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Culture-free diagnostics of *Pseudomonas aeruginosa* infection by silver nanorod array based SERS from clinical sputum samples

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Abstract

Pseudomonas aeruginosa can cause major infection in immunocompromised patients, and successful antibiotic treatment of the infection relies on accurate and rapid identification of the infectious agents. Here, we reported a culture-free diagnostic method based on the surface-enhanced Raman spectroscopy (SERS) of pyocyanin (PCN), a major biomarker of *P. aeruginosa*. This platform can detect PCN as low as 5 ppb or 2.38×10^{-8} mol L⁻¹ in both aqueous solutions and spiked clinical sputum samples. It has also been used to dynamically monitor the excretion of PCN by *P. aeruginosa* during its growth. The presence of PCN has been detected by SERS in 15 clinical sputum samples, which indicates *P. aeruginosa* infection, with 95.6% sensitivity and 93.3% specificity. The system can advantageously process multiple specimens rapidly, overcomes the need for bacterial culture and diagnostic microbiology assays, and have widespread implications in the early detection of *P. aeruginosa* infection.

Keywords

Pseudomonas aeruginosa; pyocyanin; surface-enhanced Raman spectroscopy; silver nanorod arrays; biomarker

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Introduction

Bacterial infections can be life threatening. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes major infection not only in cystic fibrosis (CF) patients¹ but also in chronic obstructive pulmonary disease (COPD) and in critically ill patients in intensive care units, inducing ventilator associated infections². It is a major nosocomial pathogen with significant morbidity and mortality (40–60%) in immunocompromised patients and in certain hospital units like burn and palliative care³. *P. aeruginosa* is the most common bacterial isolate that persists in the CF lung, contributing to declining lung function^{1, 4}. Successful antibiotic treatment of the infection relies on accurate and rapid identification of the infectious agents. However, the current diagnostic techniques based on bacterial culture usually take more than three days to obtain accurate results³. Therefore, there is an urgent need to develop novel methods for rapid identification of the infecting agent.

As a rapid and non-invasive optical technique, Raman- and surface-enhanced Raman spectroscopy (SERS)-based techniques have been utilized in the identification of bacteria including *P. aeruginosa*. In general, detection of bacteria using SERS can be conducted by examining the spectra of the bacterial cells, or the presence of the bacterial biomarker. The Raman spectra of sputa collected from CF patients with *P. aeruginosa* infection were first reported by Rusciano *et al*⁵. Despite high accuracy (95%) in sample differentiation, low sensitivity and significant interference from background fluorescence were observed. Spectral interference from sample matrix was also reported in other studies on Raman spectroscopic identification of *P. aeruginosa*^{6, 7}. These studies all adopted the strategy to detect whole bacterial cells. Unfortunately, this strategy has encountered a number of challenges in addition to the almost inevitable interference from fluorescence and signal of non-specific molecules^{5–7}. Since the bacterial Raman signal is believed to originate from cell envelope which have similar structures and chemical components among most bacterial species⁸, it is difficult for Raman spectroscopy to differentiate between closely related species, especially from a cocktail of bacteria, without secondary labeling. Moreover, in order to remove potentially interference effects from matrix or buffer residues, cells must be repeatedly rinsed and resuspended in water, which could induce cell rupture due to disrupted osmotic balance, and the assay reproducibility could be hampered as a result of inconsistent sample constitution. Owing to these reasons, Raman-based detection of whole bacterial cell presents numerous challenges for clinical diagnostics.

An alternative approach is to indirectly detect the presence of bacteria by identifying the biomarkers released by the organism in the matrix fluids. Since the biomarker molecules are unique to bacterial species, detection of specific bacteria from a mixture could easily be accomplished. Unfortunately, due to the difficulties in identifying appropriate biomarkers or high detection limits, there is still a lack of literature in SERS detection of bacterial biomarkers, especially from real clinical samples. Calcium dipicolinate is the only bacterial biomarker successfully detected by SERS thus far, which indicates the presence of *Bacillus* spores^{9, 10, 11}. The fluorescent pigments that previous researchers attempted to remove from the sample are confirmed as important biomarkers released by *P. aeruginosa* during its metabolism^{5, 12}, but these biomarkers has not been detected by SERS. Among these pigments, pyocyanin (PCN) is a major virulence factor secreted by *P. aeruginosa*, and is

also required for establishing infections. PCN is a redox-active blue green pigment, thus it exerts cytotoxic effects and subverts host defenses. PCN also plays an important role as a terminal signaling factor for the quorum sensing of *P. aeruginosa*¹³, and PCN-deficient *P. aeruginosa* strains have been found to lose virulence^{14, 15}. PCN in clinical samples (e.g., sputa and *P. aeruginosa* isolates) has been extensively studied to understand its roles in *P. aeruginosa* infection among CF patients^{12, 16–18}. The concentration of PCN in the airways of CF patients was determined and found in a range between 0.9 and 16.5 ppm¹⁹, and *in vitro* secretion of PCN by clinical isolates of *P. aeruginosa* was also reported²⁰. The presence and concentration of PCN in clinical specimens are traditionally determined through extraction of the pigment into chloroform or by high performance liquid chromatography (HPLC)²¹. But the time-consuming, expensive, and complex lab procedures have largely limited the use of HPLC as a diagnostic tool in clinical settings.

To the best of our knowledge, there is no report on the Raman or SERS detection of PCN as a biomarker to indicate *P. aeruginosa* infection in clinical samples. In this study, we have successfully identified the SERS spectra of PCN both theoretically and experimentally, and designed a strategy to rapidly detect the presence of *P. aeruginosa* in biofluid samples without the need to culture or isolate the bacteria. The detection can be completed within 1.5 h of sample receiving, and in conjunction with chemometric analysis, this non-invasive method is capable of detecting PCN from *P. aeruginosa* at as low as 5 ppb in the sputa of CF patients with 95.6% sensitivity and 93.3% specificity, which could be employed to facilitate early diagnosis and treatment in CF infections.

Methods

Reagents and bacterial strains

PCN was obtained from Cayman Chemicals (Ann Arbor, MI). Luria-Bertani (LB) broth was obtained from Sigma Aldrich (St Louis, MO). *P. aeruginosa* strain PAO1, which contains the pSMC21 plasmid constitutively expressing green fluorescent protein (GFP), was a kind gift from Dr. George O'Toole, Dartmouth Medical School. *P. aeruginosa* strain PA14 was kindly donated by Dr. Joanna Goldberg, Emory University School of Medicine.

SERS substrate fabrication

The bacterial SERS spectra were acquired using silver nanorod (AgNR) array substrates fabricated by the oblique angle deposition technique using a custom-designed electron beam evaporation (E-beam) system^{22–24}. Briefly, microscopic glass slides (BD, Portsmouth, NH) were cleaned with piranha solution (80% sulfuric acid, 20% hydrogen peroxide v/v) and rinsed with deionized (DI) water. The slides were then dried with a stream of nitrogen gas before being loaded into the E-beam system. A 20-nm titanium film and then a 200-nm silver film were first evaporated onto the glass slides at a rate of ~ 0.2 nm/s and 0.3 nm/s, respectively, monitored *in situ* by a quartz crystal microbalance (QCM). The substrates were then tilted to 86° with respect to the incident vapor and AgNRs were grown at a deposition rate of ~ 0.3 nm/s until the QCM thickness reading reached 2000 nm. AgNR substrates have previously been shown to provide SERS enhancement factors of >10⁸, a relative standard

deviation (RSD) of 10% within the same substrate, and a RSD of 15% among different substrates^{22–24}.

Bacterial cultures

P. aeruginosa strains PAO1 and PA14 were grown on LB agar and isolated colonies were seeded into 10 ml of LB broth, which were then incubated at 37 °C. A bacterial growth curve was generated as previously described²⁵. Briefly, an 800 µl aliquot of freshly grown bacterial suspension was inoculated into 10 ml of LB medium, mixed well, and the optical density (OD) was measured at wavelength $\lambda = 600$ nm. The broth was incubated at 37 °C with frequent shaking, and the OD₆₀₀ was measured every hour.

PCN release and quantitation

PCN release from *P. aeruginosa* growing in LB broth was quantitated using the A691 method as previously described²⁶. Briefly, 0.5 ml of *P. aeruginosa* strain PAO1 culture was removed and centrifuged at 18,000 g for 3 min. An aliquot of 200 µl of bacteria-free supernatant was transferred into a clear 96-well plate in duplicate, where the OD was read at $\lambda = 691$ nm using the Bio-Tek Synergy 2 Microplate Reader. The concentration of released PCN was estimated using a calibration curve generated by serial dilutions of pure PCN in LB broth. Sterile LB broth without bacteria was used as a blank control for PCN quantitation.

Preparation and handling of clinical sputum samples

Clinical samples used in this study were purchased from the Emory + Children's Center for Cystic Fibrosis and Airways Disease Research, CF Discovery Core (Atlanta, GA). These frozen aliquots of processed sputum samples were obtained by a non-invasive method from subjects with CF after signing informed consents with Institutional Review Board approval. Sputum processing was performed by the staff²⁷ and as discussed in detail in the Supplementary Note 1.

The processed sputum samples used in this study were completely de-identified except for the infection status of the subjects. A total of 15 sputum samples were examined: five samples from CF subjects positive for *P. aeruginosa* infection (PA+), 5 samples from CF subjects positive for both *P. aeruginosa* and *S. aureus* (PA+/SA+), and 5 samples from CF subjects tested negative for *P. aeruginosa* infections (PA–). One of the PA+/SA+ samples was tested positive for *Proteus mirabilis* and *E. coli* infection as well. One of the PA– samples was positive for *Burkholderia cepacia*, and two of the PA– samples were positive for *Stenotrophomonas maltophilia*, but they were all negative for *S. aureus* infection.

At the day of experiments, the processed sputum samples were thawed at room temperature and PCN was extracted by chloroform²⁸. Briefly, 100 µL of processed sputum was mixed thoroughly with 50 µL of chloroform and then centrifuged at 10,000 g for 2 min. The resultant chloroform layer was transferred to a new tube. Sputum samples were handled in a BCL-2 biological safety cabinet following all biological hazard precautions including gloves and protective clothing. Used and discarded biological samples were placed in a 10% chlorine jar and left to be inactivated for at least 6 h prior to final disposal.

SERS Measurements and Data Analysis

A 1 μ l droplet of sample was applied onto the AgNR substrate after the contaminants on the substrate surface were removed using a customized Ar⁺ plasma generator²⁹, and the samples were dried inside the plasma generator chamber to eliminate any possible contamination from the environment. SERS spectra were acquired using a portable Raman system (ProRamanL 785A2, Enware Optronics Inc., Irvine, CA) with a 785 nm diode laser as the excitation source. The power of the laser at the sample was set to $\sim$ 60 mW. SERS spectra over the range of $\sim$ 300 – 1800 cm^{-1} were collected with a 5-s exposure time from 10 randomly selected spots across the substrate surface, and 3 substrates for a sample. Spectral analysis was performed using Origin 8.5 (OriginLab Corporation, Northampton, MA). Partial least square-discriminant analysis (PLS-DA) was conducted using PLS Toolbox (Eigenvector Research, Inc., Wenatchee, WA) on the Matlab 2010b platform (MathWorks, Inc., Natick, MA).

Results

Experimental and theoretical SERS fingerprints of PCN

Unique spectral fingerprints of *P. aeruginosa* pigment pyocyanin (PCN) are successfully identified at 10 ppb using SERS on AgNR substrates, as shown in Figure 1A. The signature SERS peaks of PCN are summarized in Supplementary Table S1, and these peaks are used to indicate the presence of PCN in more complex samples. The Raman vibrational modes corresponding to these peaks are also identified through density functional theory (DFT) calculation of theoretical Raman spectrum of PCN. The details of the DFT calculation are provided in Supplementary Note 2. The estimated Raman shifts and their corresponding vibrational modes of the PCN-Ag complex are summarized in Supplementary Table S1, and the spectrum is compared with the experimental observation in Figure 1.

In Figure 1, the corresponding Raman peaks from theoretical and experimental SERS spectra are denoted with the same Greek symbols. Despite a general peak shift from the theoretical calculation, the experimental SERS spectrum of PCN agrees well with the DFT spectrum in both relative peak position and relative peak intensity. For example, the most intense peak at $\nu = 1352 \text{ cm}^{-1}$ in the experimental spectrum, which corresponds to the C-C stretch, C-N stretch, and C-H in-plane bend modes on the aromatic ring, is also the most intense peak in the DFT spectrum at $\nu = 1395 \text{ cm}^{-1}$. The strong double peaks at $\nu = 1593$ and 1613 cm^{-1} in the experimental SERS spectrum match well with the double peaks at $\nu = 1622$ and 1634 cm^{-1} in the DFT spectrum. The experimental spectrum in general has fewer peaks compared to the theoretical spectrum, as some of the adjacent peaks merge into broader and more intense peaks in the experimental spectrum, particularly when the peaks are attributed to similar vibrational modes. For example, the peaks at $\nu = 396, 400$ and 413 cm^{-1} in the DFT spectrum, which are attributed to CH wag and C=O wag, C-H wag and C=O wag, and CH₃ wag, respectively, become a single peak at $\nu = 396 \text{ cm}^{-1}$ in the SERS spectrum. These adjacent peaks become difficult to distinguish experimentally due to the limited resolution of the instrument.

Using PCN aqueous solutions we determined the limit of detection (LOD) to be 5 ppb or $2.38 \times 10^{-8} \text{ mol L}^{-1}$ as the spectral features of PCN identified in Supplementary Table S1 are recognized at this level. Furthermore, the peak intensities are found to be dependent on PCN concentration C_{PCN} . Highly similar SERS spectra are obtained across a wide PCN concentration range, from 5 ppb to 50 ppm (Supplementary Figure S2). Intensity of the most dominant and consistent PCN peak at $\nu = 1352 \text{ cm}^{-1}$, I_{1352} , is used to quantitatively indicate the overall spectral intensity. Figure 2 shows that I_{1352} increases with increasing C_{PCN} , until it reaches a maximum value at $C_{\text{PCN}} = 10 \text{ ppm}$. Here each data point represents the average signal from 30 spectra collected from three different substrates. The standard deviation is represented by the error bar, and the RSD of the spectra ranges from 12 to 19% across the different C_{PCN} , as shown in Supplementary Figure S3. When $C_{\text{PCN}} > 10 \text{ ppm}$, a multilayer of PCN may be formed on the AgNR surface, which results in a slight decrease in I_{1352} as the SERS signal starts to quench. The relationship between I_{1352} and C_{PCN} follows the power law in the range 5 ppb $< C_{\text{PCN}} < 10 \text{ ppm}$: $\log I_{1352} = (0.66 \pm 0.05) \log C_{\text{PCN}} + (3.54 \pm 0.08)$, $R^2 = 0.96$, where R^2 is coefficient of determination which represents the goodness of the fitting. Similar SERS spectra are also observed with LB broth spiked with PCN (Supplementary Figure S4A).

Quantitation of PCN secretion from *P. aeruginosa* during growth

Since PCN is secreted by *P. aeruginosa* during its growth, it is reasonable to believe that PCN will be present in the culture media. *P. aeruginosa* strains PA14 and PAO1 were inoculated in LB broth and incubated overnight, followed by centrifugation and filtration of the LB culture medium to remove any bacterial cells. Due to the interference from the complex constituents of the LB medium such as carbohydrates and proteins, signal from PCN is not detected directly in the medium. However, when the broth is diluted with DI water to 1:100, unique PCN fingerprints are clearly recovered (Supplementary Figure S4B). Three significant SERS peaks at $\nu = \sim 1088, 1125, \text{ and } 1352 \text{ cm}^{-1}$ are observed in both *P. aeruginosa*-inoculated LB broth and PCN-spiked LB broth with minor peak shifts (Supplementary Figure S4A). This confirms that the SERS fingerprints of *P. aeruginosa*-inoculated broth mainly originate from PCN produced by the bacterium; therefore PCN could be used as a major biomarker for *P. aeruginosa* infection.

In order to validate this SERS-based indirect detection method for *P. aeruginosa*, strain PAO1 was inoculated in LB broth, and 1 ml of the culture was harvested from 0 to 48 h. After removing the cells by centrifugation and filtration, the samples were diluted 1:100 with DI water, and then measured for their SERS response. As shown in Figure 3A, the SERS spectra of diluted bacterial growth broth collected at incubation time $t = 0, 1, 3, 6, 8,$ and 10 h share similar spectral features with signature peaks at $\nu = 410, 517, 544, 592, 1118, 1352, 1593, \text{ and } 1613 \text{ cm}^{-1}$, consistent with the characteristic peaks of PCN, whereas all these peaks are absent in the spectrum of the plain LB broth.

In order to further quantify the analysis, a calibration curve is established for I_{1352} and C_{PCN} using 1% LB broth spiked with PCN at different concentrations (Supplementary Figure S5). The 1% LB broth is used to account for possible interference from the medium when all broth samples are diluted 1:100 in DI water. The relationship between I_{1352} and C_{PCN} in 1%

LB broth solution also follows the power law when $5 \text{ ppb} < C_{\text{PCN}} < 10 \text{ ppm}$, $\log I_{1352} = (0.87 \pm 0.06) \log C_{\text{PCN}} + (3.89 \pm 0.07)$, $R^2 = 0.97$. This calibration curve is then used to calculate C_{PCN} at different t , and the obtained C_{PCN} is plotted against t in Figure 3B. It is interesting to note that C_{PCN} shows a monotonically increase with t , indicating PCN accumulation in the broth during bacterial growth. A positive PCN detection is assumed when I_{1352} is significantly different from the spectral fluctuation of the LB broth control^{29, 30}, which is estimated as $\overline{C_{\text{PCN}}} + 3\sigma$ of the control sample (dash line in Figure 3B). $\overline{C_{\text{PCN}}}$ is the average PCN concentration, and σ is the standard deviation of the measurements due to the fluctuation of SERS signals. All the samples after $t = 8 \text{ h}$ have C_{PCN} higher than spectral fluctuation, therefore are identified positive for PCN. The C_{PCN} during bacteria growth (black square) are also compared with the cell density obtained by OD_{600} (blue dots) shown in Figure 3B. It is interesting to note that the $C_{\text{PCN}}-t$ curve follows a typical bacterial growth pattern: In the first phase of growth ($t < 8 \text{ h}$), the concentration of PCN remains below detectable levels, which corresponds to the lag phase of growth, in which bacterial cells begin to adapt to new environmental conditions, repair macromolecular damage, and synthesize cellular components necessary for growth. As a secondary metabolite, PCN is synthesized and released in limited amount at this early stage. Beyond 8 h , the number of bacterial cells increases exponentially, and PCN is also synthesized and secreted into the growth medium rapidly. During the exponential growth phase, C_{PCN} shows a linear relationship with t ($R^2 = 0.95$). After 30 h of growth, the depleting nutrients in the broth lead to a reduced or even negative growth rate as confirmed by the blue OD_{600} curve in Figure 3B. During this stationary phase, PCN as a quorum sensing agent is continuously synthesized and released, but at a lower rate, which translates into a slowly increasing C_{PCN} ³¹.

In parallel to the SERS and OD_{600} measurements, the amount of PCN released into the medium was also directly quantified using the optical density (absorbance) of the samples at $\lambda = 691 \text{ nm}$ according to the previously published A_{691} method²⁶. As shown in Figure 3B, the C_{PCN} obtained by SERS is in good agreement with that obtained by the A_{691} method, which again validates that SERS can be used to reliably and accurately detect and quantitate PCN in biological fluids.

PCN detection in processed sputum obtained from CF subjects infected with *P. aeruginosa*

To translate our findings, we investigated the feasibility of using SERS-based diagnostic method to rapidly detect *P. aeruginosa* infection in clinical specimens. For a proof-of-concept, we examined 15 clinical sputum samples obtained from CF subjects. When examining clinical samples, two difficulties may be encountered: interference from the complicated and heterogeneous nature of biological fluids, and the range of the concentrations of *P. aeruginosa* and PCN in the sputum samples. As Figure 2 demonstrates, the C_{PCN} can only be quantitatively determined between 5 ppb and 10 ppm . The maximum detectable C_{PCN} in *P. aeruginosa* culture medium can be as much as 60 ppm , so diluting the bacterial growth broth 1: 100 with DI water not only reduces the interferences, but also move the C_{PCN} into detectable range, as discussed in the previous sections. However, when the same procedure is used for processed sputum samples, no PCN SERS peaks were

observed due to the dense matrix and volume dilution of processed sputum (Supplementary Figure S6). We hence adopted an alternative strategy of extracting PCN directly with chloroform from processed sputum, as shown previously in studies using HPLC²⁶. The chloroform extraction method is quick and such an extraction along with measurement of the processed sputum samples only takes about 5 minutes. SERS spectra were first collected from chloroform-treated sputum control spiked with different concentrations of PCN, as shown in Figure 4A. The spectra show all the previously identified characteristic PCN peaks, with additional peaks from the sputum matrix at $\nu = 1078, 1186, 1203$ and 1593 cm^{-1} . The peaks at $\nu = 1186$ and 1593 cm^{-1} are at the same position as the PCN peaks, which gives interferences to PCN detection. Fortunately, the most intense PCN peak at $\nu = 1352 \text{ cm}^{-1}$ is absent in the sputum of control sample; hence it can still be used to indicate the presence of PCN. I_{1352} from PCN is corrected by I_{1078} from the sputum matrix and plotted against C_{PCN} in Figure 4B, and a relationship between I_{1352}/I_{1078} and C_{PCN} is observed: $\log I_{1352}/I_{1078} = (0.94 \pm 0.03) \log C_{\text{PCN}} - (0.79 \pm 0.04)$, $R^2 = 0.994$. This calibration curve with a LOD of 5 ppb is used to estimate the C_{PCN} in sputum samples.

The SERS spectra of the extracted chloroform layers from the 15 processed clinical sputum samples obtained from CF patients were collected. As shown in Figure 5A, using the aforementioned calibration curve, we obtain that C_{PCN} ranges from 18.7 to 64.9 ppb in the PA+ samples, 5.1 to 21.5 ppb in the PA+/SA+ samples, and 1.1 to 2.9 ppb in the PA- samples. All the PA+ and PA+/SA+ samples have an average $C_{\text{PCN}} > 5$ ppb, the established LOD, whereas all the PA- samples have an average $C_{\text{PCN}} < 5$ ppb, which validates the LOD of the SERS method. In general, PA+ samples tend to have greater C_{PCN} than the PA+/SA+ samples, possibly because less PCN will be secreted by PA in the patient's airway in the presence of competition from *S. aureus*. Figure 5B shows the representative SERS spectra of PA+, PA-, and a PA- sample spiked with 10 ppb of PCN. The PA+ and PCN-spiked PA- samples show characteristic PCN peaks at $\nu = 1352$ and 1613 cm^{-1} , which are absent in the PA- samples. Therefore we conclude that the differences between PA- and PA+ samples are indeed introduced by the presence of PCN in PA+ samples.

In order to robustly discriminate the different types of sputum samples, partial least square-discriminant analysis (PLS-DA) was performed on the SERS spectra collected above. PLS-DA uses a linear combination of the predictor variables to project the original data to a new set of coordinates in order to generate a positive/negative prediction. It is often used to classify samples based on known characteristics. The results of the PLS-DA are shown in Figure 6. The sputum samples from subjects with PA infections (PA+ and PA+/SA+) are assigned as PCN+, and the samples from subjects without PA infections (PA-) are denoted as PCN-. Using PLS-DA, 95.6% sensitivity and 93.3% specificity between the PCN+ and PCN- samples have been achieved. The sensitivity is defined as the probability that a test will indicate disease among those with the disease. Specificity is defined as the fraction of those without disease who will have a negative test result. The calculation of sensitivity and specificity of PLS-DA is detailed in Supplementary Note 3. The loading score plot can be used to examine how the discrimination is achieved, because the wavenumbers that contribute most to the discrimination will have the greatest absolute value in the loading score plot. The most significant peaks used to discriminate PCN+ and PCN- in clinical

sputum samples are peaks at $\nu = 1078, 1352$ and 1613 cm^{-1} (Supplementary Figure S7). The peak at $\nu = 1078 \text{ cm}^{-1}$ is the most significant peak from sputa, and the peaks at $\nu = 1352$ and 1613 cm^{-1} are two major peaks from PCN as previously discussed. Four spectra from the positive samples are misclassified as negative, and all of these four misclassified positive samples are PA+/SA+, probably because of the lower concentration of PCN in those samples as discussed above. These data suggest that SERS can be used to discriminate between sputum samples with and without *P. aeruginosa* infection with high accuracy.

Discussion

P. aeruginosa is one of the most common pathogens found in CF airway. The presence of PCN in biofluids indicates active infection with *P. aeruginosa*, which aids timely and effective therapeutic decisions. Early detection and identification of *P. aeruginosa* infections in CF patients will help in successful antibiotic treatment that prevents biofilm formation in CF airways and nosocomial infections in non-CF patients, which leads to a positive impact in healthcare settings by shortening hospital stays. Being able to diagnose *P. aeruginosa* infection without time-consuming bacterial culture relies on the extremely low detection limit of PCN that has been achieved through our SERS-based detection system. Such extremely low concentrations of PCN are comparable to the levels commonly found in the biological fluids. PCN has been found as low as 1.3×10^{-5} to $8.1 \times 10^{-3} \text{ mol L}^{-1}$ in wounds and urine specimens, and 3×10^{-6} to $2.7 \times 10^{-3} \text{ mol L}^{-1}$ in human ear secretions³². These concentrations are well above the SERS LOD, which is 5 ppb or $2.38 \times 10^{-8} \text{ mol L}^{-1}$. However, if a sample has $C_{\text{PCN}} > 10\text{ppm}$, the presence of PCN can only be qualitatively determined. But through serial dilutions and redundant measurements, the C_{PCN} can still be determined quantitatively. For example, assuming that the C_{PCN} in a clinical sample is around 1000 ppm, we can dilute the original samples into four solutions with DI water: original sample, 10^{-1} , 10^{-2} , and 10^{-3} dilutions. Then the SERS spectra from all 4 samples will be obtained simultaneously. If original, 10^{-1} , and 10^{-2} dilutions samples give the similar PCN signal intensity while 10^{-3} dilution gives significantly lower SERS signal, we will quantitatively estimate the C_{PCN} in 10^{-3} dilution sample using the calibration curve, and then calculate the C_{PCN} in original samples.

PCN is toxic to host cells, so detecting PCN at very low concentrations is also important to alleviate PCN toxicity³³. Our results also reveal that *P. aeruginosa* secretes PCN during the stationary growth phase and it is correlated to the PCN's ability to promote the biofilm formation via ferrous iron acquisition³⁴, and the release of necessary DNA³⁵. The biofilm formation is important in clinical settings where central lines, ventilator related intubations and urinary catheters are used.

SERS detection of the biomarker PCN in sputum proves a novel and inexpensive tool for rapidly detection of *P. aeruginosa* infection and has a potential for developing into a point-of-care device. The use of biomarkers in bacterial SERS detection may also provide insights in the detection of other nosocomial pathogens such as *S. aureus* and *Acinetobacter*. This SERS based detection platform can advantageously process multiple specimens rapidly, overcome the need for bacterial culture, and preclude the need for sophisticated instrumentation and large consumption of samples and reagents. Future studies will be

focused on further lowering the LOD of the SERS method, extending the detection platform for other bacterial biomarkers, and integrating with more robust sample preparation procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Buchanan PJ, Ernst RK, Elborn JS, Schock B. *Biochem Soc Trans.* 2009; 37:863–867. [PubMed: 19614608]
2. Crouch Brewer S, Wunderink RG, Jones CB, Leeper KV Jr. *Chest.* 1996; 109:1019–1029. [PubMed: 8635325]
3. Agodi A, Barchitta M, Cipresso R, Giaquinta L, Romeo MA, Denaro C. *Intensive Care Med.* 2007; 33:1155–1161. [PubMed: 17503016]
4. Lamont IL, Konings AF, Reid DW. *Biometals.* 2009; 22:53–60. [PubMed: 19130260]
5. Rusciano G, Capriglione P, Pesce G, Abete P, Carnovale V, Sasso A. *Laser Physics Letters.* 2013; 10:075603.
6. Willemse-Erix DF, Jachtenberg JW, Schut TB, van Leeuwen W, van Belkum A, Puppels G, Maquelin K. *J Biophotonics.* 2010; 3:506–511. [PubMed: 20572285]
7. Willemse-Erix DFM, Scholtes-Timmerman MJ, Jachtenberg J-W, van Leeuwen WB, Horst-Kreft D, Bakker Schut TC, Deurenberg RH, Puppels GJ, van Belkum A, Vos MC, Maquelin K. *Journal of Clinical Microbiology.* 2009; 47:652–659. [PubMed: 19109462]
8. Efrima S, Zeiri L. *J Raman Spectrosc.* 2009; 40:277–288.
9. Zhang X, Young MA, Lyandres O, Van Duyne RP. *J Am Chem Soc.* 2005; 127:4484–4489. [PubMed: 15783231]
10. Cheng HW, Huan SY, Wu HL, Shen GL, Yu RQ. *Analytical Chemistry.* 2009; 81:9902–9912. [PubMed: 19928907]
11. Cowcher DP, Xu Y, Goodacre R. *Analytical Chemistry.* 2013; 85:3297–3302. [PubMed: 23409961]
12. Rada B, Leto TL. *Trends in microbiology.* 2013; 21:73–81. [PubMed: 23140890]
13. Winstanley C, Fothergill JL. *FEMS Microbiol Lett.* 2009; 290:1–9. [PubMed: 19016870]
14. Visca P, Imperi F, Lamont IL. *Trends Microbiol.* 2007; 15:22–30. [PubMed: 17118662]
15. Usher LR, Lawson RA, Geary I, Taylor CJ, Bingle CD, Taylor GW, Whyte MK. *J Immunol.* 2002; 168:1861–1868. [PubMed: 11823520]
16. Hunter RC, Klepac-Ceraj V, Lorenzi MM, Grotzinger H, Martin TR, Newman DK. *American Journal of Respiratory Cell and Molecular Biology.* 2012; 47:738–745. [PubMed: 22865623]
17. Fothergill JL, Mowat E, Ledson MJ, Walshaw MJ, Winstanley C. *Journal of Medical Microbiology.* 2010; 59:472–481. [PubMed: 20019149]
18. Fothergill J, Panagea S, Hart C, Walshaw M, Pitt T, Winstanley C. *BMC Microbiology.* 2007; 7:45. [PubMed: 17521417]

19. Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW, Cole PJ. *Infect Immun*. 1988; 56:2515–2517. [PubMed: 3137173]
20. Caldwell CC, Chen Y, Goetzmann HS, Hao Y, Borchers MT, Hassett DJ, Young LR, Mavrodi D, Thomashow L, Lau GW. *The American Journal of Pathology*. 2009; 175:2473–2488. [PubMed: 19893030]
21. Hunter RC, Klepac-Ceraj V, Lorenzi MM, Grotzinger H, Martin TR, Newman DK. *Am J Respir Cell Mol Biol*. 2012; 47:738–745. [PubMed: 22865623]
22. Chaney SB, Shanmukh S, Dluhy RA, Zhao YP. *Appl Phys Lett*. 2005; 87:031908.
23. Driskell JD, Shanmukh S, Liu Y, Chaney SB, Tang XJ, Zhao YP, Dluhy RA. *J Phys Chem C*. 2008; 112:895–901.
24. Liu YJ, Chu HY, Zhao YP. *The Journal of Physical Chemistry C*. 2010; 114:8176–8183.
25. Zughaier SM, Tangpricha V, Leong T, Stecenko AA, McCarty NA. *J Investig Med*. 2013; 61:1018–1025.
26. Reszka KJ, O'Malley Y, McCormick ML, Denning GM, Britigan BE. *Free Radical Biology and Medicine*. 2004; 36:1448–1459. [PubMed: 15135182]
27. Hector A, Jonas F, Kappler M, Feilcke M, Hartl D, Griesse M. *Respiration*. 2010; 80:393–400. [PubMed: 20029169]
28. Hassett DJ, Charniga L, Bean K, Ohman DE, Cohen MS. *Infection and Immunity*. 1992; 60:328–336. [PubMed: 1730464]
29. Wu X, Xu C, Tripp RA, Huang Y-w, Zhao Y. *Analyst*. 2013; 138:3005–3012. [PubMed: 23563168]
30. Wu X, Gao S, Wang JS, Wang H, Huang YW, Zhao Y. *Analyst*. 2012; 137:4226–4234. [PubMed: 22832405]
31. Dietrich LE, Price-Whelan A, Petersen A, Whiteley M, Newman DK. *Molecular microbiology*. 2006; 61:1308–1321. [PubMed: 16879411]
32. Reimer Å. *Acta Oto-laryngologica*. 2000; 120:86–88.
33. Gloyne LS, Grant GD, Perkins AV, Powell KL, McDermott CM, Johnson PV, Anderson GJ, Kiefel M, Anoopkumar-Dukie S. *Toxicology in Vitro*. 2011; 25:1353–1358. [PubMed: 21596130]
34. Wang Y, Wilks JC, Danhorn T, Ramos I, Croal L, Newman DK. *Journal of Bacteriology*. 2011; 193:3606–3617. [PubMed: 21602354]
35. Das T, Manefield M. *PLoS ONE*. 2012; 7:e46718. [PubMed: 23056420]

Figure 1.

Raman fingerprints of PCN. **(A)** Experimental SERS spectrum of PCN ($C_{\text{PCN}} = 10$ ppb) based on AgNR substrates. **(B)** The theoretical Raman spectra of PCN-Ag complex calculated by DFT. The corresponding major peaks in SERS and DFT are denoted in the same Greek symbols.

Figure 2.

The plot of the SERS peak intensity I_{1352} of PCN as a function of PCN concentration C_{PCN} in log-log scale. Red line indicates the apparent linear fitting in the range of 5 ppb C_{PCN} 10 ppm.

Figure 3.

PCN release and quantitation. **(A)** SERS spectra of *P. aeruginosa* strain PAO1 culture broth harvested at $t = 0, 1, 3, 6, 8,$ and 10 h, respectively. LB broth incubated without bacteria was used as a control. **(B)** Change in PCN concentration C_{PCN} and cell density with respect to incubation time t . Black squares indicate C_{PCN} calculated from SERS measurements. Red rhombuses indicate C_{PCN} estimated using the A691 method. Blue dots indicate cell density determined by OD₆₀₀. The dash line is the threshold value for positive PCN identification.

Figure 4.

PCN spiked processed sputum samples. (A) Representative SERS spectra of sputum samples inoculated with 0 ppb – 1 ppm of PCN. The red Greek symbols indicate the peaks from PCN, and the black lines and numbers indicate the peaks from sputa. (B) SERS peak intensity ratio I_{1352}/I_{1078} as a function of PCN concentration C_{PCN} in log-log scale, and the corresponding linear fitting curve.

Figure 5.

Clinical sputum samples. **(A)** Estimated PCN concentration in clinical sputum samples. The dashed line indicates the LOD of $C_{PCN} = 5$ ppb. **(B)** SERS spectra of three representative sputum samples extracted by chloroform: PA-, PA- spiked with 10 ppb PCN, and PA+.

Figure 6. PLS-DA plot of SERS spectra of the clinical sputum samples. The thick horizontal line defines the critical value for PCN+ and PCN- discrimination.