PBS washing of samples following their 30 min exposure to different bacterial solutions of *E. coli* and *L. lactis*. Fig. 5 shows the results obtained for samples exposed to a PBS solution, 10^6 CFU/ml of *L. lactis* (control experiment) and *E. coli* at 10^4, 10^6 and 10^8 CFU/ml. Since the biosensor is exposed to bacteria diluted in a PBS solution, it is important to know the biosensor response to the PBS solution alone. This serves to determine the biosensor baseline. A stabilizing PL signal is observed after the PBS washing step ($t \geq 40$ min) for samples exposed to *E. coli* solutions, which indicates the source-limited supply of a negative electric charge. This is in contrast to the PBS solution, which is responsible for a continuous decay of the PL signal as shown in Fig. 5. The differences in the PL signal observed at 120 min of the experiment could be used to calibrate the biosensor response. In particular, a 1.55 times stronger signal for 10^4 CFU/ml in comparison to the PBS baseline could be used to define the current detection limit of our biosensor. This difference increases to 2.17 and 3.0 for *E. coli* concentrations of 10^6 and 10^8 CFU/ml, respectively. It is worth mentioning that the PL signal from the sample exposed to 10^8 CFU/ml would continue to increase if no washing step was applied. For comparison, the dynamics of the response to 10^6 CFU/ml of *L. lactis*, following the washing step is, as expected, similar to that of the PBS solution. This experiment confirms that the response of the investigated biosensing architectures to *E. coli* is indeed specific.

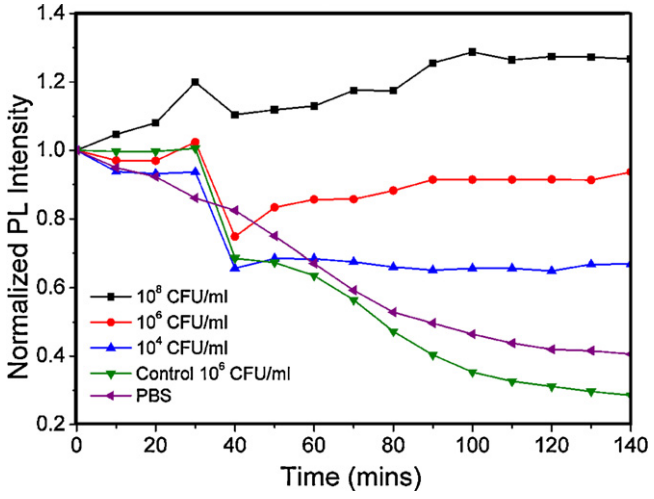

Fig. 5. Normalized PL intensity dependence for different concentrations of bacterial solutions observed *in situ* with SAM-based architectures. The samples were rinsed after 30 min of exposure to the bacteria. The results are shown without subtracting the PBS related signal.

4. Conclusion

We have investigated a novel method of detecting *E. coli* using PL emission from GaAs/$Al_{0.33}Ga_{0.67}As$ microstructures capped with a 5-nm thick GaAs layer. The functionalization of the samples was achieved by using either alkanethiol SAMs, or glutaraldehyde-based aldehydization of the surface of a thin Si_3N_4 film deposited atop the GaAs surface. The negative electric charge of the bacteria immobilized on the surface of antibody-functionalized microstructures contributed to the increased PL emission from GaAs. The samples exposed to different concentrations of bacteria allowed monitoring the dynamics of the bacteria immobilization observed over a period of several hours. The results indicate that the investigated method allows detection of *E. coli* at 10^4 CFU/ml within less than 120 min. The antibody-based architecture of the method makes it possible to address detection of numerous biomolecules, including pathogenic strains of bacteria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2011.07.010.

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