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Surface-enhanced raman spectroscopy for detection of hydrogen cyanide in breath from children colonised with *P. aeruginosa*

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There is a need for a fast and non-invasive tool to detect *Pseudomonas aeruginosa* airway colonisation in cystic fibrosis (CF) patients unable to expectorate. Fifty CF children and 19 controls aged 5-17 years were included in the feasibility study. A surface-enhanced Raman spectroscopy (SERS) nanochip optimised for detection of trace amounts of the *P. aeruginosa* biomarker hydrogen cyanide (HCN) was mounted inside a Tedlar bag, which the patient breathed into. The SERS chip was then analysed in a Raman spectrometer, investigating the C≡N peak at 2131 cm⁻¹ and correlated with sputum cultures. One new *P. aeruginosa* colonisation occurred during the trial period. The C≡N peak intensity was enhanced in this sample in contrast to the subject's 3 other samples. Three additional patients had intense C≡N SERS signals from their breath, but no *P. aeruginosa* was cultured from their sputum. It is concluded that SERS spectroscopy can be developed into an easy to use hypersensitive clinical prescreening method for detection of HCN in human breath.

Introduction

Pseudomonas aeruginosa airway infections are the major cause of morbidity and mortality in cystic fibrosis (CF) patients^{1,2}. The methods that are being used in the CF clinic to discover *P. aeruginosa* in non-expectorating children are invasive, because either a swab needs to enter through the mouth and into the pharynx or a tube is inserted through the nose and into the larynx to obtain a sample. Even with these procedures there is a low sensitivity, and it has been estimated that approximately 75% of all newly discovered *P. aeruginosa* infections are already persistently established³. Therefore, there is an urgent need for a sensitive, fast, cheap and non-invasive diagnostic tool to detect early *P. aeruginosa* airway colonisation in CF children who are unable to produce sputum or expectorate properly. *P. aeruginosa* emits the poisonous gas hydrogen cyanide (HCN), which can be an advantage combating other microorganisms in the lungs^{4,5}. HCN is considered a potential *P. aeruginosa* biomarker, and it was

detected at median values of 13.5 ppb in children's breath using selected ion flow tube mass spectroscopy (SIFT-MS)⁴. In the SPACE study, Gilchrist and co-workers measured HCN in exhaled breath of CF children, also using SIFT-MS⁶. At present time, SIFT-MS has not been optimized to meet the need for fast detection of bacteria, and the measurements were made after up to 24 hours, whereby some of the exhaled HCN may have been lost. Additionally, in the SPACE study, chronically infected patients were included, whose *P. aeruginosa* may have ceased producing HCN. Previously we have shown that at some point during adaptation *P. aeruginosa* can stop producing HCN, which can explain why the biomarker cannot be detected in the breath of all CF patients with a chronic airway infection⁷.

Between C and N of cyanide there is a triple bond, which gives rise to a distinct peak in a Raman spectrum at 2131 cm⁻¹. Raman spectroscopy is a fast and non-invasive analytical technique extensively used for molecular fingerprinting. The technique identifies analytes by differentiating their molecular vibrational frequencies in the probed Raman scattering signal. However, the limit of detection for traditional Raman spectroscopy is in the range of millimolar concentration⁸, impeding the use of the technique for detecting molecules at lower concentrations. To indicate *P. aeruginosa* infection in the breath of patients with CF, detection levels of HCN below 20 ppb are required⁴. As an advance of Raman spectroscopy, surface-enhanced Raman spectroscopy (SERS) probes the enhanced Raman scattering signal from analytes sitting on the surface of noble metal nanostructures⁹. In addition, the affinity of cyanide towards gold¹⁰ makes SERS very suitable for detecting trace amount of HCN. Recently, we have demonstrated the fast identification and quantification of gas-

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phase HCN down to 18 ppb on gold coated silicon nanopillar substrates¹¹, which are manufactured in large-format by a cost effective process¹². We have also applied the SERS method to detect HCN in emissions from *P. aeruginosa* cultures isolated from CF patients⁷. Our results indicate that SERS on the nanopillar substrate may have significant potential towards fast, cheap and non-invasive detection of *P. aeruginosa* lung colonisation from the breath of children with CF. In this feasibility study, we wanted to use the gold (Au)-coated silicon (Si) SERS substrate for direct detection of HCN in the breath of CF children with an early *P. aeruginosa* colonisation.

Materials and Methods

Subjects and study design

Fifty children with CF (median age 11 years, range 5-17 years) and 19 non-CF, age-matched controls were included in the study. CF patients were excluded if their latest total IgG measured by an Enzyme-Linked-Immorbent Assay (ELISA) against *P. aeruginosa* antigens was above 2.39 ELISA units, or if the number of precipitating antibodies against *P. aeruginosa* was ≥ 2 , meaning that they had a chronic *P. aeruginosa* infection¹³, except from two CF patients who were included to compare *in vitro* results⁷ to *in vivo* breath analysis. A total of 50 CF children were followed for four months at the CF centre at Rigshospitalet. Each month the child exhaled into a 3L Tedlar bag with the SERS substrate mounted inside, which was analysed a few hours later. The intensity of the cyanide peak at 2131 cm^{-1} was compared to culture of sputum samples obtained via expectoration or endo-laryngeal suction.

The controls were patients and siblings seen in the CF outpatient clinic at Rigshospitalet during the study period, which was January-April 2016. Controls were not included if they had CF, primary cilia dyskinesia (PCD) or asthma. Enrolment took place during the entire study period, and each subject was tested between 1 and 5 times. Controls were only tested once. The controls only delivered breath and no sputum sample.

The SERS substrate

The disposable SERS substrate was fabricated on a single-side polished Si wafer using 3 min reactive ion etching (RIE) with alternating SF_6 bombardment and O_2 passivation for protection of the emerging nanopillars, followed by a 1 min O_2 plasma cleaning step. Up till 5 days prior to use, Au was evaporated onto the Si nanopillars, forming 225 nm caps used for SERS detection (Figure 1a). On the day of exposure the wafer was cut into 5x10 mm^2 substrates using a diamond cutter and tweezers. The substrates were cleaned by immersion into ethanol (Absolute grade, CHROMASOLV R, Sigma-Aldrich, St. Louis, MO, USA) for 3 min followed by H_2O (Molecular Biology Reagent grade, Sigma-Aldrich, St. Louis, MO, USA) for 3 min and left to dry on a tissue whereby the pillars would lean to enable SERS detection (Figure 1b). For easy handling and transportation the SERS substrate was mounted inside a small Petri dish, using double sided adhesive

tape (Scotch), with a Post-It on top, the tacky side facing up for holding the substrate. The wall of the Petri dish protected the delicate SERS substrate so it would not get in contact with the Tedlar bag and be scratched (Figure 1c).

The collection device

Three L disposable Tedlar sample bags with polypropylene fittings (SKC, Pennsylvania, USA) were used for breath collection, with one large corner cut off, so the Petri dish containing the SERS substrate could be mounted inside (Figure 1d). The bag was closed with a clamp (Scentroid, Ontario, Canada). Part of a disposable latex tube (Philips Respironics, Colorado, USA) was used to connect the inlet of the Tedlar bag to the PEP flutter (Intersurgical, Berkshire, UK) that the child would blow through, equipped with a 3.5 mm resistance (Wellspect Healthcare, Mölndal, Sweden).

Sample collection

The child filled the Tedlar bag with 1-5 deep exhalations. The bag was closed and left for 15 minutes before evacuation in a laminar air flow bench. The lid was put on the Petri dish, which was then secured tightly in a small zipper bag for transportation. A short questionnaire was also filled in, regarding present well-being, coffee intake and smoking, because it is known that these stimulants can influence the amount of HCN in the breath^{14,15}.

Raman measurements

SERS measurements were recorded using an FT-Raman instrument (Bruker VERTEX 70, Bruker Optik, Ettlingen, Germany), equipped with a 1064 nm laser and an indium gallium arsenide (InGaAs) detector. Samples were measured in a 180 degrees backscattering geometry using a laser power of 200 mW with 32 scans at a resolution of 4 cm^{-1} . Each sample was measured at 5 different positions on the substrate.

Data analysis

The five spectra obtained from different positions on the SERS substrate were averaged in order to increase the signal to noise ratio. The average per sample was subsequently pre-processed using Standard Normal Variate (SNV)¹⁶. SNV eliminates off-sets and slopes from spectra with similar spectral features aiding visual inspection as well as reducing complexity of later data analysis. Principal component analysis (PCA)¹⁷ was performed using the PLS_toolbox (Ver. 7.9.5, Eigenvector Research, USA) in Matlab 2015a (MathWork, USA) on the pre-processed data both on the full spectral range and a reduced data set with only the HCN peak around 2131 cm^{-1} . In figures 2 and 3, the background was subtracted and spectra were shifted up for clarity.

Ethical approval

The local ethics' committee approved the feasibility study, which was assigned the number H-15016027. Informed consent was obtained from the parents or from the children aged 15 years or older.

Results

Demographics

A total of 50 CF patients and 19 controls were included in the study (Table 1). With 4 months and 50 CFs, about 2 new cases of *P. aeruginosa* colonisation were expected (according to the outcome of⁶), but a few patients with chronic infections were included, leaving fewer naïve cases.

Table 1. Demographics of included CF patients and controls

CF patients		
Median age, years (range)	11 (5-17)	
Gender, number (%)	Males 23 (46)	Females 27 (54)
No. of visits by CF patients in total: 87		
Controls		
Median age, years (range)	8 (5-17)	
Gender, number (%)	Males 12 (63)	Females 7 (37)
No. of visits by the controls in total: 19		
Underlying diseases of the controls: Other phacomatoses, Secondary pulmonary condition, Cough, Pneumonia, Pulmonary atelectasis, Stridor, Pulmonary hemoptysis, Acute bronchitis.		

Comparison of *in vitro* and *in vivo* SERS

Previously, *in vitro* SERS measurements were made on *P. aeruginosa* cultures isolated from chronically infected paediatric CF patients in the outpatient clinic⁷. Two of these patients were also included in the study because their isolates represented two different trends in the *in vitro* study. In the *in vitro* study, all first (wild type-like) isolates emitted detectable HCN. After it had turned into a chronic infection, subject A had a *lasR* mutated *P. aeruginosa* isolate which still produced HCN (Figure 2a, red spectrum), while subject B's *lasR* mutated *P. aeruginosa* isolate had turned off the expression of HCN (Figure 2b, red spectrum). Therefore, it was interesting to investigate whether this trend was also seen *in vivo*. The isolates were obtained five years before, so the bacteria could possibly have mutated further in the meantime. As seen in the figures' blue spectra, no HCN was detected from their breath. Patient A: Both the wild type-like (green) and the *lasR* mutated isolate (red) emitted HCN, which can be seen from the intense C≡N peak at 2131 cm⁻¹, whereas no HCN was detected from the patients' breath (blue). Patient B: The wild type-like isolate emitted HCN, which did neither the *lasR* mutated isolate, nor the breath sample. SERS on headspace of Luria Broth (LB) growth medium is included as reference (black). There is a background peak in the SERS spectra close to where the cyanide peak is located.

Although chronically infected with *P. aeruginosa*, patient A only cultured *Stenotrophomonas maltophilia* and no *P. aeruginosa* from sputum, whereas patient B cultured both mucoid and non-mucoid *P. aeruginosa*.

Four cases of elevated cyanide signal

There was one new case of *P. aeruginosa* during the 4 months' trial period. Patient C was a 15 year-old male with a positive sputum culture at his 3rd out of 4 visits during the four months. Figure 3 shows the SERS spectra from his four visits, with a more intense triple bond signal about 2131 cm⁻¹ from visit no. 3. Additionally, the red peak is slightly shifted towards higher wave numbers, away from the background signal. After antibiotic eradication therapy between the third and fourth visit to the outpatient clinic, the background peak is back to where it started (green). The intensity is increased at visit no. 3, and the peak is slightly shifted towards higher wave numbers.

Three additional times during the trial period the SERS triple bond signal was elevated in breath samples, but none of these patients had *P. aeruginosa* cultured from their sputum. In one of the cases, the Tedlar bag had been left for 60 min before the SERS substrate was taken out, and it turned out that this had also been the case in the 3rd visit of patient C, and it is uncertain whether this had an effect on the peak intensity. Two of the incidents were from children whose parents were smoking in-door at home, which may have been detected on the chip. One of them cultured yeast, while the other only cultured non-pathogenic oropharyngeal microorganisms from the sputum samples.

Discussion

We explored whether the SERS chip could be used to detect the biomarker HCN in the breath of CF children with an early *P. aeruginosa* airway colonisation and whether our findings could be correlated to the findings in sputum cultures. Due to a limited number of includable patients, only one new case of *P. aeruginosa* was cultured during the study period, and it was most likely detected on the SERS chip. In the SERS measurements from the patient's other visits, only the background peak was present. Three additional cases of intense CN signal occurred in children, whose sputum cultures were *P. aeruginosa* negative. There was a dependency of exposure time on peak intensity, and it seemed like passive smoking could also be detected from the children's breath.

The reason to include two patients with chronic infections was that we wanted to compare our *in vitro* results⁷ on their first colonising strain and later *lasR* mutated isolate to *in vivo* breath analysis, because these cannot be expected to be the same¹⁸. Patient A, whose *lasR* mutated *P. aeruginosa* isolate emitted HCN, was 8 years old; and at the time the strain was isolated she was 3. An explanation to why no HCN was detected in the breath of this patient could be that the bacteria had mutated even further since the isolate was cultured back in 2011. Even though this CF patient has about 25 precipitating antibodies against *P. aeruginosa* today, no *P. aeruginosa* had been cultured from her last 3 sputum samples. Only when naso-laryngeal suction was performed, mucoid *P. aeruginosa* was cultured; and it can be questioned whether it came from the patient's sinuses, and also whether young patients are able to expectorate properly.

In the *P. aeruginosa* case cultured during the trial, the position of the Raman peak was not outside the normal shift seen across patients; however, the patient specific shift (assuming a given patient has an individual peak position) can be an indication. More similar cases are necessary to investigate or reject this hypothesis. Two of the 3 additional patients with positive cyanide SERS signal were children whose parents were smoking in-door at home. The third patient had an immediate microscopy result indicating few Gram-negative rods, but when the final microbiological result came, it was *Moraxella catarrhalis*, which is a diplococcus, that can hardly be mistaken for a Gram-negative rod. The question is, whether Gram-negative rods were present in the beginning, but not cultured subsequently. Another observation was, that these children can be exposed to passive smoking as they enter the CF centre, which is located on the main floor, where people are standing right outside, smoking, because this is prohibited inside the hospital.

A major drawback of the applied method is the background signal on the Au SERS substrate, which has also been reported elsewhere²¹, and it seems to be enhanced by the warm and moist breath. The three cases with intense triple bond signal and negative sputum culture were followed for five months after study termination, and none of them cultured *P. aeruginosa* during the follow-up period, suggesting the SERS results must have been false positives. Regarding the control subjects, these need to be selected more carefully. Some were too sick, and some could have had *P. aeruginosa* in their lungs, since their sputum was not cultured. Ideally, the study period should have been longer or more patients should have been included to obtain more new positive *P. aeruginosa* cultures. In the SPACE study, Gilchrist and co-workers followed 233 CF children for 24 months, and 71 children cultured *P. aeruginosa* positive. With 50 CF children followed for 4 months, an estimate of two new *P. aeruginosa* cases were expected. To ease logistics and keep it to one centre, primary ciliary dyskinesia (PCD) patients could have been included, too, because it is known that the evolution of *P. aeruginosa* follows the same mutational steps in PCD as in CF²².

In the SPACE study, 21 chronically infected patients were included, who had not been cultured positive in their swab or sputum samples for the last 12 months⁶, and it can be questioned what the probability would be to culture positive during the trial period. In future studies we will suggest to consider using a more sensitive reference method, such as PCR instead of just sputum culture to detect whether *P. aeruginosa* is present in the sputum sample or not. Another issue regarding bacterial culturing is the transportation conditions of the sputum samples which could be optimised to better secure survival of the microorganisms. Samples should be either cooled down or kept at 37°C and transported immediately to the clinical microbiology laboratory and not wait up till 24 or 48 hours due to internal mail delivery. The PCA analyses did not show strong trends in the pre-processed spectral data, and it is important to remember that the spectral measurements as well as the sputum culturing results carry large uncertainties

due to exposure time differences, delayed analysis of sputum samples, possibly leading to increased number of false negative results, etc., making it challenging to relate the two.

The CN peak intensity seemed to depend on exposure time, and in future studies the device could be left for e.g. 60 minutes for complete exposure to occur. In the *in vitro* study, 15 min exposure time was applied, which was sufficient, and it is a learning to take to the next clinical trial. Another way to overcome the exposure time issue could be to condensate the breath directly onto the SERS substrate. Disposable devices for this are available (like the R-Tube from MESM Ltd.)¹⁹, but it is uncertain if patient compliance would be affected by having to breathe into a "chimney" device like this instead of into the applied "balloon-like" device which most of the children thought was fun. It could also be considered to make the substrate alkaline, so that a maximal amount of the cyanide would be expected to stay on the Au surface (according to Henry's Law²⁰).

Conclusions

The present study, has demonstrated that Surface Enhanced Raman Spectroscopy (SERS) can be developed into a very useful and sensitive method for early detection of *P. aeruginosa* in CF patients. However, the method is highly sensitive to other sources of HCN wherefore more *P. aeruginosa* positive patients must be tested in future trials in order to prove whether this method can be used for diagnosis.

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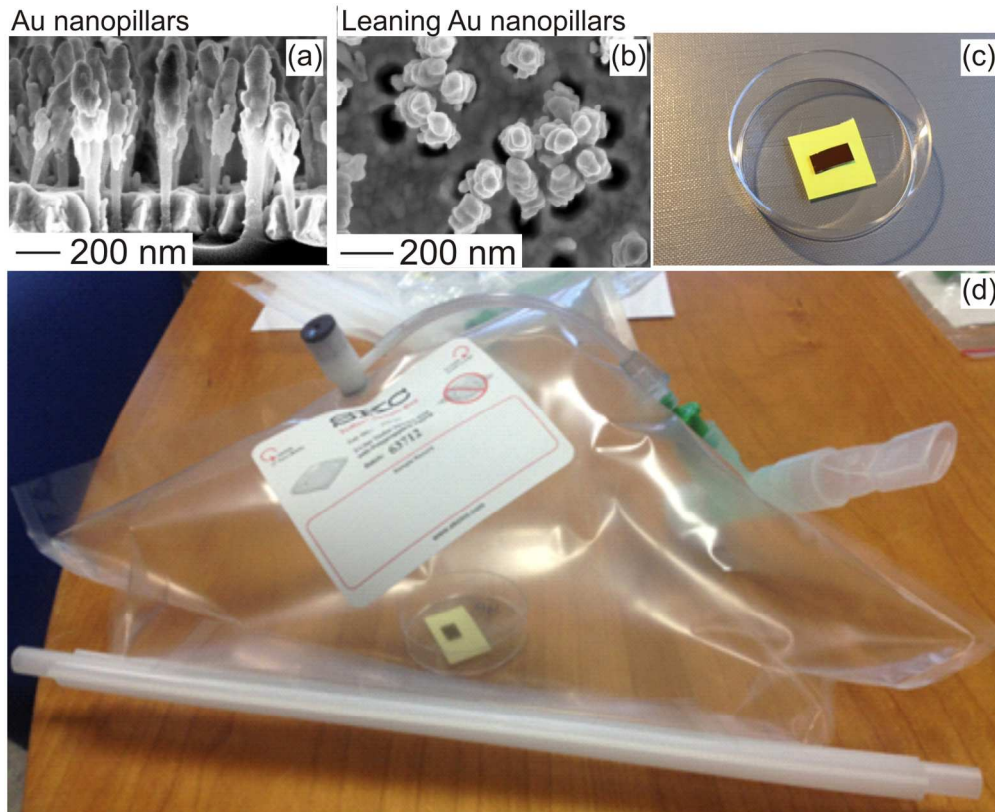
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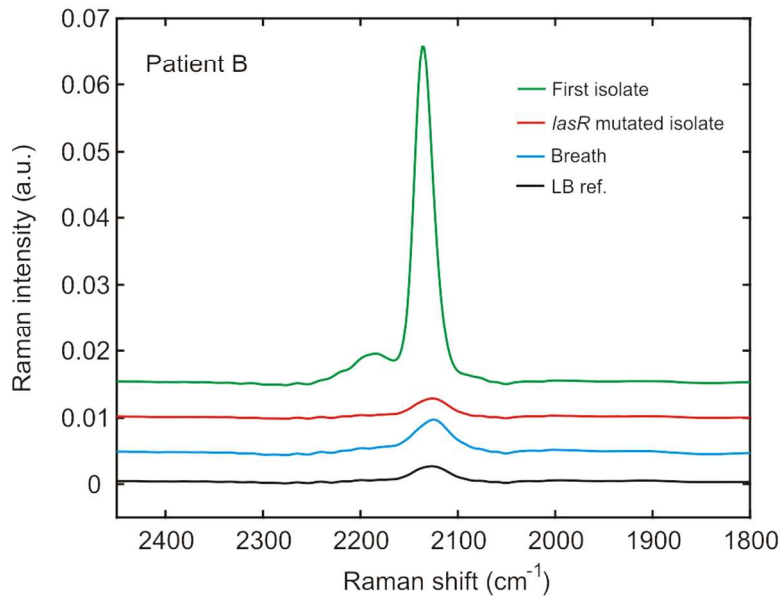
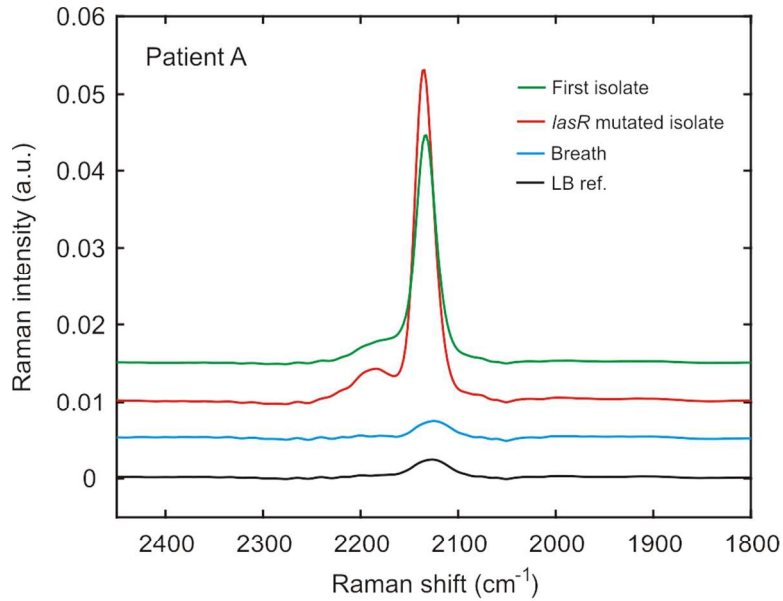
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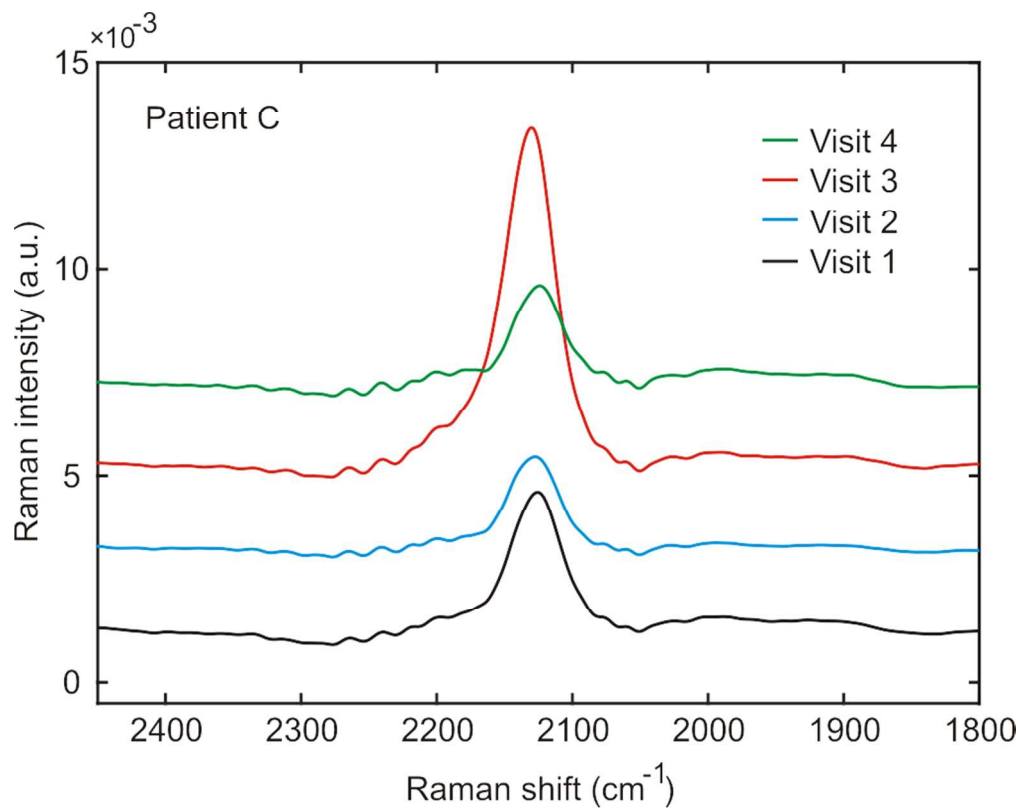
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The SERS substrate. a) Scanning Electron Microscopy (SEM) image of the vertically standing nanopillars prior to leaning. The Si nanopillars are capped by gold, and the underlying Si substrate is covered by a gold film. b) Top-view SEM image of two clusters of nanopillars after leaning. c) Petri dish with a gold (Au) SERS substrate mounted inside. d) The filled collection device with the SERS chip inside, being exposed to exhaled breath. The tip of the PEP flutter is seen to the right. The fitting on top and the clamp at the bottom keep the Tedlar bag closed.



In vitro and in vivo SERS on *P. aeruginosa* isolates and breath from two CF patients who today are chronically infected. Patient A: Both the wild type-like (green) and the *lasR* mutated isolate (red) emitted HCN, which can be seen from the intense $\text{C}\equiv\text{N}$ peak at 2131 cm^{-1} , whereas no HCN was detected from the patients' breath (blue). Patient B: The wild type-like isolate emitted HCN, which did neither the *lasR* mutated isolate, nor the breath sample. SERS on headspace of Luria Broth (LB) growth medium is included as reference (black). There is a background peak in the SERS spectra close to where the cyanide peak is located.



Zoom on the triple bond peak about 2131 cm^{-1} from patient C's visits. The intensity is increased at visit no. 3, and the peak is slightly shifted towards higher wave numbers.