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Bacillus spp. Cells Captured Selectively by Phages and Identified by Surface Enhanced Raman Spectroscopy Technique †

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Abstract: Surface Enhanced Raman Spectroscopy (SERS), has been employed as a rapid and accurate tool for recognition and classification of different microorganisms. The vibrational spectrum intrinsically provides the fingerprint of the molecular structure of microorganisms, without sample preparation. The characterization of *Bacillus* spp., used as a simulants of the dangerous *Bacillus anthracis* by means of SERS technique has been carried out. The positive results on the use of the SERS substrate functionalized with phages specific for bacteria are showed. This work is a part of the RAMBO project (Rapid Air particle Monitoring against BiOlogical threats) funded by EDA (European Defense Agency) whose goal is the development of an advanced "detect to warn sensor" of cells and spores of dangerous bacteria, with high performance, good selectivity and reliability.

Keywords: sensors; SERS; bacteria; phages; *Bacillus* spp.

1. Introduction

Rapid detection and identification of the microorganisms in the environment including aerosol, water and food, is a major issue for public health and industrial processes. The main goal of many research studies is the development of a device that needs negligible or not sample preparation and can give, in in situ conditions, fast and effective detection. The techniques generally used, e.g., in vitro cultivation, or biotechnology assays like Enzyme-linked Immunosorbent Assay (ELISA) or Polymerase Chain Reaction (PCR) even if are essential for genetic identification [1,2] are time consuming and are not fully handy for onsite detection [3]. Instead, in this contest, biosensors are the most favorable candidates for fast and real time identification of different kinds of microorganisms, allowing their use as a first alarm sensor. Surface Enhanced Raman Spectroscopy (SERS), thanks to its ability to identify single molecules by using their intrinsic vibrational fingerprint [4] has been

successfully used as a label-free biosensing technique for rapid identification of bacteria and related spores [5–8]. The efficiency and selectivity of the SERS substrate in capturing biological targets can be guaranteed by means of specific receptors, as phages, peptides, aptamer etc. [9]. In the framework of the RAMBO (Rapid-Air particle Monitoring against BiOlogical threats) project of the European Defense Agency (EDA), an innovative sensor, based on the combination of two sensing techniques, is proposed. The SERS system, in combination with specific receptors, has the role of first alarm sensor of the bacteria target, while the second sensor, represented by DNA-based technique, PCR, confirms of the nature of the detected warning.

A feasibility study of the use of such system, combining SERS and PCR, for the detection of *Bacillus anthracis* has been carried out. The experiments have been performed with *Bacillus thuringiensis* (BT) employed as the simulant of the *B. anthracis*, (classified in the biosafety as level 3). *Bacillus atrophaeus* (BG) cells were also used for both interferent and simulant of *B. anthracis*. With the purpose to enhance the selectivity of the sensor, suitable selection of receptors to capture bacteria has been done. Gamma-phages, specific for *Bacillus cereus group* that includes BT [10] were chosen. The selectivity for BT was even better investigated in presence of BG cells. Moreover, since the infective dose, peculiar for each *Bacillus* spp., is in the range 10^4–10^{11} cells/mL [11,12] the sensitivity of the SERS sensor and its limit of detection (LoD) was also investigated within this range.

2. Materials and Methods

2.1. Bacteria Samples and Inoculation on SERS Substrate

Bacillus thuringiensis (BT) (ATCC® 10792™) and *Bacillus atrophaeus* (BG) (ATCC® 9372™), provided by the Military Institute of Hygiene and Epidemiology (Warsaw, Poland) were utilized respectively, as a simulant of the *Bacillus anthracis* and as interfering sample. In order to determine the limit of detection (LoD), different concentration of cells suspension, form 10^2 to 10^8 CFU/mL, were inoculated on functionalized SERS sensor (1 drop of 20 μL). After 45 min, period useful to allow the bonding with phages receptors, the bacteria suspension was deeply rinsed with pure sterile water (MilliQ, Millipore, Darmstadt, Germany) to remove unlinked cells. Raman spectra were acquired after drying at room temperature.

2.2. SERS Substrates and Functionalization

Commercial Klarite® (Renishaw Diagnostics Inc., New Mills, GL12 8JR, United Kingdom) and GaN/Ag-Au (https://www.unipress.waw.pl/growth/files/sers-ihhp.pdf) were used as SERS substrates. Their surfaces were coated using carboxy aniline electrodeposition thought cyclic voltametry (3 cycles, from 0 to 2 V vs. Pt at of 200 mV/s). Then, the grafted carboxylic acid function was activated using carbodiimide (EDC 0.4 M + NHS 0.05 M for 8 min at room temperature). The phage were, then, brought into contact with the activated surface (over-concentrated in acetate buffer 0.1 M, pH 6 for 16 min at room temperature).The activated surface was then neutralized using 1 M ethanolamine pH 8.5. Between all different steps, the SERS surface was washed with milliQ water and air dried.

2.3. Instrument Set-Up and Data Collection

SERS measurements were performed with an integrated table-top Raman microscope (BW&Tek). The laser source emits at 785 nm (linewidth < 0.3 nm), spectral range span between 200 and 3500 cm^{-1}, the CCD array set 2048 pixels. Spectra were acquired at laser power 60 mW, integration time 20 s, objective 40X (spot size ~40 μm). In order to clearly identify the interfering blank signal coming from both clean cartridge and phages, before to collect spectra of the bacteria, the spectra of the substrate were acquired. Then on 30 different points of the whole inoculated area tree spectra were collected. The range between 350 and 2200 cm^{-1} was considered for the characterization of the spectra being the so-called Raman "fingerprint" region. Spectra were baseline-corrected [13] and normalized between 0 and 1 prior to perform any multivariate statistics or classification.

2.4. Data Processing

Statistical analysis was performed using Matlab software. To reduce the dimensionality of such complex datasets (e.g., the number of pixel) and highligth the minimal spectral differences between different classes (e.g., spectra from the clean substrate vs spectra from the contaminated substrate) a Principal Component Analysis (PCA) [14,15] was applied to sets of blank and positive measurements to compare their PCs. The analysis were limited to the first 3 PCs. Firstly a 'Background Cloud' (BC) was computed applying PCA to the measures of the clean substrate (Background measures, BM). This cloud has and ellipsoidal shape with its center on the average first 3 PCs of the BM and semiaxes equal to 3 σ_{BM}, σ_{BM} being the standard deviation. Then each new Raman spectrum to check was projected in the PC-space and then compared with the BC. If the projection of this spectrum in the PCs space is out of the BM the measure is considered anomalous and a warning is generated.

3. Results

Figure 1 shows the average of the spectra acquired on Klarite® SERS substrates functionalized with Phages before (blue line) and after inoculation with increasing concentration of BT cells (different color lines) Among spectra relative to inoculated and not inoculated samples, few and weak differences are recognized. Nevertheless certain variation, especially in intensity, and some bacterial peaks, around 500 to 600, 700 to 800 and 1200 to 1400 cm^{-1} can be noticed.

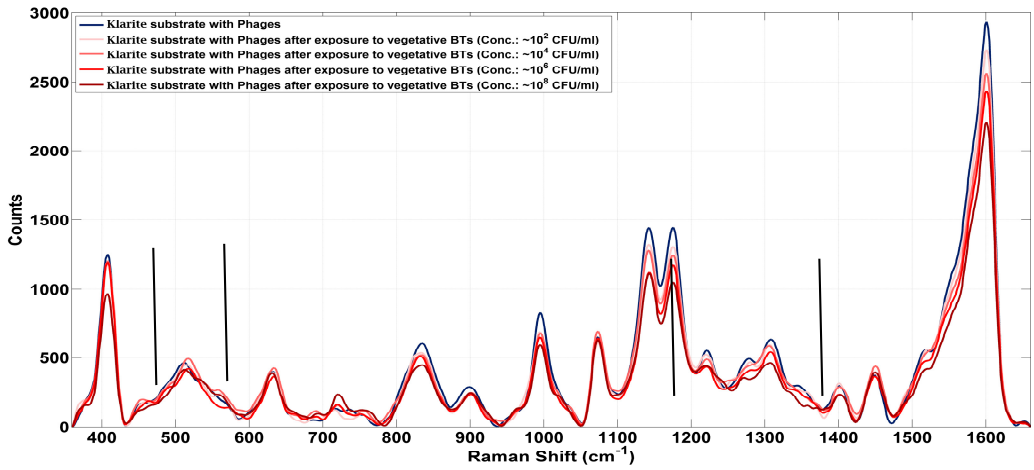

Figure 1. SERS spectra acquired on the Klarite® SERS substrates functionalized with specific phages substrate (blue line) compared to the same Klarite® SERS substrates inoculated with *Bacillus thuringiensis* (BT) cells at increasing concentration.

In order to stress characteristic peaks and to evaluate the LoD of bacteria captured by phages, PCA was performed. The score plot of the spectra shows that, when the cell concentration increases, two groups, represented by functionalized clean substrate and substrate inoculated with BT, are significantly characterized. The first LoD is revealed starting from at 10^4 CFU/mL until to 10^8 CFU/mL were the two clusters are definitely separated. That result describes also the progressive linkage with whole surface covered by phages. In that contest the effective tie between BT and phages, even in presence of comparable bacteria as BG, was demonstrated. An experiment was performed with a mixed cell suspension of both BT and BG cells. The results shown in Figure 2 prove such strict interaction BT/phages. In fact the score plot from Raman spectra of the SERS substrate before and after inoculation with the BG are grouped togheter while BT cells are clustered separately.

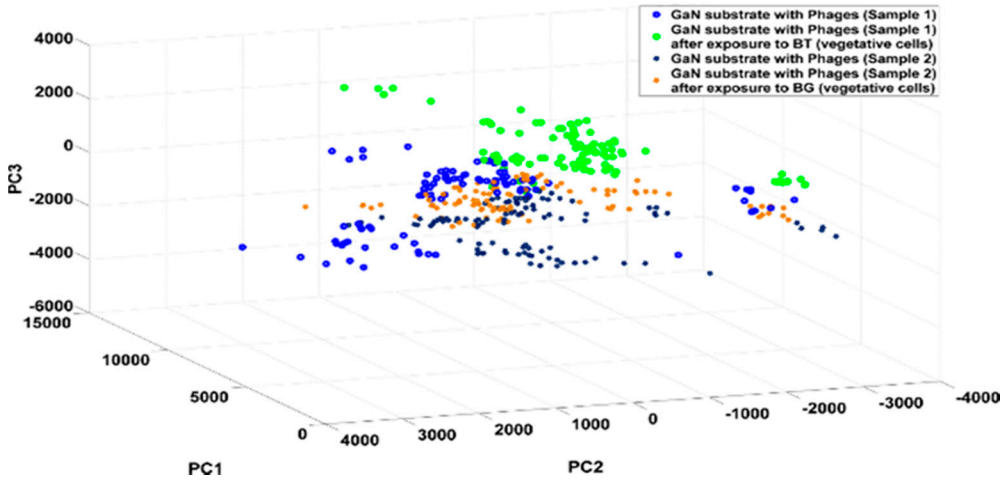

Figure 2. Principal Components (PC) score plot of the SERS spectra acquired on the clean functionalized GaN substrate (black and blue dots) and the same functionalized GaN substrate inoculated with *Bacillus thuringiensis* (BT, green dots) and *Bacillus atrophaeus*(BG, brown dots) cells.

4. Discussion

In this work SERS has proven to be a rapid and effective identification tool for *Bacillus* spp. cells. PCA has been demonstrate to be an efficient method to discriminate among few and weak spectral signal from bacteria at concentrations down to of 10^4 CFU/mL. This data is comparable to the literature data about claiming doses of *Bacillus* spp. That result to be infective if higher to 10^4 CFU/mL [11,12]. This result was synthesized by the unambiguous separation of the spectra of clean and contaminated substrates in two distinct groups in the PC space. Finally, the perfect interaction between BT and specific phages was also demontrate when the substrate was inoculated with other bacteria, like BG, used as interferent. SERS sensors functionalized with gamma-phages can be proposed as a first "detect to warn" on line sensors.

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Conflicts of Interest: The authors declare no conflict of interest.

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