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Short communication

Antimicrobial warnericin RK peptide functionalized GaAs/AlGaAs biosensor for highly sensitive and selective detection of *Legionella pneumophila*

M. Amirul Islam[a], Walid M. Hassen[a], Azam F. Tayabali[b], Jan J. Dubowski[a,*,1]

[a] *Interdisciplinary Institute for Technological Innovation (3IT), CNRS UMI-3463, Laboratory for Quantum Semiconductors and Photon-based BioNanotechnology, Department of Electrical and Computer Engineering, Université de Sherbrooke, 3000, boul. de l'Université, Sherbrooke, Québec J1K 0A5, Canada*

[b] *Biotechnology Laboratory, Environmental Health Science and Research Bureau, Healthy Environments and Consumer Safety Branch, Environmental Health Centre, Health Canada, Ottawa, Ontario K1A 0K9, Canada*

HIGHLIGHTS

- Warnericin RK antimicrobial peptide (AMP) biosensor developed for *L. pneumophila* (*Lp*).
- Warnericin RK AMP captures *Lp* more efficiently than polyclonal antibodies.
- Detection of *Lp* at 10^3 CFU/mL demonstrates the potential of AMP-functionalized biosensors.

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ABSTRACT

Detection of pathogenic *Legionella pneumophila* by culture-based methods is not efficient in predicting outbreaks of the Legionnaires' disease. The main problem is the relatively slow time-to-result and the inability of some culture media to support the growth of viable bacteria. One strategy to alleviate these issues is developing biosensors functionalized with mammalian antibodies designed to capture bacteria. However, mammalian antibodies are known to suffer from batch-to-batch variations, as well as limited stability, which reduce the consistent utility of antibody-based biosensors. In an attempt to address this problem, we investigated antimicrobial peptides (AMPs) for capture of *L. pneumophila* with GaAs/AlGaAs biochips. The Fourier-transform infrared spectroscopy measurements revealed that the peptides were covalently immobilized on the 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide activated −COOH terminals of mercaptohexadecanoic acid self-assembled monolayer functionalized GaAs surface. The efficiency of the specific interaction between the peptide and *L. pneumophila*, *E. coli*, *B. subtilis* and *P. fluorescens* was investigated with fluorescence microscopy and a digital photocorrosion GaAs/AlGaAs biosensor. We found that the warnericin RK peptides exhibited ~5 times greater binding affinity towards *L. pneumophila* than to the other bacteria investigated. Furthermore, detection level as low as 10^3 CFU/mL was possible with the proposed biosensor architecture. We argue that a biosensor based on warnericin RK AMP peptides offers an attractive alternative solution in comparison to antibody-based devices towards detection of *L. pneumophila*.

1. Introduction

Biosensor-based detection of *Legionella pneumophila* (*L. pneumophila*) is relatively fast, requires minimal technical knowledge for the user, and can be adapted as potentially portable devices [1]. In the past few years, a variety of biosensing methods, such as optical [2], piezoelectric [1] and electrochemical [3] have been proposed to detect *L. pneumophila*. Recently, a photoluminescence (PL) monitored digital photocorrosion (DIP) biosensor using GaAs/AlGaAs nanoheterostructures has proven attractive for rapid and sensitive detection of *E. coli* [4] and *L. pneumophila* [5] bacteria.

The efficiency of bio-recognition elements is crucial to the operation of biosensors [6]. Several bio-recognition elements such as antibodies, carbohydrates, aptamers, peptides have been widely used for capturing bacteria on the biosensor surface [1]. Among these, antibodies are commonly used since they can be selected to be highly specific to the target [7]. However, antibody-based biosensors suffer from instability and non-specificity to pathogens under harsh environments [8].

* Corresponding author.
E-mail address: jan.j.dubowski@usherbrooke.ca (J.J. Dubowski).
[1] www.dubowski.ca.

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Furthermore, antibodies are prone to batch-to-batch variation [9], which may result in inconsistent biosensor calibration. Recently, studies have shown that antimicrobial peptides (AMPs) could be employed as bio-recognition elements as an alternative to antibodies [7,10]. AMPs have been extensively investigated for their antibacterial action towards *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [11,12] as well as for *L. pneumophila* [13,14]. However, to the best of our knowledge, AMPs have not been used as bioreceptors to detect *L. pneumophila* in biosensing platforms. AMPs contain multiple domains that bind with specific bacterial or fungal cell envelope moieties [8]. The stability of AMPs is considerably higher than that of typical globular proteins, especially antibodies [8,15]. Therefore, AMPs could be considered as a replacement of typical polyclonal antibody-based biosensing architectures. The bio-recognition mechanism of an AMP based GaAs biosensor has been schematically illustrated in Fig. S1.

The warnericin RK peptide is highly active against *L. pneumophila* [16]. Although the exact interaction between peptide and bacteria is not clearly understood, it has been proposed that the peptide may attach to the target cell surface through electrostatic interaction between the positively charged peptide and the negatively charged bacteria, followed by the specific interactions of peptide with a specific but as yet unknown surface membrane component. According to Verdon et al. [17], warnericin RK and delta-lysin I display the same antibacterial spectra, which is largely restricted to the *Legionella* genus. In further analyses [16], these authors observed that the warnericin RK range of antimicrobial activity is due to the presence of phosphatidylcholines (30 % content) lipid on the surface of *Legionella* membrane. However, in a different study, Marchand et al. [18] found that the amino acid residues at the position 14 for warnericin RK were of the major importance for bactericidal as well as lytic activities to *L. pneumophila*.

Prompted by the intriguing prospect of employing AMP for *L. pneumophila* biosensing, we have functionalized a GaAs/AlGaAs DIP biosensor with this moiety and investigated its capacity to detect *L. pneumophila*. We demonstrate that the investigated biosensing architecture offers attractive both sensitivity and selectivity to detect *L. pneumophila* in water environment.

2. Methodology

2.1. Sample preparation

The samples prepared for Fourier-transform infrared (FTIR) absorption measurements were cut from an undoped GaAs (100) bulk wafer (WV 23084). The 2 mm × 2 mm chips were cleaned following the previously established procedure [4,19] in an ultrasonic bath using acetone, OptiClear, acetone, and isopropanol sequentially for 5 min each and blown dry with high purity compressed nitrogen gas. Thereafter, the samples were etched in 28% NH_4OH for 2 min at room temperature for removing native oxides from the surface of GaAs and immediately dipped in the degassed ethanol. Following this step, the etched samples were immersed in 1 mM of 16-mercaptohexadecanoic acid (MHDA) thiol for 20 h. After the thiolation step, the biochips were sonicated in deoxygenated ethanol for 1 min and rinsed with degassed ethanol to remove unbound molecules. Thereafter, the thiolated samples were incubated for 30 min in the 0.4 M EDC (1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimnide) – 0.1 M NHS (N-HydroxySuccinimide) solution (1:1) to activate −COOH terminals. Following this procedure, the samples were immerged for 1 h in 0.1 mg/mL of warnericin RK AMP synthesized by GenScript Corporation, Piscataway, USA. The concentration of a peptide solution was chosen to match that of the *L. pneumophila* antibody solution (0.1 mg/mL). However, it is understood that a more comprehensive investigation will be necessary to optimize the biosensor performance. The functionalized samples were incubated with *L. pneumophila*, JR32 *E. coli* ATCC 25922, *Bacillus subtilis* ATCC 60514 (*B. subtilis*) and *Pseudomonas fluorescens* ATCC 13525 (*P. fluorescens*) at 10^6 CFU/mL, each, for 2 h.

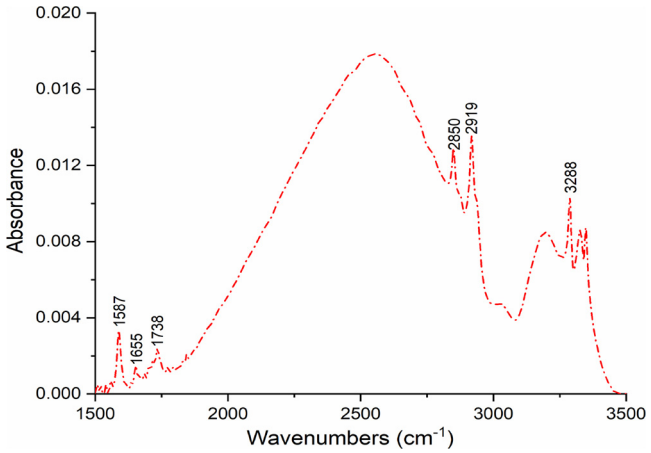

Fig. 1. FTIR spectrum of a MHDA/EDC-NHS/AMP biofunctionalized GaAs (001) chip.

The DIP biochips were prepared using an undoped wafer comprising stack of GaAs/$Al_{0.35}Ga_{0.65}As$ nanoheterostructures (Wafer D3422). The cross-sectional view of the wafer has been presented in Fig. S2. The biofunctionalization procedure of the biochip GaAs surface was carried out in the same manner as that employed for the biofunctionalization of the bulk GaAs (001) samples described above.

The sources of materials and reagents used for this study, sample preparation and measurement techniques for optical microscopy, FTIR and PL measurement have been conducted according to Sharma et al. [20]. A brief description of these procedures, has been provided in the Supplementary data section.

3. Results and discussions

3.1. FTIR analysis

The molecular conformation of the peptide binding architecture on the surface of GaAs was evaluated by collecting FTIR absorption spectroscopy spectra (Fig. 1 and Table S1). The intense bands recorded at 2919 cm^{-1} and 2850 cm^{-1}, as shown in Figs. 1 and S3, are assigned to the $-CH_2$ asymmetric and symmetric vibrations, respectively. The observed FTIR characteristics of these vibrations suggest formation of a high quality MHDA self-assembled monolayer (SAM) [21]. The absorbance bands at 1655 cm^{-1}, 1587/1738 cm^{-1} and 3288 cm^{-1} are assigned to the amide I, amide II, amide III and amide A bands of AMP, respectively [7,15,22]. Similarly, the peptide immobilized through the C-terminal and with free N-terminal region shows a characteristic peak at 1655 cm^{-1} [23,24]. The band observed at 1738 cm^{-1} is the C═O stretching mode of lateral chain functions and of some hydrolysed ester functions [25]. The peaks at 1655 cm^{-1} and 1738 cm^{-1} suggest the presence of a characteristic helical conformation of the surface-conjugated peptide [24,26].

Furthermore, the peak at 1587 cm^{-1} is likely related to the presence of N—H bending mode for Amide II [27]. These results suggest that the investigated peptide bound covalently on the EDC-NHS activated MHDA SAM. We note that, as expected, the peptide related absorbance peaks have not been observed for the MHDA/EDC-NHS modified GaAs unexposed to AMP as presented in Fig. S4.

3.2. Interaction of bacteria with the warnericin RK functionalized GaAs (001) surface

To evaluate the attachment/binding efficiency of warnericin RK, the AMP functionalized GaAs chips were incubated for 2 h in either *L. pneumophila*, *E. coli*, *B. subtilis* or *P. fluorescens* at 10^6 CFU/mL. As presented in Fig. 2, the non-functionalized GaAs surface (Fig. 2a)

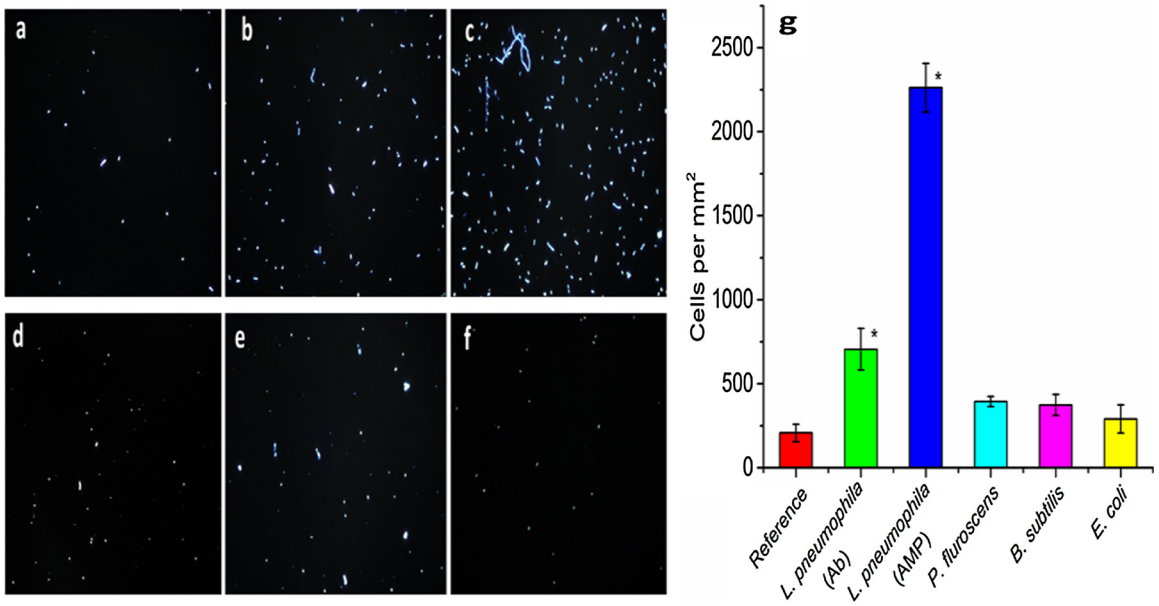

Fig. 2. (a) The attachment efficiency of *L. pneumophila* to the non-functionalized surface of GaAs, (b) antibody functionalized surface of GaAs, (c) AMP functionalized surface of GaAs, and the attachment efficiency of the AMP functionalized surface of GaAs for (d) *P. fluorescens*, (e) *B. subtilis*, and (f) *E. coli*. Averaged surface coverage for different bacteria is shown in (g). The asterisks indicate significantly different values compared to reference ($p < 0.05$) as determined by the Students *t*-test ($n = 3$).

captured a small number of *L. pneumophila* compared to the antibody and warnericin RK functionalized surfaces (Fig. 2c and 2c, respectively). Furthermore, the low capture efficiency of *E. coli*, *B. subtilis* and *P. fluorescens* by the warnericin RK AMP functionalized GaAs is well illustrated in Fig. 2d–f. The average number of quantified bacteria, as summarized in Fig. 2g, shows that the peptide-coated surface captured ~2261, ~394, ~373, and ~290 cells/mm^2 of *L. pneumonphila*, *E. coli*, *B. subtilis* and *P. fluorescens*, respectively, while the peptide-free surface (*i.e.*, background) exhibited ~207 cells/mm^2. Thus, these results illustrate a 5–6 times greater capture efficiency of the warnericin RK functionalized GaAs surface of the biochip towards *L. pneumophila* than to *E. coli*, *B. subtilis* and *P. fluorescens*. The variation of binding efficiency could be attributed to the specificity of peptide bacteria interactions [15,28].

In the present study, the warnericin RK functionalized surface of GaAs was found to be highly specific to *L. pneumophila* probably due to the presence of the unique lipid composition of the *L.pneumophila* bacterial membrane [18]. Verdon et al. [16] investigated the sensitivity of the warnericin RK to *L. pneumophila* and found that the presence of branched-chain fatty acids on the surface of bacteria play a crucial role in the sensitivity of bacteria to these peptides. Furthermore, *L. pneumophila* contains a high proportion (30 %) of phosphatidylcholines that are conventionally prevalent in eukaryotic cells [16]. As such, these lipids are very specific to the *Legionella* genus [28], and may explain the strong interaction observed between warnericin RK and the *Legionella* bacterial membrane.

3.3. Detection of L. pneumophila with DIP GaAs/AlGaAs biosensor

An example of a series of temporal PL plots collected for the GaAs/AlGaAs DIP biochips exposed to *L. pneumophila* solutions in the range from 10^3 to 10^6 CFU/mL at 0.1x PBS, and the calibration plot obtained from 3 repetitions are presented in Fig. 3a and b, respectively. Delayed positions of PL maxima were observed with increasing concentrations of bacteria, which is consistent with the response of a DIP GaAs/AlGaAs biochip exposed to negatively charged bacteria suspended in a water environment [4,5,19]. The limit of detection at 10^3 CFU/mL has been estimated based on the time dependent positions of PL maxima determined for 3 independent runs with an error nearest to that of the positions of PL maxima for 3 independent reference runs in a 0.1XPBS solution. Although the specificity of this method warrants an extensive future research, the results demonstrate that the sensitivity of a warnericin AMP-functionalized *L. pneumophila* biosensor is by one order of magnitude better than that reported by an antibody-based DIP biosensor [5], while comparable to that of DIP detected *L. pneumophila* decorated with sodium dodecyl sulfate [29], as well to some other recently published results (Table S2).

It is important to note that antibodies have been widely used as biorecognition elements for bacterial detection solely based on their specific interaction with bacterial antigens. However, apart from the specificity, the design of the detection architecture should include other practical considerations. In electrochemical [1] and DIP biosensors [5], the excessive distance of the antibody from the sensing surface could decrease the detection performance due to reduced electrostatic interaction and/or inefficient charge transfer between the biosensor and an analyte. Furthermore, antibodies might not demonstrate stable performance in harsh environments. In contrast, AMPs could offer attractive biosensing solutions, as their small molecular size allows for efficient charge transfer, and they exhibit high stability compared to typical mammalian antibodies [30,31]. Furthermore, AMPs can be manufactured with high reproducibility [1]. The specific detection of *L. pneumophila* with a warnericin RK biosensor as reported here should not be surprising in view of some recent studies claiming the highly selective nature of AMPs-based biosensors. For instance, Hossein-Nejad-Ariani et al. [32] reported the high binding affinity of a Leucocin A (Leu A) functionalized gold microelectrode to *Listeria monocytogenes* in comparison to 4 other bacteria. In another study, Mannoor et al. [8] observed that a gold electrode functionalized with magainin I AMP permitted ~4-fold higher binding affinities to the *E. coli* O157:H7 compared to *E. coli* ATCC 35218 and *Listeria Monocytogenes*. To the best of our knowledge, the present study demonstrates for the first time the attractive application of warnericin RK AMP for highly selective detection of *L. pneumophila* with a photonic biosensor.

4. Conclusions

The present study demonstrates the application of AMP for direct *in situ* detection of *L. pneumophila* in a water environment. The molecular

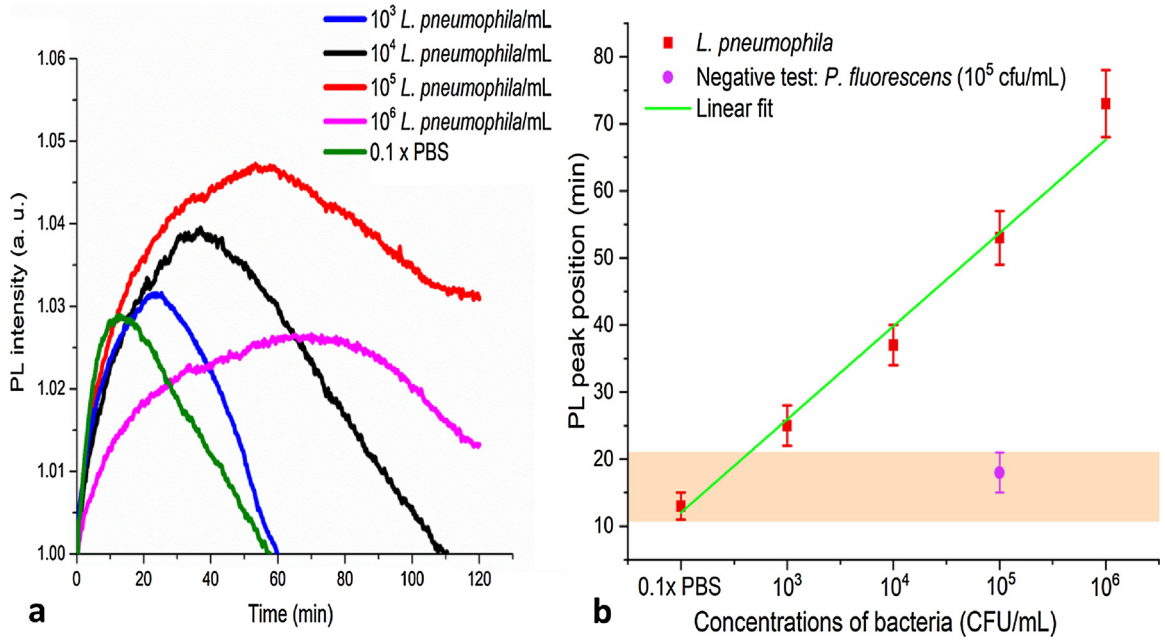

Fig. 3. (a) Normalized PL intensity for MHDA/EDC-NHS/AMP functionalized GaAs/AlGaAs DIP biochips (wafer D3422) exposed at 0.1 x PBS to different concentrations of *L. pneumophila*. (b) PL peak positions *vs.* different concentrations of *L. pneumophila* bacteria. The PL peak positions obtained for *L. pneumophila* were statistically different compared to either 0.1x PBS and *P. fluorescens* treated surface ($p < 0.05$) as determined by the Students *t*-test (n = 3).

orientation of the warnericin RK AMP on the GaAs (001) surface, examined using FTIR analysis, indicated covalent interaction of AMP with the GaAs surface. The specificity experiments demonstrated that *L. pneumophila* was captured with ~5-fold greater binding affinity than JR32 *E. coli* ATCC 25922, *Bacillus subtilis* ATCC 60514, and *Pseudomonas fluorescens* ATCC 13525. Detection sensitivity of the biosensor was demonstrated between 10^3 – 10^6 CFU/mL. The detection limit at 10^3 CFU/mL is one order of magnitude better than the previously reported direct detection with an antibody-based GaAs/AlGaAs DIP biosensor [5]. Further investigations are still required to address the specificity, sensitivity, reliability, as well as shelf lifetime of the proposed warnericin RK AMP biosensor. Nevertheless, our results provide the evidence for a potentially attractive application of a warnericin RK AMP biofunctionalized DIP biosensor for highly sensitive and specific detection of *L. pneumophila* in water samples, and pave the way towards the development of a robust biosensor operating in harsh environments.

CRediT authorship contribution statement

M. Amirul Islam: Conceptualization, Investigation, Methodology, Writing - original draft. **Walid M. Hassen:** Investigation, Methodology. **Azam F. Tayabali:** Formal analysis, Writing - review & editing. **Jan J. Dubowski:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2019.107435.

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