Review

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The potential of SERS as an AST methodology in clinical settings

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Abstract: The ability to identify and characterize microorganisms from tiny sample volumes in a rapid and reliable way is the first and crucial step in the diagnostics of microbial infections. Ideal analytical techniques would require minimal and low-cost sample preparation, permit automatic analysis of many serial samples, and allow rapid classification of present microorganisms against a stable database. Current practice, however, is far from this ideal; a typical analytical procedure might require a few days. Delayed laboratory results might lead, for example, to progress/spread of the infection, more serious condition of the patient, even death, prescription of inappropriate antibiotics that could be ineffective against causative agents and may as well contribute to the emerging problem of drug resistance in microorganisms. Several studies confirmed that surface enhanced Raman scattering (SERS) is capable of a rapid identification and discrimination of biological samples including medically relevant bacteria. A typical spectrum contains a wealth of information indicative of the cellular content of nucleic acids, purine bases, proteins, carbohydrates, and lipids. Such a spectrum functions as a cellular ‘fingerprint’ and serves as a sensitive indicator of the physiological state of the cell which in turn enables to differentiate cell types, actual physiological states, nutrient conditions, and phenotype changes. Consequently, the focus of this review is on the SERS spectra of bacteria which result from secreted metabolic substances – the purine bases – which are a common feature in the label-free SERS research related to clinical

diagnostics of pathogens. Here is the review of the current status of SERS applications on bacteria. A special attention is given to the efforts of profiling antimicrobial susceptibility at clinically relevant species, which in turn has a great potential for use in routine point-of-care (POC) tests. Thus, early and accurate infection disease management can be provided at the bedside or at remote care centres.

Keywords: biosensor; infection disease management; surface enhanced Raman scattering; vibration spectroscopy.

1 Introduction

An early and accurate detection of microbial pathogens in the human samples (cerebrospinal fluid, blood, urine, stool, wound smears, sputum, swabs, etc.) is the most crucial step for clinical microbiology laboratories in infection disease management, including severe conditions like bacteremia and sepsis. As noted by Sweeney et al. [1], sepsis is frequently used synonymously with bacteremia (presence of microorganisms in the blood), however, sepsis and bacteremia occupy an important but narrow overlap.

Sepsis—extreme response of a human body to an infection causing damage to body’s own tissues and organs—is in the medical literature represented as a common and lethal syndrome. Recently the outcomes have improved, however the mortality remains high. No specific antisepsis treatments exist. Thus, the management of patients relies mainly on an early recognition that allows correct therapeutic measures to be started rapidly, including the administration of appropriate antibiotics [2]. It is obvious that the prompt initiation of appropriate antibiotic is crucial. The guidelines of Surviving Sepsis Campaign suggested that a treatment with antibiotic should ideally be started within 1 h of the diagnosis of severe sepsis or septic shock [2]. The principal goal of antimicrobial therapy is eradicating the underlying infection and resolution of its clinical impact while trying to avoid adverse effects and the development of drug resistance [3].

Inglis and Ekelund [4] suggested that definitive “Antimicrobial susceptibility testing” (AST) with corresponding

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antimicrobial amendment should be available within an 8 h window (e.g., the same working day). Therefore, truly rapid AST methods must be integrated into the clinical laboratory workflow to ensure maximum impact on the clinical outcomes of sepsis [4].

Moreover, recently Reddy et al. [5] noted that, frustratingly, only a little progress has been made in the past three decades of development of diagnostics and therapeutics for sepsis. There are some non-point-of-care (POC) devices on the market which can detect pathogens directly from the blood without the culture step in 3–5 h [5, 6]. Thus, if the pathogen identification and the AST could be performed directly from whole blood in 60 min, these devices could eliminate the need for the blood culture, enable immediate tailored antibiotic treatment, and eliminate the current blanket approach of application of broadspectrum antibiotics. It has been shown that such an optimized treatment can directly translate into a reduced length of stay, reduced mortality and an improved patient care [5]

In sepsis, the underlying infection of blood stream is mainly caused by one microorganism [7–10]. However, microorganisms associated with sepsis are often resistant to antimicrobials and their populations can be persistent [11–14]. Moreover, they can commonly form biofilms. The recent study by Dengler Haunreiter et al. [15] pointed out that the biofilm formed by the *Staphylococcus epidermidis* was not cleared by the antibiotics used in a patient. Consequently, the resistance to antibiotic evolved over time and in turn *S. epidermidis* bacteremia was observed, despite a full antibiotic treatment. Liu et al. [16] showed that microbes slowed by one drug can rapidly develop a resistance to another.

Finally, here comes the role of the diagnostic microbiology which stands at the epicenter of the tests for sepsis in patients [2]. In most instances the bloodstream infections are intermittent and the circulating microbial loads are low, typically between one and hundreds of colony-forming units (CFU) per mL [2]. Thus, the gold standard is conventional culture-based system where a sample is taken (Figure 1) from a patient in order to identify the causative agent, it is further cultured with liquid cultivation medium. This is followed by AST, e.g. disc diffusion, gradient diffusion, and broth microdilution assays. Here a method which can offer significant advantages of speed and sensitivity over conventional culture method is needed.

There have been some advances made in the microbiological diagnosis of sepsis which focused on the fast identification and AST determination. We would like to mention mainly the advances in nonculture-based diagnostics such as matrix-assisted laser desorption/

Figure 1: In order to determine bacterial pathogens in a small amount of blood is taken from a vein with a needle. Consequently, in an ideal case, pathogens are identified quickly and appropriate antibiotics are prescribed. Here is shown surface enhanced Raman scattering (SERS) as a potential method of choice which excels in speed and sensitivity. Figure courtesy of Gabriela Samkova.

ionization mass spectrometry (MALDI-TOF MS) or nucleic acid-based systems such as polymerase chain reaction (PCR)-based, transcription-mediated, loop-mediated isothermal, or helicase-dependent amplifications of the molecular targets [2, 17]. Thus, bridging this time gap with the new technology is in the best interests of all patients with treatment-unresponsive sepsis, whether it is due to multidrug-resistant bacteria or not. This is an area all stakeholders, including funding agencies need to concentrate their efforts on [18].

We believe that Raman-based techniques could also contribute and play an important role in the fast microbiological diagnostic (Figure 1). Raman spectroscopy/SERS is slowly becoming a commercial venture because of the identification of its competitive position amongst other analytical techniques used for pathogen identification. This was highlighted in the two recent publications based on the data from Raman4Clinic COST Action (lead by Prof. Jurgen Popp) [19, 20]. Recently, Raman-based techniques have been recognized and acknowledged in the dedicated microbiological/sensors journals as a possible method of choice for fast diagnostic [17, 21, 22], to name a few.

Here we would like to show successful, “real-world”, example of Raman spectroscopy – problem linked to the fast whole blood analysis. For this, Yuan et al. [23] reported a sensitive SERS biosensor to detect three bacterial pathogens in whole blood before the blood transfusion. In this case, SERS instrument is based on a magnetic separation and SERS tags. This method also succeeded in the detection of bacteria from the real blood samples of clinical patients who were infected with bacteria.

While the success of Raman spectroscopy and SERS in the analysis of biological samples is now unquestioned, a search of the published SERS literature reveals that the sparse amount of publications addresses a direct determination of AST so that the identity of the bacteria can be rapidly matched with antibiotic sensitivity and the correct dose of antibiotics. Several broad SERS/Raman spectroscopy review papers which illustrate theory for optimal SERS and different experimental considerations can be followed in recent studies [24–29], to name a few. For reviews more focused on advances in microbiology readers are directed to excellent reviews [30–37].

Presented review is selectively focused on the capability of SERS to the instance where highly sensitive, specific, repeatable, rapid, inexpensive, and compact POC platforms for bacterial species determination and for AST testing are required. Thus, results described in this review may lay the foundations for building a Raman-based diagnostic instrument capable of real-time, *in vivo*, *in situ*, characterization of pathogens.

For this specialized instrumentation must be design and developed. Thus, one possible solution could be to use a microfluidic device based on surface acoustic waves (SAW) which can be combined with SERS spectroscopy (this is described in more details in Section 3). This microfluidic platform would enable bacterial separation and identification from selected body fluids or blood samples. Consequently, in this review we present SERS as suitable and reliable technique for possible use in clinical settings. We show that very low detection limit (as low as 1 CFU/mL) can be achieved.

Findings which have been published from early 2016 will be summarized. It is due to the fact that in this year Liu et al. [38] pioneered a new term for SERS-based application in microbiology targeted for AST determination of bacteria cultured from blood samples of sepsis patients – “SERS-AST”. Therefore, for this review, advances in the field of clinical applications have been tracked in the scientific databases from 2016.

2 Methods and targeted applications

2.1 Raman spectroscopy and SERS

In the following section a short background on SERS and Raman spectroscopy will be provided. We found many SERS review papers which explain the theory and principles of the techniques in detail so that the readers can be

directed to excellent reviews mentioned above. For this reason, we decided to use slightly different way of presentation and in turn we will focus on some of the experts' comments which we think will add a nice flavor into understanding why research teams worldwide use the SERS technique as a potential candidate with impact in clinical routines and show a little bit of the main driving force behind the SERS experiments dealing with pathogens.

Raman spectroscopy – a current area of research into improving the speed and accuracy of the identification process of different strains of bacteria in a clinical environment is using the phenomenon of the inelastic scattering of light, Raman spectroscopy. In modern Raman spectroscopy the benefits of this method with regards to the typing of bacteria are at the forefront of research into combating problems such as the formation of bacterial biofilms on implant surfaces. It has been shown in several studies [39–41] that Raman microspectroscopy is capable of rapid identification and discrimination of biological samples including medically relevant microorganisms (bacteria, yeast). This experimental technique employs a laser beam that is focused with a microscope objective in order to excite and collect Raman scattering spectra from a small volume of the sample. In principle, Raman spectroscopy requires measurement times on the order of minutes, and sample preparation can be short and extremely economical.

Specially engineered systems (e.g. microfluidic platforms) can be designed and assembled for each analytical problem in order to obtain reliable Raman signal. Consequently, combining data from Raman spectroscopy with pattern recognition algorithms/chemometric allow fast decisions to be made concerning the selection and dosing regiment of antibiotic which can then be immediately used to cure ill patients.

Surface-enhanced Raman scattering (SERS) has become a mature analytical technique that significantly increases the Raman scattering cross section. The ability of SERS to detect extremely low concentrations of analytes leads to a wide range of applications in microbiology. The signal enhancement in SERS is connected with the excitation of localized surface plasmon resonances in metal nanoparticles (NPs). SERS is capable of single molecule sensitivity [42] with signal enhancement factors up to 10^{15} . The SERS effect, first observed on molecules adsorbed on roughened silver surfaces by Fleischmann [43] (with currently over 6500 citations on Web of Science) is currently the subject of intense research. Recently, for various SERS applications, different sophisticated substrates are exploited such as metallic nanostructured surfaces, nanoantennas of different shapes, colloidal NPs, and clusters of metal spheres [44]. Recent progress in SERS

is focused on bacteria from the priority list ranking of top 15 antibiotic-resistant bacteria published by Tacconelli et al. in Lancet [45] which in turn show SERS as a POC solution with possible impact in standard clinical routines.

2.2 SERS – the method of choice for clinical use

In order to better explain this issue we would like to briefly present some expert views presented by Faraday Discussions. This journal provides an important record of the current international knowledge and opinions in the field of Raman spectroscopy [46, 47].

Also, we would like to mention an important issue which is the SERS enhancement mechanism – the effects underlying SERS – which are still not fully understood. However, as we will see in the following section this should not discourage scientists from not performing new exciting experiments. Moreover, nearly all scientific papers mentioned in this review target only experimental parts of the research without explaining detailed theoretical background of the SERS mechanisms.

Thus, we will highlight some interesting remarks from the expert views, namely from the Faraday Discussions [47]. We will follow the question asked by Dr Aggarwal: *„Is the enhancement factor (EF) a product of the enhancement factors due to the chemical and electromagnetic mechanisms, assuming that both mechanisms are contributing?“* In order to answer this question Professor Graham commented on this question in general discussion: *“...knowing all the mechanisms of enhancement are not necessary to use SERS in anger. Central dogma of SERS proposed as: Metal plus adsorption of molecule—shine light and measure!”* As already mentioned, nearly all papers presented in this review broadly followed recommendation made by prof. Graham and prove that SERS is the method which is sensitive enough to be used for clinical use which in turn shows that SERS is a suitable and reliable tool to be incorporated to POC instrumentation targeting microorganisms.

Additionally, we would like to note that Jarvis et al. [48] as early as in 2008 mentioned that – *“SERS offers a very valuable and powerful additional approach in the ‘whole-organism fingerprinting’ toolbox for the identification of bacteria to the strain level.”* At that time there were nearly 100 bacterial SERS articles, based on using the search term ‘SERS’ and ‘bacter*’ or ‘microb*’ in Web of Knowledge [48]. We have found out that the recent number of SERS articles according to the same search used by Jarvis [48] is 926 articles [Web of Science search on January 6, 2021] with more than 100 contributing research institutions worldwide.

Therefore, in this review we will mention only the research groups which, in our opinion, are closely related to the research on bacteria (determination of AST) and brought novel technology portfolios for clinical diagnostics. The WoS database tells us that the most contributing teams for this issues are led by Wang YL (Academia Sinica Taiwan), Popp J. (Leibnitz Institute), Haisch C. and Niessner R. (Technical University Munich), Goodacre R. (University of Manchester), and Premasiriy WR (Boston University), just to mention a few.

2.3 Reliability of SERS (standardization) for bioanalysis from experimental point of view

Standardization of SERS was previously followed in the report by Witkowska et al. 2019 [49]. The researchers suggested that SERS-based analysis of bacteria suffers from the lack of a standard SERS detection protocols which could be employed throughout laboratories to produce repeatable and valuable spectral information. More specifically, the authors proposed several factors influencing the spectrum and signal enhancement during SERS studies conducted on the bacterial species e.g. different culturing conditions, types of culture media, and method of biological sample preparation.

In the same year standardization issue was revisited in the study of Bell et al. [50]. The authors pointed out to a large number of variables which occur in a typical SERS measurement which in turn make it very difficult to compare the data from different studies [50]. Consequently, experimental results obtained in different laboratories world-wide by researchers using SERS can differ significantly. The main driving force of the international team of scientists with long-standing expertise in SERS was to address key parameters and pitfalls that are often encountered in the literature. To that end, this study provided a series of recommendations on the three crucial issues: (i) on the characterization of solid and colloidal SERS substrates by correlative electron and optical microscopy and spectroscopy, (ii) on the determination of the SERS enhancement factor (EF), including suitable Raman reporter/probe molecules, and finally (iii) on good analytical practice.

Recently, in 2020, Fornasaro et al. [19] reported on the first interlaboratory SERS study in a notable manuscript with more than 40 coauthors who participated in already mentioned recent COST Action – Raman4Clinics in an effort to overcome the problematic perception of quantitative SERS methods. This study involved as many as 15

laboratories and 44 researchers worldwide to tackle the task if a quantitative SERS method can be consistently implemented by different laboratories. Thus, methodology to assess the reproducibility and trueness of a quantitative SERS method was defined as well as different methods were compared. To the best of authors knowledge this study is a first large effort toward a “standardization” process of SERS protocols proposed by leading Raman laboratories. We would like to note that at the same year Guo et al. [20] published a similar report – based on finding in the same COST action – on a large-scale and cross-laboratory round robin tests devoted to Raman spectroscopy experiment which were designed by researchers from approximately 50 institutes in Europe.

In order for the reader to get the feeling about the real SERS experiments it should be noted that for a given

bacterial strain, SERS spectra are very sensitive to the small changes in experimental conditions (e.g. different excitation laser) and the substrates used. As an illustrative example Yan et al. [51] observed a significant effect shown in Figure 2. The results of SERS spectra which are significantly different because of the difference in centrifugation speed during samples preparation are obvious in this case [51] (Figure 2).

2.4 Different SERS approaches – label-free and label-based

In 2015, Bonifacio et al. [52] (experienced Raman/SERS group at the University of Trieste) illustrated that SERS can

Figure 2: Surface enhanced Raman scattering (SERS) spectra of single *Escherichia coli* cells after centrifugation treatment. (A) Comparison of SERS spectra of single *E. coli* cells after centrifugation with different centrifugal forces; (B) Transmission electron microscopy (TEM) micrograph of *E. coli* cell with AgNPs after centrifugation at 3000 rpm; (C) TEM micrograph of *E. coli* cell with AgNPs without centrifugation [51].

Figure 3: Diagram schematically showing the differences between a label-free surface enhanced Raman scattering (SERS) approach and a SERS immunoassay using labels (label-based). In label-free SERS, the spectroscopic signal results from all those analytes which adsorb on the SERS substrate (direct detection), whereas in the SERS immunoassay the spectroscopic signal results from the labels on a SERS tag (label-based, indirect detection) that specifically binds to a target analyte. Reprinted with permission from Bonifacio et al. [52]: Springer Nature, Copyright (2015).

Figure 4: Time-lapse surface enhanced Raman scattering (SERS) spectra for methicillin-susceptible and -resistant *S. aureus* (MSSA and MRSA) with and without oxacillin treatment.

(A) *S. aureus* (MSSA, ATCC 29213); (B) clinical isolate of MRSA with (red curves) and without (black curves) oxacillin treatment; Black and red curves represent the mean SERS spectra, while gray and light red curves represent their corresponding standard deviation. A HeNe laser emitting at 632.8 nm served as the excitation source [38].

be performed in case of qualitative or quantitative detection of analytes either directly or indirectly, employing two completely distinct approaches presented in Figure 3.

When SERS detection is direct (i.e. **label-free approach**), the observed bands come directly from the sample, whereas in the indirect approach, the spectroscopic signal arises from one or more SERS labels (**label-based**) i.e. chemical species having an intense, stable, and well-recognizable SERS spectrum used to signal the presence of the analyte of interest. The systems constituted by such labels adsorbed on one or more metal NPs are often referred to as SERS labels, markers, or tags.

Label-free SERS is basically based on simple mixing pathogens with NPs followed by “shining light and measuring” either directly in the solution or on the SERS substrate where drop of the solution is placed and dried. As was mentioned this approach could impact the SERS signal when the contact of cells and selected NPs is not in the SERS particles proximity. As an illustration let us present the work by Chisanga et al. [53] who developed an *in situ* synthesis of hydroxylamine-reduced AgNPs directly onto the cell wall of bacteria, resulting in higher bacteria detection sensitivity than the conventional method using a simple mixture of particles with bacteria. Recently, Hickey

et al. [54] found that label-free approaches to SERS bacteria analyses are better suited for biochemical characterization and label-based approaches are beneficial when accounting for individual cells among population.

For this review, more importantly, label free approach can be further divided into two different SERS approaches to monitor changes in bacteria metabolism. This can be introduced in the cultivation stage when bacteria are cultivated/exposed to different stress conditions e.g. antibiotics: (i) **Direct cell wall** study, and (ii) **detection of secreted metabolites**. This review is mainly focused on detection of secreted metabolites because this method was used for the pioneering work related to AST determination. Details can be found in the following paragraphs

2.5 Antibiotic susceptibility testing with label-free SERS based on the detection of purine metabolites as biomarkers

Detection of secreted metabolites – small molecules, slightly soluble in water, can be found in the bacterial supernatant of the purine degradation pathway such as

adenosine monophosphate (AMP), adenine, guanine, hypoxanthine, xanthine, and uric acid. These metabolites are secreted in response to the starvation conditions or physiological stress induced during the sample preparation for SERS study [55–58] or after the stress introduced by antibiotic treatment. We will focus on this and show that this approach has been broadly followed by different groups in the following section.

2.5.1 Overview of pioneering work for AST determination using label-free SERS

The desire to create bacteria identification SERS based instrument capable of rapid pathogen detection and identification was pioneered and first report showing bacterial discrimination using SERS was presented by Goodacre and Jarvis in 2004, mentioned in excellent review by the same group [48]. The technique has been further developed by the dedicated SERS groups mentioned in the previous paragraph “SERS – the method of choice” involved in bacterial research.

However, out of the research groups involved in SERS bacterial research it was the Wang group that, for the first time, introduced the term SERS-AST in September 2016 by Scientific Reports, Liu et al. [38]. Thus, it is highlighting that the SERS technique is capable of AST determination.

In the study, the group from the National Taiwan University and the Academia Sinica exploited previous knowledge and followed the previously published SERS papers targeting bacteria [59, 60]. Wang’s group [38] described in detail how susceptible strain of bacteria reacts when exposed to an antibiotic. The group found out that the intensity of specific SERS biomarkers is significantly decreased in 2 h after the reaction with antibiotic, which is illustrated in Figure 4. These findings have been further exploited for rapid AST test. As was previously mentioned, the group termed this procedure as SERS-AST [38] to emphasize the full capacity of SERS.

In order to emphasize the speed of the introduced SERS-AST technique authors presented a schematic comparison between the SERS-AST method and the standard broth dilution (BD). Briefly, the two methods start with the two standard steps – (i) overnight bacterial culture and, (ii) subsequent adjustment of bacteria concentration. However, the following step in the SERS method – inoculation with different antibiotic – took only 2 h, which is much faster than the BD method with an overnight inoculation. Authors discovered that, when a susceptible strain of bacteria is exposed to an antibiotic, the intensity of specific biomarkers in its SERS spectra drops evidently in a few hours. Here, as the SERS “marker” spectrum at 730 cm^{-1}

was selected—the SERS biomarker signal of gram-positive bacteria. Note that 730 cm^{-1} line has been recently used by other group and this will be discussed further in this review.

SERS signal for samples treated with the antibiotic was compared with that of reference samples which were without the added antibiotic. This large study included experiments with four different bacteria *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* which were determined by SERS-AST method and compared with the standard agar dilution (AD) method.

Two years later, in 2018, the Wang group published a follow up research [61]. In this subsequent study, the attention of the group shifted towards the analysis of the biomolecules responsible for bacterial SERS biomarkers found earlier Gram-positive *S. aureus* (SERS marker at 733 cm^{-1}) and Gram-negative *E. coli* (SERS marker at 660, 725 or 742 cm^{-1}) which have been identified as purine derivative metabolites involved in bacterial purine salvage pathways. Using ultra performance liquid chromatography/electrospray ionization-mass spectrometry (UPLC/ESI-MS), the time dependences of the concentrations of these molecules were measured during cells starvation. Note that bacteria were incubated in water – which is essentially the situation facing the bacteria when they are going through the washing and SERS measurement procedures used in previous SERS-AST study [38]. Surprisingly, a single *S. aureus* and *E. coli* cells were found to release millions of adenine and hypoxanthine into the water environment in 1 h. Based on the SERS spectra and quantitative UPLC/ESI-MS measurements of the bacterial supernatants, Wang research group confirmed that the molecules responsible for the biomarkers in the SERS spectra of *S. aureus* are adenine, guanine, and AMP; while that for *E. coli* are adenine, hypoxanthine, guanine, and xanthine.

To be noted is that, there are two possible mechanisms for the accumulation of nucleobases in the bacterial supernatants during water incubation period. The nucleobases could be released from live cells in response to stress, or they could be leaked out from physically ruptured dead cells. To differentiate between these two mechanisms, the viability of bacterial cells after different water incubation time was performed in this study. Moreover, these results confirmed findings presented in the following paper published by Premasiri et al. [55] in 2016.

Now we return to 2016 once again – specifically to Liu et al. in 2016 [38], Premasiri et al. [55] investigated the contribution of the dominant molecular species contributing to the SERS spectra of bacteria excited with a laser at

785 nm. They identified metabolites of purine degradation: adenine, hypoxanthine, xanthine, guanine, uric acid, and AMP. These results provide the biochemical basis for further development of SERS as a rapid bacterial diagnostic and illustrate how SERS can be applied more generally for metabolic profiling as a probe of cellular activity. Which is why we demonstrate this very interesting finding in more details.

The observed SERS spectra of bacteria result from above mentioned metabolites that have been secreted during the sample preparation and manipulation – bacterial strains were cultivated overnight before subculture and harvested during the log phase by centrifugation of 2 mL of culture. Consequently, washed four times with 2 mL of deionized Millipore or distilled water. The concentration of these molecules is largest in regions closest to the cells from which they are secreted. However, the metabolites are found in the supernatant solution as well.

Researchers found that the supernatant spectra were nearly identical to the corresponding bacterial cell SERS spectrum. Consequently, the main finding was that bacterial SERS signatures are not due to structural bacterial cell wall features. Thus, they must arise from small molecules sufficiently water soluble at biological concentrations, which have been secreted from the bacterial cells and collect in the exogenous regions of these organisms.

Very interesting results are the best fits of the 10 bacterial SERS spectra shown in Figure 5 resulting from a linear combination of the six purine component illustrated in Figure 6. The best fits are displayed in Figure 7. Here, nearly all vibrational features and their relative intensities, seen in each of these bacterial SERS spectra, are captured by this fitting procedure.

It is apparent that (i) adenine makes the overwhelmingly dominant contribution to the *Streptococcus pneumoniae* TIGR4 and *S. aureus* NCTC 8325 SERS spectra, (ii) hypoxanthine makes the largest molecular contribution to the SERS spectra of *Bacillus anthracis* Sterne, *Enterococcus faecium* DO, and *Enterococcus faecalis* ATCC 29212, and (iii) high uric acid contributions are observed only in the *Pseudomonas putida* S16 and *A. baumannii* ATCC 17978 SERS spectra.

In the consequent study of Premasiri et al. [62] SERS spectra of 12 bacterial strains (urinary tract infection (UTI) clinical isolates) grown in urine were targeted [62]. Again, spectra for selected bacteria are shown to be primarily due to seven purine components: adenine, hypoxanthine, xanthine, guanine, AMP, uric acid, and guanosine (Figure 8). Researchers followed the steps shown in Figure 9. As can be seen from the schematic diagram in the last step 1 μ L of this resulting bacterial suspension was

Figure 5: Surface-enhanced Raman spectroscopy (SERS) spectra of 10 bacterial species excited at 785 nm on Au NP substrates. The spectra are averages of four to six individual spectra and normalized to the intensity of the strongest feature in each spectrum. Gram-positive (plus sign) and gram-negative (minus sign) types are indicated. Reprinted with permission from Premasiri et al. [55]: Springer Nature, Copyright (2016).

pipetted directly onto the SERS substrate and allowed to dry for ~5 min before SERS spectral acquisition.

Consequently, partial least squares-discriminant analysis (PLS-DA) classification treatment was employed which enabled strain level identification with >95% sensitivity and >99% specificity. It should be noted that here no growth or cell culturing is required, which in turn is time demanding step at traditional bacterial identification methods. Consequently, UTI diagnosis can be accomplished in less than an hour via SERS with antibiotic specificity. Thus, presented SERS-based technology could be used at POC as a fast (under 1 h), growth-free, relatively low cost diagnostic tool.

Independently, the Munich group by Kubryk et al. [56] in 2016 also confirmed the origin of the SERS band around 730 cm^{-1} used in paper by Liu et al. [38]. The main driving force behind these experiments was to clarify the origin of the band which appears in SERS spectra of different bacteria. The mentioned band was usually assigned to

Figure 6: Surface-enhanced Raman spectroscopy (SERS) spectra of 20 μM aqueous solutions of the indicated purine components of bacterial SERS spectra. The spectra have been offset for viewing and are normalized to the maximum peak intensity of the 20 μM adenine solution. AMP adenosine monophosphate. Reprinted with permission from Premasiri et al. [55]: Springer Nature, Copyright (2016).

glycosidic ring vibrations, adenine or to CH_2 deformation. In this illustrative study Kubryk employed a stable isotope approach with cells grown on unlabeled (^{12}C , ^{14}N) and labeled (^{13}C , ^{15}N) carbon and nitrogen sources in different combinations. The position of selected bands of polysaccharides, phospholipids and adenine-related substances depend on their different molecular structure. Thus, determination of the spectra origin of stable isotope labeled bacteria was possible. For this four samples of *E. coli* were cultivated on unlabeled (^{12}C , ^{14}N) and labeled (^{13}C , ^{15}N) carbon and nitrogen sources in different combinations and the spectral changes could be recorded. Authors concluded that the band at 730 cm^{-1} can be assigned to adenine (in-plane ring breathing mode). It was noted that another compounds may also contribute to this band which contains adenine such as FAD, NAD, etc.) as well as different products of the purine degradation pathway (e.g. hypoxanthine).

The report by Weiss et al. 2019 [57], which is a joint paper by Niessner (Munich) and Wagner (Vienna) groups

involved in SERS investigations, further exploited the origin of microbial SERS signals and parameters which affect reproducibility of SERS spectra. SERS signals from six phylogenetically diverse microorganisms representing different cell compositions were analyzed to investigate the variability and reproducibility of SERS technique in different physiological conditions of the cells.

Authors also focused on the strong signal at 730 cm^{-1} in SERS spectra of *E. coli* cells and concluded that the SERS intensity of the marker represents the starvation-induced release of substances related to the metabolic activity [38, 56, 61, 62]. Thus, metabolically inactive and dormant cells would not release purine derivatives which explain that slow growing microorganisms might not exhibit the characteristic SERS signal at 730 cm^{-1} . It was concluded that the effect of physiological state of cells must be taken into the account; otherwise an incomplete understanding may give rise to pitfalls in the characterization and evaluation, and to misinterpretations.

This identification of metabolically inactive and dormant cells via SERS could work in the favor of recent findings by Dengler Haunreiter et al. [15]. Here, resistance to antibiotic evolved over time and in turn *S. epidermidis* bacteremia was observed despite the full antibiotic treatment in a patient who developed a breakthrough bacteremia. Antimicrobial tolerance could be achieved by a small subpopulation of bacteria dormant cells which can survive the antibiotic treatment. Here, as mentioned above, SERS should be able to detect this subpopulation of dormant cells and also different subpopulations of cells such as resistant, tolerant, susceptible, and persistent.

The exhaustive study [63] illustrates the quantitative differentiation of bacteria labeled with isotopes. When bacteria is incorporated with varying isotopic ratios the SERS spectral bands displayed clear redshifts that were used as the basis for quantitative characterization of bacteria at the molecular level.

The findings of this study also highlight the importance of the choice of protocols (e.g. microbial sample preparation and NP synthesis) and instruments (laser wavelength) used for the bacterial SERS analysis, which may consequently reveal or suppress specific spectral features. For example, Kubryk and colleagues [56] used hydroxylammonium chloride solution as the reduction agent and employed a 633 nm laser to acquire SERS spectra of isotopically labeled bacterial cells. These authors reported major shifts occurring at 733 and 1330 cm^{-1} , whilst in this study using sodium borohydride as the reduction agent and a 532 nm laser, the peak at 733 cm^{-1} was, surprisingly, not detected.

Figure 7: Empirically determined best fits (red) of the bacterial spectra (black) shown in Figure 5 to a linear combination of purine (adenine, hypoxanthine, xanthine, guanine, uric acid, and AMP) surface enhanced Raman scattering (SERS) spectra shown in Figure 6. Excellent fits are obtained for all bacterial SERS spectra. Reprinted with permission from Premasiri et al. [55]: Springer Nature, Copyright (2016).

That broadly confirms the finding of Jarvis et al. [48] pointing out that one of the fascinating aspects of bacterial SERS is the radical difference in chemical information observed when experimental parameters are altered. Here, the spectra obtained using laser at 785 nm, with a citrate reduced silver colloid and NaCl aggregating agent were compared to the spectrum of *E. coli* this time acquired with green excitation laser at 532 nm and a borohydride reduced silver colloid [48]. The spectra were entirely different, despite the fact that the cells have very similar surface chemistries. Thus, slight change in the experimental parameters could well be the reason why the peak at 733 cm^{-1} was not detected in the finding by Chisanga et al. [63]

2.5.2 Recent research focused on lab-on-a-chip applications suitable for POC applications

In the following section we will return to the Taiwan group which subsequently published a follow up of the previous findings by Chang et al. in 2019 [64]. Thus, the SERS-AST method was again revisited by dedicated Wang group. To address the problems related to the antibiotic susceptibility, a microfluidic system integrating membrane filtration and dedicated SERS-active substrate (MF-SERS) was developed. The main aim of this study was to perform on-chip bacterial enrichment, metabolite collection, and *in situ* SERS measurements for AST. In this study *E. coli* was

Figure 8: The relative contribution of each of the seven purines found to contribute to the bacterial surface enhanced Raman scattering (SERS) spectra is summarized in this bar graph. Strain-specific differences are evident, it can be seen how the pattern of contributing purines is more different between the four species than between the strains of a given species. Reprinted with permission from Premasiri et al. [62]: Springer Nature, Copyright (2017).

Figure 9: A summary of the sample preparation procedure for the acquisition of a surface enhanced Raman scattering (SERS) spectrum from a human urine sample spiked with 10^5 cfu/mL bacteria. A four-stage gravitation filtration system (glass wool and sequential 30-, 10-, and 5-µm pore size nylon filters) removed nonbacterial solid materials followed by centrifugation to achieve enrichment. The entire procedure took ~50 min. Reprinted by permission from Premasiri et al. [62]: Springer Nature, Copyright (2017).

used as the prototype bacterium with detection limit of 10^3 CFU/mL. The bacteria and secreted metabolites are enclosed during bacterial trapping, metabolite filtration, and SERS detection, thus minimizing possible contamination and human errors. Here, bacteria were trapped at the filter which in turn enabled only secreted metabolites to flow through the membrane filter to the SERS detection zone. The operation of the MF-SERS system is illustrated in Figure 10 and can be divided into four steps: (1) Filtration

on membrane filter, (2) washing, (3) incubation, and (4) detection. First, the bacterial solution was injected into the filtration zone at 2 mL/min and bacteria were trapped on the membrane filter. An amount of 1.5 mL of deionized water was then injected to the chamber to force the culture medium to pass through the filter. The trapped bacteria then released metabolites in the chamber filled with deionized water for 20 min (incubation at room temperature). Consequently, only the metabolite solution in the

Figure 10: MF-SERS system: (A) Schematic diagram of the microfluidic system and device; (B) scanning electron microscopy (SEM) image of SERS substrate (scale bar, 200 nm); and (C) photo of the microfluidic device. Reprinted with permission from [64], Copyright (2019) American Chemical Society.

chamber was subsequently compelled through the filter and guided into the SERS detection zone for SERS measurements. The total operational time was 30 min long.

Presented MF-SERS system improved three issues in the previous SERS-AST method: (1) Entailing prolonged culture time to obtain a large number of bacteria, (2) human errors and contamination owing to manual operations, and (3) large SERS signal variation due to nonuniform and unstable molecular adhesion on the SERS substrate.

Finally, the authors claim that employing miniature size and well-confined microenvironment allows the integration of multiple bacteria processes for bacterial enrichment, culture and determination of AST. Thus, MF-SERS systems can potentially be assembled in a parallel for determination of AST and minimum inhibitory concentration (MIC) of different antibiotics in clinical samples (e.g. whole blood or urine). The SERS substrates in this study were made of arrays of silver NPs embedded in porous anodic aluminum oxide (AAO) nanochannels.

In the middle of 2020, again, the Taiwan group came up with two excellent manuscripts published in the prestigious journals [58, 65]. The researchers followed previously explored topic of rapid antibiotic susceptibility testing of bacteria based on findings that SERS spectra of bacteria originate primarily from the metabolites of purine degradation rather than the cell wall structures: Adenine, hypoxanthine, xanthine, guanine, uric acid, and AMP [61].

This time they dedicated their research to a protocol based on SERS in order to obtain consistent AST results

from clinical blood-culture samples within 4 h [65]. This novel methodology could be used for clinical diagnostics to the benefit of patients as well as to the economy for timely administration of appropriate antibiotic therapy. The study was conducted in the National Taiwan University Hospital (NTUH). Here, again, authors stressed a linear increase in the risk of mortality for patients with e.g. bacteremia (bacteremia could be in most cases associated with sepsis) for each hour delay in antibiotic administration. Because of the lack of timely microbiological evidence, antibiotics are usually forced to start empirically, rather than precisely upon a specific target.

The main aim of this study was to apply SERS for rapid AST from clinical blood-culture samples, which in turn, is a major step for SERS application in clinical microbiology. This is an extremely challenging task because of the complex nature of clinical blood samples and pretreatment procedure of samples to separate the blood components from the bacteria has to be applied. Thus, the red blood cells containing hemoglobin were lysed using ACK (Ammonium–chloride–potassium) and consequently separated from „blood-culture isolates“ along with other constituents in blood samples affecting SERS spectra – providing that targeted bacteria are left viable – using dedicated procedure. In the experiments the group followed the protocol described in the previous SERS study on reference strains and pure clinical isolates where the spectral peaks located at 730 and 724 cm^{-1} , respectively, were identified and adopted as the SERS biomarkers of *S. aureus* and *E. coli*, based on purine metabolites. The

release of purines strongly depends on applied antibiotic – e.g. the purine biosynthesis pathway of susceptible *E. coli* strain is influenced by antibiotics while that of resistant strain is unaffected. Thus, the SERS biomarkers were used for bacteria AST determination. Bacterial samples were pipetted on the active SERS substrate based on two-dimension Ag NP array embedded in nanochannels of anodic alumina [66] and dried on a hot plate in order to perform SERS measurements. Laser at 632.8 nm was used for excitation.

The second manuscript was published in 2020 by Lab on a Chip journal dedicated to microfluidic applications [58]. Here, again, authors addressed the problem of conventional AST which usually requires a prolonged bacterial culture time and a labor-intensive sample pretreatment process. They report on the development of a microfluidic microwell device which integrates SERS so that a rapid and high-throughput AST can be achieved. Presented results show that the Microwell-SERS system can successfully encapsulate bacteria in a miniaturized microwell with a greatly increased effective bacteria concentration which in turn enables a 2 h AST on susceptible and resistant *E. coli* and *S. aureus*. This instrument integrates a SERS substrate (Microwell-SERS system) for bacteria isolation, followed by the enrichment and *in situ* AST shown in Figure 11. As a result SERS spectra of a bacteria-secreted metabolite after antibiotic treatments can be monitored and measured. Compared to the NP-based SERS probe, the nanostructure-based SERS substrate is believed to have a higher sensing uniformity and reproducibility since it has a more uniform nanostructure arrangement and would not suffer from NP aggregation issue.

Bacteria secreted metabolites were also reported by Andrei et al. [67] on generation of reproducible SERS spectra for complex structures of bacteria and their aggregation. The studies demonstrate that densely packed

bacteria deposited on ultrathin silver films (thermally evaporated Ag films) were characterized with the secretion of adenosine triphosphate (ATP) for *S. aureus* which enabled discrimination between the various strains. Silver thin films were deposited using a home-made thermal evaporator and a 7-nm-thick Ag film was chosen for the study of bacteria.

The main finding was that the signature of *S. aureus* is dominated by the signature of adenosine and/or ATP shown in Figure 12 and the SERS features of *E. coli* are mainly due to the proteins from the pili/flagella or outer membrane and to the lipopolysaccharides, which contain adenine part in the lipid layer.

In the case of *S. aureus*, a different response is observed as a function of the surface concentration of bacteria,

Figure 12: Zoom of surface enhanced Raman scattering (SERS) spectra of *S. aureus*, ATP (in red) and adenosine (in green). Reprinted with permission from Andrei et al. [67]: Springer Nature, Copyright (2017).

Figure 11: The Microwell-SERS system: (A) Schematic diagram of the system, including Raman microscopy and the Microwell-SERS device; (B) photo of the microwell device attached with a SERS substrate with the SERS-active surface (in purple) facing downward; (C) bright-field image of the microwells rehydrated with blue food dye (scale bar, 200 μm); (D) fluorescence image of 106 CFU mL^{-1} GFP-transfection bacteria confined in nine microwells (scale bar, 50 μm). Reprinted with permission from [58], Copyright © Royal Society of Chemistry 2017.

giving rise to two distinct clusters in principal component analysis (PCA). This separation highlights the secretion of ATP at high concentrations (most likely generated outside the bacteria), whereas the poor stability of bacteria at low concentrations allows for detecting the cell wall signature and/or cell lysis. To conclude, these substrates, in spite of their simple, robust, fast and easy technique of production, meet the challenges for fast single-cell analysis of bacteria using SERS.

2.6 Selected label-free SERS research related to AST determination

Previously mentioned group from Boston University, Premasiri et al. [62] published in mid of 2016 their second paper Boardman et al. [68] on detection of bacteria from whole blood. According to the authors the technology described in this publication could as well revolutionize the way bacteremic samples are screened for pathogens. In our opinion this paper has been so far underrated and we believe that this technology can still find its way to the clinical diagnostics of bacteria directly from blood.

Boardman et al. [68] developed a challenging technique for bacteremic patients using SERS to detect and identified specific bacteria directly from blood. The process has many challenges such as low concentration of bacteria (often less than 10 CFU/mL) and a complex of blood matrix containing blood cells and proteins. For this, a universal sample preparation process was designed that accepts 10 mL of whole human blood, preferentially lyses blood components while maintaining the microorganism's

viability (if any microorganisms are present), and produces an enriched output of viable microorganisms for SERS analysis (Figure 13). The lysis process was optimized to be selective for the blood components and nondestructive to the bacteria.

The only low incident laser power (1–5 mW) is required for SERS data acquisition due to the large Raman cross-section enhancement, thus enabling the development of low cost, portable, and eye-safe SERS platforms for POC diagnostics. This instrument provides physicians identification of the pathogens directly from whole, uncultured blood within 7 h. Processing whole blood circumvents the need for positive blood cultures, which can take days before definitive results are determined.

The library of SERS spectra from as many as 115 microbial strains that included clinical isolates from blood cultures ($n = 68$) and reference bacterial strains ($n = 47$) was created in this project. All SERS spectra were obtained using *in situ* grown, aggregated Au NP-covered SiO₂ substrates that were previously described by Premasiri et al. [69]

In the study by Tien et al. [70], authors reported in mid-2016 the cylindrical SERS substrate array was used to identify bacteria in the dialysate of peritoneal dialysis peritonitis. In order to achieve this goal the cylindrical SERS was fabricated by decorating silver NPs on the tip of 2-mm diameter polymethylmethacrylate (PMMA) rod. The main advantage of this chip is that SERS spectra can be acquired without drying the samples. In similar SERS experiments samples have to be dried in order to establish the close contact of bacteria with the SERS substrate.

Figure 13: Technology overview for viable microbe recovery from human blood. 10 mL of whole blood is processed to produce a 200- μ L output which is incubated briefly prior to surface enhanced Raman scattering (SERS) for identification or for integration into a standard clinical workflow. Reprinted with permission from [68], Copyright (2016) American Chemical Society.

For the determination of antibiotic susceptibility of bacteria oxacillin and vancomycin were added to MSSA and MRSA and incubated for 6–24 h. Bacteria after antibiotic treatment were loaded onto the SERS chips and the resulting Raman spectra were compared to that of bacteria without antibiotic treatment. The authors concluded that SERS may be able to shorten the time needed for bacteria identification and antibiotic susceptibility testing. In this study authors commented on the mixed bacterial flora infection that Raman shifts of many bacteria are very similar. Thus, it was found that the mixed flora infection may make bacteria detection difficult.

In the subsequent study by Dina et al. [71] the attention was shifted on rapid label-free SERS-based biosensor for bacteria detection and identification for both gram-positive and -negative bacteria that are found in bacteremia. As a novel approach the group proposed application of microscope adhesion slides for bacteria immobilization and subsequent SERS detection employing the *in situ* synthesis of silver NPs enabling close contact with the bacterial cell wall. As a part of this study was an investigation of the influence of the growth medium used for cultivation on the SERS signature of bacteria in order to have similar results obtained by Premasiri et al. [55] (described in one of previous paragraphs). After detailed monitoring of even slight differences in the bacterial membrane composition researchers did not find any indication for the theory introduced by Premasiri et al. [55] (Premasiri et al. suggested that bacterial SERS spectra are usually due to small purine-like molecules). We believe that it could be that Dina et al. [71] followed slightly different washing procedure with saline solution. In the contrary, Premasiri et al. [55] (and also research papers published by Wang group) used deionized water. Also Premasiri 2016 mentioned that a

further complication for the assignment of the chemical origins of bacterial SERS spectra is the Raman excitation wavelength dependence of these spectra. Thus, the fact that Dina et al. used either the 532 nm or 633 nm excitation lines could well partly explain the difference mentioned above. Moreover, as mentioned previously, the fact that “One of the fascinating aspects of bacterial SERS is the radical difference in chemical information observed when experimental parameters are altered.” Jarvis et al. [48] might also contribute to the explanation.

In the study of Akanny et al. [72] the SERS performance and efficiency of the developed uncoated spherical AuNPs as SERS substrate was compared with that of “standard” AgNPs for the three different bacterial strains: Gram-positive *Bacillus subtilis* and *Lactobacillus rhamnosus* GG and gram-negative *E. coli*. Authors illustrated that AuNPs employed in this study showed better enhancement over AgNPs. Authors explained this by findings based again on the facts that purine molecules highly contribute to the SERS spectra which in turn have different affinity of Au and Ag NPs. Thus, AuNPs might have stronger affinity with purine than AgNPs resulting in higher signal (Figure 14).

Authors again considered that the detected molecules are metabolites of the purine degradation and that their concentration is the largest in the extracellular regions near the outer cell walls which reflect close interaction between bacteria cell wall and NPs.

Mosier-Boss et al. [73] recently explored differences in SERS spectra of bacteria which were obtained using citrate (capped) and borohydride (uncapped, bare) generated silver NPs (AgNPs). The observed differences in SERS spectra are attributed to the manner in which these Ag NPs interact with bacteria. Capped Ag NPs can partition through the cell envelop (through the surface

Figure 14: Comparison of surface enhanced Raman scattering (SERS) spectra from *Escherichia coli* (A), *Lactobacillus rhamnosus* GG (B), and *Bacillus subtilis* (C) suspensions. Raman spectra were obtained with either gold (Au) or silver (Ag) NPs. Note that bacterial suspension concentration used for *Escherichia coli* bacteria is 10 times higher with silver NPs than with Au NPs. Reprinted with permission from Akanny et al. [72]; John Wiley and Sons, Copyright (2020).

polysaccharides of the bacterial cell) to bind to proteins, lipids, and carbohydrates found in the inner and outer cell membranes, as well as the periplasmic space between them. Thus, the resultant spectra show contributions due to these components of the cell envelope and cellular secretions.

Uncapped Ag NPs are unable to partition through the polysaccharide outer structures of the cells cannot partition inside the cell envelope and are randomly distributed outside the cells and in turn will only bind to the metabolic purine secretions. Consequently, SERS spectra recorded for uncapped Ag NPs are based on secretions primarily due to the metabolites of purine degradation and spectra obtained using capped Ag NPs exhibit features originating from the cell envelope in addition to purine metabolites. SERS spectra of *E. coli*, *S. putrefaciens*, and three strains of *Pseudomonas aeruginosa* were obtained using capped and uncapped Ag NPs.

As a conclusion authors indicated that the same purines were secreted by selected pathogens which means that their metabolic activity was similar. Thus, this study extended and validated already mentioned findings of Premasiri group [55, 62].

For analyzing the three types of bacteria *S. aureus*, *E. faecalis*, and *P. aeruginosa* a unique approach for rapid SERS-based label-free detection of bacteria was used by Dina et al. [74]. For this study the SERS-active substrate with Ag spot which was synthesized inside a hermetically sealed microfluidic channel was explored. For experimental data evaluation robust fuzzy partitions based on the fuzzy sets theory was employed. Experimental arrangement enabled single-cell detection on the SERS silver spot.

From 2016 a parallel to the studies mentioned above findings devoted to the detection of bacteria using SERS published by established groups appeared in prestigious journals. It should be noted that these studies had not been directly targeted to AST evaluation. However, these findings could be easily translated to different bacteria identification and consequently lend themselves to AST determination.

As an example, in summer 2016, interesting findings were published by Popp group from Jena dealing with a closed droplet based lab-on-a-chip (LOC) device which the group developed for the differentiation of six species of mycobacteria [75]. In order to keep a high level of safety for these pathogens closed system was introduced (Figure 15). Using the common strategy for the mechanical lysis the cell wall of mycobacteria species was lysed (cells were disrupted) using a bead-beating module. Here, an interesting illustration of spectra differences between the standard

Raman spectra and SERS is illustrated in Figure 16 – as it was mentioned in part 2.2. Here, Raman spectra nicely show information about the whole bacteria, in the contrary SERS spectra shows spectral information of the molecules which were binded to the Ag NPs. Consequently, SERS spectra monitor vibrational signals from mycolic acid which in turn enables to discriminate various mycobacteria strains involved in this study. The excitation source was a continuous-wave with a wavelength of 514 nm.

Later, in 2018, a stimulating manuscript was published by Munich group by Yang et al. [76] who developed a portable bacteria-grasping SERS chip for identification of three species of uropathogens directly from culture matrix. The chip is based on modification of positively charged NH_3 group and enables to trap the negatively charged bacterial cells through the electrostatic adsorption principle (Figure 17). Six bacteria samples can be captured simultaneously, which improves the detection speed for multiple samples.

2.7 Label-based SERS for bacteria detection and analysis

In the following part we present selected reports/findings on label-based SERS which show some potential benefits for the clinical community. Here we start with sandwich SERS which enables to detect multiple pathogens which is often the case for POC testing [77]. Consequently, two reports focusing on separation and identification of bacteria

Figure 15: Scheme of sample preparation, including the sample lysing module (bead-beating system) for the bacterial cell disruption, the internal storage container, the syringe pump system and the droplet-based microfluidic device mounted to the microscope stage. Reprinted with permission from [75], Copyright (2016) American Chemical Society.

Figure 16: (A) Mean of Raman spectra of *Mycobacterium tuberculosis* Beijing (ID 8304/09); (B) Mean of surface enhanced Raman scattering (SERS) spectra of *M. tb* Beijing (ID 8304/09); light gray: Standard deviations. Contributions from all parts of the bacterial cells can be found in the Raman spectra, while the SERS spectra of the disrupted bacterial cells are strongly dominated by contributions from mycolic acid. Reprinted with permission from [75], Copyright (2016) American Chemical Society.

from blood samples are presented which in turn lend themselves for the clinical diagnostic [23, 78]. Further, three groups present that label-based SERS is suitable for the detection of ultralow limits (as low as 1 CFU/mL) – which is very important for the detection of bacteria in patient with sepsis where number of bacteria in blood is very low [79–81]. Moreover, label-based SERS can be combined with different techniques so that the original findings can be obtained [82]. Other interesting findings were observed in [83] that the bacteria CFU increased under sub MIC antibiotic concentration. Finally, impressive

approach of label-based SERS shows enormous potential for competitive and effective capture of target bacteria [84].

2.7.1 Sandwich SERS assay

Out of the successful applications of label-based SERS for bacterial diagnostics, the report by Kears et al. [77] (prof Goodacre group) is an excellent study of three bacterial pathogens (*E. coli*, *Salmonella typhimurium*, and methicillin-resistant *S. aureus*). These species were successfully isolated and detected, with the limit of detection at 10 CFU/mL. Moreover, the group presented SERS spectra obtained from multiple pathogen detection.

Essentially, main approach was a sandwich SERS assay in which they combine SERS active Ag NPs functionalized with bacteria specific recognition molecules (antibodies) with lectin functionalized magnetic NPs enabling the capture and isolation of bacteria through magnetic separation (Figure 18). It is the use of lectin functionalized magnetic NPs which makes this separation step novel and efficient in capturing and isolating bacteria from a sample matrix.

The total analysis time was ~1 h. This is comparable with other bionanosensors which have been developed, however this approach is unprecedented because it can detect multiple pathogens simultaneously in this time.

Raman reporters used for multiple pathogen detection:

- (i) 7-dimethylamino-4-methylcoumarin-3-isothiocyanate (DACITC) – *S. typhimurium*, with a characteristic peak at 535 cm^{-1}
- (ii) 4-(1H-pyrazol-4-yl)-pyridine (PPY) – MRSA, with a characteristic peak at 955 cm^{-1} ,
- (iii) Malachite green isothiocyanate (MGITC) – *E. coli*, with a characteristic peak at 1616 cm^{-1}

2.7.2 Pathogens in blood

In a subsequent study, Li et al. [78] reported on AST of pathogens in blood. This study is based on the magnetic separation of bacteria from the blood samples using polyethyleneimine-modified magnetic microspheres

Figure 17: Preparation schematic of portable chip for bacteria-capture and detection [76].

Figure 18: Schematic illustrating the single-plex and multiplex detection assay.

Assay format: (A) Lectin (Con A) functionalized silver coated magnetic nanoparticle (Ag@MNPs) will bind to bacteria, and the presence of the magnet will allow for magnetic separation of the bacteria from the sample matrix. (B) Surface enhanced Raman scattering (SERS) active silver nanoparticles (AgNPs) functionalized with a biorecognition molecule (antibody; Ab) and a unique SERS reporter are added. The mixture is shaken for 30 min before application of a magnet for a further 30 min and collection of the sample. Any unbound matrix is gently removed and the sample subsequently resuspended in deionized water. (C) The sample is then interrogated with a 532 nm laser beam and a SERS signal obtained (green spectrum). When no target is present, the functionalized AgNPs will be washed away; thus, they will not bind to bacteria so a minimum SERS signal is obtained (red spectrum). (D) Multiplexing step: 3× AgNP conjugates each functionalized with a different Raman reporter and an antibody (which is specific for a bacterial pathogen) are added together with three bacterial pathogens and Con A (which binds to all three bacteria) functionalized Ag@MNPs. In the same way as the single-plex systems, magnetic separation allows for the samples to be concentrated and analyzed via a 532 nm laser. A SERS spectrum is obtained which contains characteristic peaks from the three Raman reporters and thus can be used to confirm the targets are present. Reprinted with permission from [77], Copyright (2017) American Chemical Society.

(Fe₃O₄@PEI) to capture and enrich the pathogens rapidly upon receiving the blood culture bottle. The first step was the creation of the spectral library of pathogens and their drug-resistant strains as reference spectra in order to identify pathogens separated from the blood samples. In the second step, the PEI-modified magnetic microspheres were used to separate the bacteria from clinical samples, and the magnetic microspheres/bacterial complexes were cultured overnight on normal blood and drug-resistant culture plates to form a single colony. In the third step, the target single colony was selected from the culture medium and mixed with the high performance SERS reinforcing material (AgNPs) and then added to the Si wafer. After the mixture was naturally air dried, SERS spectrum was detected, and the bacterial spectrum was compared with the standard spectrum measured in our first step to determine whether it was the corresponding pathogenic bacteria.

Using this methodology the drug resistance of *S. aureus*, *A. baumannii*, *P. aeruginosa* and their resistant

strains (obtained from 77 common pathogenic bacteria in clinical blood infection) can be identified and monitored quickly. The successful implementation of this detection scheme could provide a new solution for the rapid detection of clinically important pathogenic bacteria and drug susceptibility test of pathogens which in turn could replace currently used methods.

The main driving force of another excellent research jointly performed by Munich group (Niessner and Haisch) by Yuan et al. [23] was to develop a SERS sandwich strategy for the sensitive detection and discrimination of *E. coli*, *P. aeruginosa*, and *S. aureus* directly in blood samples.

In this study, a new biosensor based on the sandwich structure employing antimicrobial peptides (AMPs) functionalized magnetic NPs as “capturing” probes for bacteria isolation and gold coated silver decorated graphene oxide (Au@Ag-GO) nanocomposites modified with 4-mercaptophenylboronic acid (4-MPBA) as SERS tags was used. Authors noted that AMPs as a capture element for the SERS detection of bacteria

Figure 19: Schematic illustration of the operating procedures for bacterial detection via a surface enhanced Raman scattering (SERS) sandwich strategy, in which AMP modified magnetic Fe_3O_4 NPs were utilized in the bacteria capture and 4-MPBA modified Au@Ag-GO nanocomposites were used as SERS tags.

(A) AMP modified Fe_3O_4 NPs were cultured with a bacterial sample matrix, which included bacteria, blood cells or other interference; (B) the Fe_3O_4 NPs@bacteria complex was magnetically separated from the sample matrix; (C) blood cells or any other interference were removed; (D) 4-MPBA modified Au@Ag-GO nanocomposite SERS tags were cultured with the Fe_3O_4 NPs@bacteria complex to form a sandwich structure; (E) the Fe_3O_4 NPs/bacteria/SERS tags sandwich structure was magnetically separated and detected by the Raman spectrometer; (F) different kinds of bacteria were discriminated according to their Raman “fingerprints”; and (G) 4-MPBA can be used as an IS to correct the SERS intensities [23].

has not yet been reported before. In order to improve the stability of Au@Ag NPs the combination with graphene oxide (GO) was used which in turn makes the SERS active substrate more durable and beneficial for further chemical modification.

If bacteria is present in the blood (bacteremia or sepsis), the sandwich structure is created and SERS spectra can be consequently analyzed. Thus, in the “real-world” experiments the AMPs modified Fe_3O_4 NPs@bacteria complex were magnetically separated from the blood, and mixed with 4-MPBA modified Au@Ag-GO nanocomposites, summarized in Figure 19.

Using suggested methodology, *E. coli*, *S. aureus*, and *P. aeruginosa* were discriminated with limit of detection (LOD) of 10^1 CFU mL^{-1} , respectively. This novel method was

further used in the detection of bacteria from clinical patients who were infected with bacteria and the results showed that 97.3% of the real blood samples (39 patients) can be classified effectively. Moreover, authors noted that the AMP modified Fe_3O_4 NPs have good antibacterial activities, so that the instrument can be used for three tasks – (i) capture, (ii) discrimination, and (iii) inactivation of bacteria in the storage of blood.

2.7.3 Ultralow detection limits

The label-based approach offers very low detection limit (as low as 1 CFU/mL) however, methodology can be at some instances quite complex as it is presented by You et al. [79]. Here, authors employed gold nanoparticle-

coated starch magnetic beads (AuNP@SMBs) that were prepared by *in situ* synthesis of AuNPs on the surface of starch magnetic beads (SMBs). In the next step the AuNP@SMBs were mixed with 4GS (linker protein) and incubated for 30 min. The specific affinity of the 4x-GBP (gold binding peptide) domain of the linker protein toward the gold surface was utilized for the spontaneous conjugation of 4GS with the AuNPs present on the surface of SMBs. The aggregation of immuno-AuNP@SMBs in the presence of target bacteria, followed by coupling of the captured target bacteria with SERS tags, provided an effective hotspot to amplify the Raman signals. The results suggest that the SERS-based detection system proposed in this study is highly sensitive and can detect target bacteria as low as 1 CFU/mL with excellent specificity.

Li et al. [80] developed a SERS sensor based on aptamers for the sensitive and simultaneous detection of different food pathogens – *E. coli* O157:H7 and *S. typhimurium* with high detection sensitivity (<8 CFU/mL). Presented biosensor benefits from the flexible combination of aptamers and Raman reporters in novel SERS tags with possible detection of other foodborne pathogens.

Jabbar et al. [81] proposed an innovative SERS platform for the detection of *E. coli* bacteria where plasmonics (Ag@PdNPs) have been formed on the gradient porosity silicon (GPSi) by simple immersion plating process. The main aim of this study was to enhance the activity of bimetallic SERS substrate for the detection of a single cell of pathogenic bacteria. The main idea behind this study is the superior structural characteristics of the GPSi in formation of (Ag@PdNPs) bimetallic NPs. The ultralow concentration of about 1 CFU/mL of *E. coli* was detected.

2.7.4 SERS in combination with different techniques

The research presented by Chisanga et al. [82] has been motivated by rapid detection of *Campylobacter jejuni* which is a major cause of foodborne gastroenteritis worldwide. Samples were tested with a battery of techniques, including ultraviolet, Raman spectroscopy, SERS, and MALDI-TOF MS to provide complementary biomolecular information capable of differentiating bacterial classes. Raman spectroscopy provided whole-bacterium fingerprint, SERS detected structural moieties and bond linkages located specifically on the bacterial cell surface.

Moreover, researchers for the first time employed label-free SERS involving simple mixing and *in situ* synthesis of AgNPs (two different *in situ* methods for silver nanoparticle (AgNP) production) to probe molecular dynamics of pathogenic bacteria to complement data obtained from Raman and MALDI-TOF MS instruments.

Chemometric treatment of data was employed to differentiate biochemical differences and phenotypic information from different locations in the cell–cell wall versus cytoplasm based on cell envelope or intracellular molecular dynamics. In this study, again, particularly for the three mutants highlighted more intense SERS bands at 730 cm^{-1} (adenine moiety in flavin adenine dinucleotide [FAD]) located in the cell wall were observed. Authors concluded that Raman, SERS, and MALDI-TOF MS are powerful metabolic fingerprinting techniques capable of discriminating clinically relevant cell wall mutants of a single isogenic bacterial strain.

2.7.5 Inhibitory effect of antibiotic

Fu et al. [83] illustrated an elegant approach to rapid AST and MIC determination. For this, the researchers employed combination of aptamer, silver NPs (*in situ* synthesis), and bacteria to the final product – Bacteria-aptamer@AgNPs. The SERS spectra of *E. coli* O157:H7 with different concentration of tigecycline and *S. aureus* with different concentration of vancomycin have been recorded in 2 h. Here, 2 μL of sample (Bacteria-aptamer@AgNPs) suspension was dropped on a fluted glass slide and consequently analyzed.

It should be pointed out that the researchers found interesting behavior when monitoring the SERS spectra peak at 735 cm^{-1} enhancement. It was observed that the bacteria CFU increased under sub MIC antibiotic concentration. The authors concluded that this is probably due to the fact that antibiotic at sub MIC concentrations stimulate the bacterial reproduction. There is an assumption that antibiotic at sub MIC has a short inhibitory effect on bacterial respiration, bacteria still have higher activity, and the number of bacteria is increasing. This is an important finding because this effect is not always taken into the account in the experiments of other groups using SERS for bacteria study.

2.7.6 Impressive approach inspired by nature

An impressive approach – enlightened by the physiology and predation of octopus – for detection of pathogenic bacteria in case of sepsis was presented by Lv et al. [84]. The captured bacteria (*S. aureus*) can be identified by the SERS method with a detection limit as low as 10 cells/mL^{-1} . In this work, a facile method based on a novel synthetic method to mimic the octopus-like architecture in order to capture bacterial pathogens was introduced – octopus-like multiarm structure (MAS). Consequently, MAS-ligand conjugates (MAS-Ls) were prepared so that octopus-like polymeric arms and ligands distributed along each arm behave as octopus' suckers. Manufactured biomimetic

arms have a diameter of about 30 nm and are easy to curl, and move around freely, so that target pathogens can be easily captured. The ligands modified on each arm provide multivalent binding sites for the bacterial cell. Thus, the octopus-like multivalent scaffold architecture has enormous potential for the competitive and effective capture of target bacteria. In the final step the MAS-Ls-binding bacteria (MAS-Ls-*S. aureus*) is characterized by SERS spectra, as illustrated in Figure 20. Here, the peak at 735 cm^{-1} was employed and for very low concentration of *S. aureus* of 10 CFU/mL, could still be distinguished, demonstrating this system to be highly sensitive without the interference from other background pathogens present in the sample demonstrated in Figure 20.

3 Conclusions and outlook in finding solutions for clinical use

Imagine this wishful scenario in a clinical practice: A sick person enters a hospital emergency ward with signs of sepsis. The doctor collects a specimen from this person – in an ideal case, the doctor could quickly and simply analyze the sample in the examination room, which in turn should provide him with enough information for identification of causative agent. In this scenario, a POC instrumentation

that could directly identify the pathogens and provide antibiotic-susceptibility information from e.g. whole blood in minutes is required. Consequently, rapidly identified pathogens can be tested for antibiotic sensitivity so the clinician could prescribe tailored antibiotics. Here, in this review we show how the SERS technique could possibly contribute to the development of the biosensor which would enable quick and convenient POC testing for the clinical diagnostics of body fluids, bacteria, cells, and tissues. POC tests are laboratory tests designed to be used directly at the site of patient care, which may comprise physicians' offices, outpatient clinics, intensive-care units, emergency rooms, hospital laboratories, and even patients' homes [85].

For the future work on innovative strategies for fast testing of pathogens it is obvious that cultivation of bacteria is time demanding and must be avoided in the process of sample analysis. Thus, it is necessary to separate pathogen cells directly from blood which is taken from patient at POC. However, as was mentioned in the introduction – in most instances bloodstream infections are intermittent and the circulating microbial loads are very low, typically between one and hundreds of CFU per ml. Plus we need to consider intraspecies variability of microorganisms. An ideal device that can preprocess samples (body fluids and namely blood) and consequently quickly separate bacteria for subsequent analysis directly on the compact microfluidic device would provide LOC solution as *in vitro*

Figure 20: Scanning electron microscopy (SEM) images of (A) MAS-Ls-*S. aureus* and (B) the local enlarged images of the interaction between the arms and bacteria cell. Pseudocolored cell in image (a) shows the topographic interaction between the captured cell and MAS-Ls. (C) Schematic illustration of the specific capture of *S. aureus* by MAS-Ls. (D) Enrichment of 102 *S. aureus* cells by 0.5 mg MAS-Ls in varied volumes. (E) surface enhanced Raman scattering (SERS) detection of target bacteria after capture and separation: SERS spectrum of MAS-Ls binding with *S. aureus*, *L. mono*, *E. coli*, *S. flexneri*, and blank samples, respectively, with bacteria concentration of 107 CFU mL⁻¹. Reprinted with permission from [84], Copyright (2019) American Chemical Society.

Figure 21: Illustration of the microfluidic platform based on surface acoustic waves (SAW) – with indicated pressure node (green sinusoid) – sorting flowing bacteria directly from blood to dedicated microfluidic chip with microchambers (left and right channels) for further bacteria recognition and consequent analysis under defined antibiotic. Red blood cells, white blood cells, and platelets are directed to the middle channel. In the chip parallel laminar flows of the cultivation medium and antibiotic solution of known concentration can be maintained along with the use of optical tweezers to manipulate and sort identified cell to the different chambers. Illustration on the right shows microfluidic chip with microchambers which shows how identified bacteria can be placed to the separate microchambers for further detailed analysis either with SERS or with Raman spectroscopy. Figure courtesy of Gabriela Samkova.

platform. One potential solution we would like to highlight could be a microfluidic device based on SAW combined with SERS/Raman spectroscopy in microfluidic chip for bacterial separation and identification from selected body fluids or blood samples (Figure 21).

Such an instrumental combination would enable very fast analysis of bacteria – on the chip bacteria could be separated from blood in less than 1 h and consequently analyzed by SERS. Thus the procedure starting from blood collection from patient to full bacteria analysis including AST should be the limit of 1 h. This is in agreement with “The guidelines of Surviving Sepsis Campaign” as mentioned in the introduction – antibiotic should ideally be started within 1 h of the diagnostic of severe sepsis.

The driving force behind SAW is that the cells and particles (red blood cells, platelets etc.) not targeted for analysis will be fully eliminated from the fluid [86]. This can be realized by using different microfluidic designs based on acoustofluidic separation. Here, size-dependent separation of cells/microparticles is performed using the SAW field (employing pressure nodes) – particles are deviated and sorted from the original flow due to the interaction with an acoustic force. Thus, size-dependent separation can be performed directly on the microfluidic chip before the cells are analyzed using SERS platform. Such an early diagnosis would help the doctors to promptly decide at POC on the appropriate therapy and allow the application of tailored antibiotic treatment for improved patient’s outcome. In this way polymicrobial sepsis could also be early recognized because single cells are targeted for cell analysis.

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