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Short communication

# A novel high sensitive surface plasmon resonance *Legionella pneumophila* sensing platform

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ABSTRACT

In the present work a novel high sensitive strategy for the detection of *Legionella pneumophila* bacterium exploiting grating-based surface plasmon resonance is presented.

*L. pneumophila* is usually detected by microbiological bacterial culture that often suffer from the need of dedicated microbiology laboratories, highly specialised personnel, and long analysis times. Indeed novel approaches for the detection of *L. pneumophila* bacterium have been currently developed. In this context GC-SPR under azimuthal control demonstrated its ability in detecting specifically down to 10 CFU (colony forming unit) of *L. pneumophila*, a concentration beyond the Italian legal limit for high risk hospital environment, resulting a valid technology with a detection sensitivity up to 1000 folds higher than fluorescence assays here adopted as validation technology. These results represent a promising starting point for the development of a scalable sensing prototype for the direct detection of Legionella in water and air samples in working environments that could be used also by non-specialised personnel.

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## 1. Introduction

*Legionella pneumophila (L. pneumophila)*, the causative agent of Legionnaires' disease and Pontiac fever [1], was first recognised in 1977 following an outbreak of acute pneumonia in Philadelphia. Among the 42 different species of *Legionella* described in literature, not all associated with human disease [2], *L. pneumophila* is the most often detected specie in diagnosed cases and it is responsible for more than 90% of cases of Legionnaires' disease [3]. *L. pneumophila* bacterium is diffused in aquatic habitats, especially in potable water, air conditioning, hot and cold water systems, cooling towers, evaporative condensers, spa/natural pools, healthcare facilities and, more in general, in high accommodation capacity structures [4]. The standard technique for the detection and monitoring of this pathogen is the microbiological bacterial culture, which is based on the *in vitro* selective growth of bacteria (ISO 11731: 1998 and ISO 11731-2: 2008). Whereas this approach is very accurate in identifying low contents of vital proliferating Legionella bacteria, it has essentially three disadvantages: (1) it requires dedicated microbiology laboratories, (2) it must be performed by highly specialised personnel only, and (3) time needed for test results is typically one week [5].

To overcome these issues, new sensing strategies were recently explored, all aimed at reaching high sensitivity and low detection limit, selectivity towards target pathogen detection, short analysis time, ease of use also for non-specialised personnel and compact and portable device development. An optical biosensor, based on imaging ellipsometry (IE), has been developed for the multiple detection of various pathogens such as *E. coli* O157:H7, *S. typhimurium*, *Y. enterocolitica*, and *L. pneumophila*, with a detection limit of $10^3$–$10^7$ CFU/mL [6]. Moreover the use of an optical biosensor based on SPR for the detection of *L. pneumophila* in artificially contaminated waters with a sensitivity of $10^5$ cells/mL was also demonstrated [7]. Some other preliminary SPR-based methods recently appeared in the Legionella biosensing scenario, all of them leading to a minimum concentration of *L. pneumophila* of $10^3$ [5] or $10^1$ CFU [8]. At the present the Italian legal limit of *L. pneumophila* in a high-risk hospital environment is $10^2$ CFU/L, and

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accurate methods for bacterium detection under this limit are not currently established.

In this context we report the development of a SPR-based device and a *L. pneumophila* detection strategy based on the highly sensitive azimuthally-controlled grating-coupling (GC-SPR) SPR technology [9] with the aim of showing the proof of concept of a sensor with a sensitivity higher than the standard adopted methods and suitable for future applications to be used also by non-specialised personnel. The GC-SPR technology has been widely applied to different biological and chemical targets, including proteins [9], nucleic acids [10,11] and explosive molecules [12], demonstrating its high versatility and capability of adaptability to the diverse applications. Moreover GC-SPR is a promising approach for the development of bench and portable real-time SPR setup [13]. We realized a gold sinusoidal plasmonic platform combining standard micro- and nano-fabrication techniques and we tested the sensor in the presence of different concentrations of *L. pneumophila* down to 10 CFU, exploiting direct and indirect antibody assays as sensing approach. The sensor output was collected through plasmonic reflectivity measurements and fluorescence-based microarray technology was used as standard technique for our system validation. The GC-SPR technology showed a 1000-fold enhanced Legionella detection sensitivity with respect to the standard fluorescence-based system.

## 2. Materials and methods

All the reagents and solution components were purchased from Sigma-Aldrich (St. Louis, MO, USA), if not otherwise specified. The water was of bi-distilled (dd-$H_2O$) or Milli-Q grade.

### 2.1. Sensing surface preparation

Plasmonic substrates were fabricated by combining laser interference lithography and soft lithography, according to a previously optimised protocol for the fabrication of a 500 nm-pitch and 40 nm-peak-to-valley amplitude gold sinusoidal grating. Briefly, a sinusoidal photoresist master was prepared using a Lloyd's configuration LIL lab-made setup. The master was then replicated onto a glass-supported thiolen resin (Norland Optical Adhesive–NOA-61) layer via replica molding process using a polydimethyl siloxane mold with the original grating master geometry. [11,12]

For flat substrates a metal layer was deposited onto a microscope glass slide. Both types of substrates (nanostructured and flat) were coated by a chromium (5 nm)/gold (40 nm) bilayer. Antibodies (0.1–1 mg/ml) in protein microarray print buffer (0.1 M sodium phosphate, 0.3 M NaCl 0,01% Triton X100, pH 7.2) were covalently bound to EDC-mediated activation of a previously assembled carboxyl thiol-polyethylene glycol layer [11].

Virostat (Portland, ME, USA) IgG α-*Legionella pneumophila* polyclonal antibody (#6051) and Abnova (Taipei, Taiwan) IgG α-*Legionella pneumophila* rabbit polyclonal antibody (#PAB13999) were selected as bioreceptor layer. *L. pneumophila* antibodies were obtained immunising with a whole cell preparation of *L. pneumophila* (ATCC #33152).

For microarray slides printing, a microarray spotter (Versarray Chipwriter Pro System, BioRad Laboratories, Hercules, CA, USA) was used, following the supplier protocol.

### 2.2. Labelling and binding protocol for bacteria cells

*L. pneumophila* subsp. pneumophila (ATCC 33152) serogroup 1 (BSL2) bacteria strain was used. *E. coli* was used as negative control (BSL1).

Culture media and supplement for Legionella were the following: Legionella BCYE (Buffered Charcoal Yeast Extract) Agar Base, Legionella BCYE α-Growth Supplement. *E. coli* was cultured in TSA. All media formulations were prepared following the supplier protocol (Biolife Italia – Milano, Italy).

Bacteria colonies were picked, dissolved in physiological solution (0.9% NaCl) and washed 2 times in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4) + 0.5% FBS to remove any agar residual, and kept at 4 °C up to few days or frozen in a solution of 1X PBS, 10% FBS and 15% glycerol. For fluorescent labelling, colonies were recovered, washed and resuspended in 1X TBS/FBS buffer (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 + 0.05% FBS). $OD_{600}$ was spectrophotometrically determined and cell suspensions were diluted to the appropriate $OD_{600}$ corresponding to the desired CFU amount. Bacteria labelling fluorescence validation was performed with NHS ester (succinimidyl ester) of Alexa Fluor® 555 or 647 (Invitrogen). Known amounts of bacteria cells (typically $10^8$ CFU) were labelled with 30 μg of NHS ester Alexa (resuspended in 5 μl of DMSO) for 1 h at room temperature under gentle shaking.

Cells were washed 3 times in 1X TBS/FBS to remove the excess of unbound fluorophore and then resuspended in 1X TBS/1% BSA. The solution was incubated for 1 h at RT under gentle shaking. After incubation, solutions were removed and microarray wells were washed once with Protein washing buffer (50 mM Tris, 250 mM NaCl, 0.05% Tween 20, pH 7.0) and twice in 1X PBS. Slides were finally spin dried and analysed for fluorescence response through laser scanner. Unlabelled cells were manipulated and diluted in the same way except for the labelling step.

### 2.3. Fluorescence detection

Bacterium cells were recognised directly through the incubation of fluorescent cell labelled as described above, or indirectly, through the formation of a sandwich between captured Legionella cells and fluorescent anti Legionella labelled antibody. For each sample, 2 μg of antibody were labelled with NHS ester of Alexa Fluor® in a 1:4 optimised molar ratio. The procedure was performed for 1 h at RT and the mix was diluted in 1X TBS/1% BSA to stop the reaction. Labelled antibodies were incubated on microarray slide – previously incubated with Legionella cells – for 1 h at RT.

Fluorescent measurements on arrays were performed using a GenePix 4000 B laser scanner (Molecular Devices, Sunnyvale, CA) and the GenePix Pro software ($\lambda = 532$ nm and 635 nm). Fluorescent spot intensities were quantified using the GenePix Pro software after normalising the data by subtracting local background from the recorded spot intensities.

### 2.4. SPR measurements

For Legionella incubation on functionalised gratings and subsequent reflectivity measurements, a microfluidic cell (TLC-300 Small Volume Liquid Cell – JA Woollam and Co., Inc. – CA, USA) mounted onto a spectroscopic ellipsometer (JA Woollam and Co., Inc. – CA, USA) was adopted. An incident wavelength range of 600–800 nm, incidence angle of 70°, azimuthal orientation of 45° and polarization 140° were used as detection parameters. Measurements were performed after each experimental step: after initial gold grating cleaning, after PEGylation, antibody anchoring and surface blocking, and after bacterium incubation.

## 3. Results and discussion

### 3.1. Antibody binding evaluation and direct vs indirect fluorescent bacteria detection

Virostat and Abnova antibodies performances were evaluated directly by using fluorescently labelled bacteria, or indirectly by

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