Isolation and identification of bacteria by means of Raman spectroscopy☆

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Abstract

Bacterial detection is a highly topical research area, because various fields of application will benefit from the progress being made. Consequently, new and innovative strategies which enable the investigation of complex samples, like body fluids or food stuff, and improvements regarding the limit of detection are of general interest. Within this review the prospects of Raman spectroscopy as a reliable tool for identifying bacteria in complex samples are discussed. The main emphasis of this work is on important aspects of applying Raman spectroscopy for the detection of bacteria like sample preparation and the identification process. Several approaches for a Raman compatible isolation of bacterial cells have been developed and applied to different matrices. Here, an overview of the limitations and possibilities of these methods is provided. Furthermore, the utilization of Raman spectroscopy for diagnostic purposes, food safety and environmental issues is discussed under a critical view.

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1. Introduction

Bacterial detection is an issue-area which is of great importance for various aspects of modern life. The industrial production of food or pharmaceutical substances requires test procedures which will prevent spoiled or contaminated products from being placed on the market. The supply system for drinking water can be subject to bacterial contamination, too, and constant controls are necessary in order to ensure that the consumer won’t be exposed to any health risk. More examples of high significance can be found in the medical field. Of course, a timely identification of the source of an infection can be lifesaving for patients. But also strategies for improving hospital hygiene in general have received considerable attention lately. In addition, bacterial detection methods are of peculiar interest for the environment and agriculture, because the spread of plant pathogens for instance can not only endanger whole ecosystems, but can cause serious economic damage as well.

These examples highlight the meaningfulness of strategies which allow identifying bacteria in different environments. According to the relevance of this research area, several approaches have been developed. The current gold standard is to culture the bacteria and identify them based on their morphological and metabolically characteristics. Even though this technique is well established and reliable, it comes along with two disadvantages which are quite severe. Firstly, depending on the bacterial species culturing can take several days. Secondly, it is assumed that the vast majority of bacteria are not culturable at all and therefore not accessible with this approach. Within this context it should be noted, that in some cases only cells which are in a viable but not culturable (VBNC) state might be available. Nucleic acid based detection methods for bacteria, which usually rely on the amplification of DNA or RNA via polymerase chain reaction (PCR), provide a high sensitivity and specificity and are also very rapid. Depending on the primer design and the follow up method used for analyzing the amplified sequence, the identification of the pathogens can be achieved on genus, species or strain level. Even though PCR is a precise, efficient and rapid method, some difficulties can arise when real-world-samples are investigated. If the sample matrix contains substances inhibiting the PCR reaction, more or less complicated procedures will be necessary for extracting the target DNA. The method is also sensitive to contamination and experimental conditions.

Despite the fact that culturing and PCR are well established and widely recognized techniques for pathogen detection, further research and development of more sophisticated approaches are desirable. As previously mentioned numerous fields of applications will benefit from advances made in this challenging territory. Among other alternatives to PCR and culturing, Raman spectroscopy can be a valuable and attractive tool in pathogen diagnostics [1]. The possibility to acquire suitable spectra of single cells within seconds and the high specificity of the Raman signal are the most important characteristics of this approach and imply an enormous potential for various applications. Most importantly, Raman spectroscopy enables the culture independent detection of bacteria, since single cell measurements can be conducted. Being able to omit the culturing step completely, huge amounts of time can be saved, which is quite an important factor in many fields of application. Of course, it is also possible to apply Raman spectroscopy to cultured bacteria, if desired. The measurements can be either performed on single cells or in bulk phase. A further advantage of Raman spectroscopy is that the sample generally is not destroyed and remains available for additional investigations. This potential has been recognized quite a while ago; accordingly the detection of microbes by means of Raman spectroscopy is a well discussed topic. But, although it was often shown that vibrational spectroscopy can provide results rapidly and accurately, especially in the section of bacterial detection, it is still a method in the early stage of development [2].

The requirements for establishing a new detection system are well known. Important parameters like the time, required until the result is available, sensitivity, specificity, size, costs and handling have to be optimized and defined. Those specifications are particularly important with regard to the proposed application of the system. It is often desirable to develop a device, which can be used directly on-site. It should be kept in mind that for virtually any application not only the Raman measurements have to be conducted, but also sample preparation and the analysis of the spectra are vital parts of the process chain. In Fig. 1 a typical work flow, which involves multiple steps from sampling to the final identification, is depicted.

Raman spectroscopy exploits the effect that laser light is scattered inelastically to a small extent, when interacting with a sample [3–7]. During this process energy is transferred between the incident photons and the sample molecules. The amounts of energy correspond to specific molecular vibrations. The fact that the Raman spectrum displays the molecular composition of the investigated sample with unique specificity makes this spectroscopic technique highly attractive for various analytes. By combining a Raman setup with a microscope even very small sample volumes within the range of a few μm³, including single cells, can be investigated separately. Additionally, Raman measurements can be performed unimpededly on microbial cells, since water is a very weak Raman scatterer. Usually, visible wavelengths are used to probe microorganisms. Eventually fluorescence can hamper the measurements and dominate the spectra, since the laser light used for the Raman measurements can also excite fluorescence emission in the samples. The rather weak, but very sharp Raman bands can be masked by a broad and intense fluorescence background, appearing in the same spectral region. The choice of an appropriate excitation wavelength is one option to overcome this problem. On the one hand the wavelength can be chosen so that the excitation is far away from any absorption process, but on the other hand it is useful to select a wavelength which matches with an absorption to partially get a resonant effect. This phenomenon, known as resonance Raman spectroscopy, is additionally a method to enhance the intrinsically weak Raman process [8]. Surface enhanced Raman spectroscopy (SERS) is another well-known possibility to face this problem [9–14]. Here, metallic nanoparticles or nanostructured metal surfaces are used in order to enhance the intrinsically weak Raman signal by several orders of magnitude.

Regarding SERS based detection of bacteria, there are several possibilities, some of which are schematically depicted in Fig. 2. For example the samples can be analyzed using nanostructured arrays as shown in Fig. 2a [15–18]. Various types of metallic nanoparticles or colloids are also frequently employed for acquiring SERS spectra of bacteria (Fig. 2b) [19–29]. It is also possible to deposit the nanoparticles directly onto the bacterial cell wall as indicated in Fig. 2c [30,31]. While all the previously mentioned approaches usually aim to detect the chemical fingerprint spectrum of the bacterial cells, it is also possible to use SERS tags (see Fig. 2d, for example [32–35]), which enable a highly sensitive detection of the cells via the spectra of the Raman reporter molecules. A more detailed discussion concerning SERS based detection of bacteria and spores can be found in other reviews [36–41]. Even though using SERS for the detection of bacteria can be advantageous, it also causes some challenges. For example providing SERS substrates with reliable reproducibility is an extremely demanding task, but if
Fig. 1. A typical work flow for the Raman based identification of bacteria.
identification of bacteria from samples via comparison to a previously established database is desired, absolutely necessary. On the other hand SERS offers the possibility to identify bacteria not only based on their Raman fingerprint, but also by employing SERS tags. SERS tags are usually composed of metallic nanoparticles, which are modified with Raman reporter molecule and specific capture probes in order to recognize their target. This approach has the advantage that the enhanced Raman spectrum of the Raman reporter molecule, which is located in close proximity to the metallic nanoparticle, is detected. By choosing reporter molecules with a large Raman cross section, very high sensitivities can be achieved. Furthermore this approach has a high multiplexing potential, since numerous Raman reporter molecules with very distinct marker bands are available.

Within this review recent advances in the field of bacterial detection via Raman spectroscopy will be discussed with the goal to provide an overview of the most important trends within the last five years. This article focuses mainly on the phenotypical identification of bacteria by means of Raman spectroscopy. Thus, nucleic acid based techniques, for example hybridization assays, were not included, even though it is very well possible to use Raman spectroscopy for sequence specific DNA detection [42–46]. However, the corresponding considerations, which have to be made, regarding sample preparation, specificity and detection are entirely different from the phenotypical approach. The first part of this review is devoted to Raman compatible isolation strategies for microorganisms from complex matrices, like body fluids, food stuff or even environmental samples. The second part is focused on the identification of bacterial cells from 'real-world-samples'. Conclusively, a critical outlook summarizes the achieved results and suggests which steps have to be done in future.

2. Raman compatible techniques for the isolation of bacteria cells

It is widely accepted, that Raman spectroscopy has the potential to be a valuable and powerful tool in medical diagnostics, food safety and many other fields of application. However, the application of Raman spectroscopy to real-world samples is only advantageous if the microorganisms in the sample of interest are actually accessible for the Raman measurements. Therefore, the meaning of efficient strategies for isolating whole and intact bacteria cells from samples must not be underestimated. The development of such methods can be extremely challenging depending on the composition of the sample and the amount of bacteria cells present. Nevertheless, this issue has been addressed in various publications and different solutions have been developed.

Developing Raman compatible isolation strategies requires consideration of several aspects. Of course the constituents of the sample have a major influence on the choice of the method. Analyzing a blood sample comes along with other demands than investigating water samples. Also, the cell concentrations in the different samples have to be taken into account for choosing a suitable approach. Furthermore an important parameter is the yield of cells. Especially when a sample might contain more than one species, it should be granted that all contaminants will be isolated, in order to prevent a misdiagnosis. In general, a careful evaluation of each method is indispensable. In the following section the distinct approaches and their capabilities and limitations will be discussed. Only publications, in which the Raman compatibility of the method was successfully demonstrated, have been considered.

2.1. Cultivation

The most straightforward approach for obtaining bacteria cells from samples is of course culturing. With this technique high amounts of biomass will be available for the Raman spectroscopic analysis. Accordingly the measurements can be performed in bulk phase. Maquelin and coworkers have applied this technique with great success for different applications [47–50]. Typically the bacteria are harvested from an agar plate by using an inoculation loop. The collected biomass is then centrifuged in order to obtain a proper cell pellet, which is consequently placed on fused silica slide. After drying the Raman measurements can be performed. A drawback of this method is, that the culturing step requires several hours, which can be critical for several applications.
2.2. Filtration

Filtration is a quite common method for separating solid particles from a liquid matrix. Depending on the pore size of the filter objects up to a certain size can be removed specifically. Bacteria occur in a variety of shapes and sizes, but typically within a range of 0.5 and 5 μm. Since they are significantly smaller than eukaryotic cells, separation via filtration is an option.

Ravindranath et al. have combined nanoporous filter membranes with functionalized metallic nanoparticles for SERS based detection of bacteria [51]. Gold (Au), silver (Ag) and Ag-Au-core-shell nanoparticles were fabricated and modified with pathogen specific antibodies or aptamers. Each type of nanoparticle was labelled using a different Raman reporter molecule. By assigning the Raman labels to a certain pathogen capture probe, it is possible to distinguish between Salmonella typhimurium, Escherichia coli and Staphylococcus aureus based on the fingerprint spectrum of the Raman reporter molecules. In a typical experiment the three different types of nanoparticles were incubated with the samples containing different bacteria species in phosphate buffer saline. For specificity a filtration step using a polyvinylidene fluoride (PVDF) filter membrane was included. While the bacteria will remain on the filter surface the unbound nanoparticles will pass the membrane. The proposed platform enables species specific detection of S. typhimurium, E. coli and S. aureus. Further it is possible to differentiate between E. coli O157:H7 and non-pathogenic E. coli K12. The total detection time is lower than 45 min and the limit of detection was determined to be in the range of 10^2 – 10^3 CFU/ml for buffer solution.

Chen et al. deposited silver nanorod arrays on cellulose and aluminum oxide filter membranes in order to obtain a SERS substrate for bacterial detection [52]. Depending on the type of filter used the entire sample preparation for 10 ml of E. coli cell suspension in high purity water takes between 10 and 20 minutes. Compared to SERS detection of bacteria on nanoporous filters without the filtration step, the limit of detection was improved by two orders of magnitude and was determined to be approximately 10^5 cells/ml.

Lin et al. developed a filter-like SERS substrate based on mesoporous silica [53]. The Au nanoparticle meso-porous silica (AuNP@MS) composites were fabricated using gelatin as an organic template. The AuNP@MS powder is pressed into disk-like form. SERS fingerprint spectra of S. aureus were obtained for a cell concentration of 10^6 CFU/ml. The application is limited to small sample volumes (up to a hundred microliters).

Stöckel et al. developed an assay for Raman spectroscopic detection of Burkholderia mallei and Burkholderia pseudomallei in feedstuff [54]. For isolating the pathogens from the pelleted animal fodder two filtration steps were carried out. Gauze was used to remove crude particles from the suspension. The resulting filtrate was further purified using a filter with 0.45 μm pore size. The final suspensions were dried on nickel substrates and Raman measurements were performed. Stöckel et al. demonstrated that reliable identification of Burkholderia species based on their Raman spectra is possible.

2.3. Centrifugation

In principal, there are two techniques regarding centrifugation. Differential centrifugation exploits the effect that particles of different size or density will sediment at a different rate. Density gradient centrifugation requires a special medium in order to provide a more efficient separation of the particles. Generally, it has to be distinguished between isopycnic and buoyant density gradient centrifugation. The former one separates particles only due to their different density, while for the latter one both mass and size are the criteria. Of course the previously mentioned techniques can be employed for separating bacteria cells from a sample as well.

Zhou et al. combined centrifugation and direct deposition of AgNPs on the surface of bacterial cell walls for SERS based detection of bacteria in drinking water [31]. Sample preparation includes several washing steps using centrifugation and consequently silver nanoparticles were prepared in the bacterial suspension using a modified version of the Leopold and Lendl protocol. This approach enables fast and quite sensitive detection of E. coli in water down to a level of 10^3 cells/ml. Zhou et al. report that an even lower limit of detection (2.5 x 10^2 cells/ml) was achieved if the silver coated bacteria are applied on a chip surface and SERS mapping is used for detection. At such low concentrations, the possibility has to be taken into account, that there are no cells at all present in the sample, when very small volumes are used. Furthermore Zhou et al. demonstrated that the detected SERS spectra are suitable for discriminating between three different E. coli strains and one strain of Staphylococcus epidermidis using hierarchy cluster analysis.

Meisel et al. isolated bacterial cells from spiked milk samples via centrifugation and performed subsequent single cell Raman measurements [55]. With this approach a reliable method is given to identify and distinguish between different milk contaminants like Brucella, Escherichia, Ochrobactrum, Pseudomonas and Yersinia spp.. In order to achieve detection limits in the range of 10^3 cells/ml buoyant density centrifugation (BDC) or enzymatic milk clearing (MC) can be used. Meisel et al. investigated these two approaches in detail and found that BDC has a better Raman compatibility and provides more reliable results regarding the yield of isolated cells [56].

Stöckel et al. reported that density gradient centrifugation is also a suitable approach for isolating endospores from powder samples. Combining this isolation technique with Raman microspectroscopy the detection of Anthrax endospores and differentiation from closely related species in baking powder, gypsum, milk powder, baking soda, bird sand, washing detergent, salt and acetylsalicylic acid is enabled. Sample preparation, Raman measurements and chemometric identification can be achieved within three hours [57,58].

Kloß et al. were able to detect pathogens responsible for urinary tract infections (UTI) in real patients’ samples using Raman microspectroscopy. For sample preparation the urine was centrifuged and the remaining cells were washed twice with buffer and finally resuspended in water. By establishing a database with Raman spectra of eleven UTI relevant species it was possible to identify the bacteria in the patients’ samples correctly, which was confirmed by standard culturing. The whole process including sample preparation and identification is completed within two hours. Samples having a cell count of 10^6 cells/ml or higher can be investigated. With this kind of sensitivity a concentration range actually relevant for clinical applications is achieved [59].

For analyzing meat samples for possible contamination with pathogens centrifugation and filtration steps were combined by Meisel et al.: Spiked beef and poultry samples were floated with distilled water and the resulting extract was further purified via filtration and centrifugation. Raman spectra of the isolated bacterial cells were recorded and compared to a database, containing typical foodborne pathogens, for identification. The whole process requires approximately two hours [60].

Van de Vossenberg et al. used centrifuge tubes with a membrane having a pore size of 0.2 μm in order to concentrate bacteria from drinking water samples for further Raman spectroscopic analysis. The initial concentration in the drinking water samples was 10^3 cells/ml. It is possible to discriminate between E. coli and coliform species on strain level also the majority of the investigated Legionella strains were distinguishable [61].

2.4. Enhanced evaporation

As an alternative to centrifugation Zhang et al. have developed a microfluidic sample preparation device which uses enhanced evaporation in order to concentrate bacteria in liquid samples. Especially for point-of-care applications this approach could be of interest. The microfluidic device developed by Zhang et al. is capable of concentrating sample volumes of hundreds of microliters into nanoliters within...
Dielectrophoresis (DEP) describes the effect that a polarizable particle will move in a non-uniform electric field [63]. The electric field causes a polarization of the particle, which then will experience an attractive or repulsive force towards regions of larger field intensity (see Fig. 3). The direction of the movement depends on the polarizability of the particle itself and the surrounding medium. This phenomenon can also be exploited for separating cells [64]. Clearly, this approach has potential for being combined with Raman spectroscopic identification of bacteria cells. The electric-field based separation method allows concentrating intact bacteria cells, even in complex samples like blood or urine, and enables Raman spectroscopic investigations of the cells.

Cheng et al. report the development of a microfluidic chip for sorting and concentrating bacteria cells via dielectrophoresis [65]. The bacteria are trapped in a region of the chip, which has a roughened gold surface, enabling immediate SERS measurements. The capability of their setup is demonstrated by separating Staphylococcus aureus cells from a 1:1 mixture with human red blood cells at a concentration of $10^6$ cells/ml. At this cell concentration the enrichment procedure only requires 3 min in order to obtain a sufficient SERS signal of the bacteria cells. At lower cell counts the time needed for the process has to be increased.

Later on another microfluidic setup involving DEP, electrophoresis and electrohydrodynamics was developed by Cheng et al. as well [66]. By using different electrokinetic mechanisms simultaneously some significant improvements compared to the previous system were achieved. SERS based detection of S. aureus at a concentration of $10^4$ CFU/ml from a diluted human whole blood sample was achieved within 3 min. Distinct SERS spectra of Escherichia coli and Pseudomonas aeruginosa, isolated from a diluted whole blood sample, were also obtained. In order to apply this method for diagnosing bacteremia, an additional step of blood culturing has to be included, so that a high enough concentration of bacteria cells in the blood sample can be achieved.

A further development of Cheng et al. for the SERS based detection of bacteria is a DEP setup which allows concentrating bacteria cells from diluted blood and aggregating them with silver nanoparticles later on [67]. Like the previously introduced system the approach can be possibly used in combination with blood culturing.

In contrast to the previously mentioned approaches Schröder et al. combine dielectrophoresis with Raman microspectroscopy instead of SERS for identifying bacteria in urine samples [68]. It is demonstrated how their dielectrophoresis–Raman chip can be applied for analyzing actual urine samples from patients with single pathogen urinary tract infections. The pretreatment of the urine sample involves a filtration step in order to remove larger particles such as leukocytes or epithelial cells. Their proposed assay including sample preparation only requires 35 min and is suitable for diagnosing significant bacteria ($<10^5$ cells/ml). In this study the discrimination between E. coli and Enterococcus faecalis, which are typical pathogens causing urinary tract infections, was achieved using a statistical classification model. Patients’ samples were also analyzed using microbiological cultivations test in order to ensure that the assignment of the classification model works correctly.

Generally DEP is a suitable approach for concentrating bacteria cells in order to make them accessible for Raman measurements. Even very complex samples like whole blood can be analyzed. Currently the limit of detection for such demanding matrices is in the range of approximately $10^5$ cells/ml. For some medical applications this is already adequate. Schröder et al. performed live/dead fluorescence viability staining and found that the bacteria survive the treatment [68]. Of course the viability will strongly depend on the electric field strength and the time period, in which the cells are exposed to the electric field. It might be of interest to find out if the bacteria are not only viable, but culturable. Cheng et al. suggest that cell damage can be avoided when negative DEP is used, where the bacteria will migrate to regions of lowest field intensity [65].

### 2.6. Optical trapping

Optical trapping or optical tweezers use highly focused laser beams to move and manipulate small dielectric objects as schematically shown in Fig. 4. This technique is a well-known and useful tool for cell sorting and micromanipulation of cells.

Recently Huang et al. combined Raman microspectroscopy and optical trapping for selectively isolating microbial cells from a mixed population [69]. Based on their Raman spectrum cells can be discriminated and later on separated using a highly focused laser beam. A mixture of yeast cells (Saccharomyces cerevisiae) and two bacterial species (Escherichia coli, Pseudomonas fluorescens) was used in this investigation. The sample had a concentration of $10^5$ cells/ml and contained each species in equal amounts. More than 50% of the isolated yeast cells and approximately 40% of the P. fluorescens cells were recovered by cultivation. In order to emphasize that this approach might be suitable for studying unculturability microorganisms, genome amplification of single cells was also performed. For 2 out of 7 sorted yeast cells and 3 out of 8 sorted bacterial cells the genome was amplified correctly.

Optical trapping enables the investigation and manipulation of single cells, which makes this technique valuable for studying unculturable species. Within this context, Raman spectroscopy can be used to analyze microbial communities and identify cells of interest, which are supposed to be isolated. Even though some of the isolated cells might suffer damage from the laser power used for the Raman measurement or the optical trapping, it was demonstrated that further analysis via genome amplification and recultivation is possible to a certain extent.
cells were indeed probably the most common approach. Antibodies have the ability to employ specific capture probes. Immunocapture with antibodies is a well-known possibility to isolate bacteria from complex samples is to employ specific capture probes. Immunocapture with antibodies is probably the most common approach. Antibodies have the ability to recognize their target structure with extremely high specificity, which makes them very valuable for application in complex samples. Antibodies specific against numerous bacterial species are available.

Kenge-Momo et al. developed a fast and simple electrochemical method for functionalizing surfaces with a polythiophene. Due to reactive para-benzenesulfonyl chloride groups it is possible to modify the polymer with protein A and subsequently with a Salmonella specific antibody. Protein A allows the oriented immobilization of antibodies via their Fc (Fragment, crystallizable) region, so that the antigen binding region will remain reactive and accessible. Even though this improved antibody immobilization procedure might be valuable for developing immunosensors in general, for Salmonella based detection of bacteria, the authors concede some limitations. The Raman spectra of the polymer with protein A and the antibody can hardly be distinguished from the Raman spectra of the modified polymer with bacteria. For comparison the authors immobilized protein A and the Salmonella specific antibody on a gold surface and obtained bacterial Raman spectra which allowed identification of Salmonella [71].

Another chip based approach using antibodies for capturing bacteria was reported by Pahlow et al. Here instead of species specific antibodies, immunoglobulins against common cell wall surface structures of Gram positive and Gram negative bacteria were employed. Since the identification of the bacteria is supposed to be achieved via their Raman spectra this choice of antibodies is suitable and enables the investigations of samples containing various species. It was demonstrated that several Gram positive and Gram negative species can be isolated with the chip and that the Raman spectra of the isolated bacteria cells are appropriate for classification. The limit of detection for this approach is in the region of 10³ cells/ml in buffer solution [72].

Knauer et al. employed antibody modified glass slides for capturing Salmonella typhimurium and Legionella pneumophila from buffer solution. SERS measurements for label-free detection of the bacterial cells were performed using a silver colloid [73]. Later on this stationary detection system was modified for improving the sensitivity. The development of a microarray flow-through system, which allows characterizing microorganisms via SERS in an aqueous environment, was reported. Antibodies specific against E. coli were immobilized on the PEG-modified chip surface for immobilizing the bacteria cells. Water samples spiked with E. coli were investigated and it was demonstrated that quantification of the cell count is possible within the range of 4.3 x 10³ to 4.3 x 10⁵ cells/ml [74].

In addition to chip based isolation methods assays involving magnetic beads and antibodies (immunomagnetic separation) are also a common choice (see Fig. 5). Because of their unique properties magnetic beads are a valuable tool for fast and specific isolation of analytes from complex samples. By modifying their surface with specific capture probes analytes can be isolated from a sample simply by applying a magnet. The low technical demands and the convenient handling render them ideal for on-site and point-of-care applications.

Tamer et al. report on the synthesis of rod-shaped core-shell Fe₃O₄-Au nanoparticles. The nanorods are further modified with avidin and E. coli specific biotinylated antibodies. Detection is achieved by using additional gold nanorods, modified with a Raman reporter and E. coli specific antibodies. The signal of the Raman reporter molecules was found to correlate with the E. coli concentration in the sample for 10² – 10⁷ CFU/ml. The limit of detection is stated as 35 CFU/ml in buffer solution [75].

Wang et al. fabricated magnetic SERS nanoprobes for detecting Salmonella enterica and Staphylococcus aureus in spinach and peanut butter. Silica coated magnetic nanoparticles were functionalized with pathogen specific antibodies in order to enable the isolation from the food matrix. In the next step antibody modified gold nanoparticles were used for SERS based detection. Two different approaches were investigated by the authors. Either the gold nanoparticles can be modified with Raman reporter molecules and detection is achieved via the SERS signal of the Raman reporter or the SERS spectrum of the bacterial
cells themselves can be acquired. It was found that the Raman reporter based method provided a much higher signal intensity and thus sensitivity. For both investigated food matrices a detection limit of 10^3 CFU/ml was achieved [76].

Fan et al. introduce popcorn-shaped plasmonic gold nanoparticles with a magnetic core. The multifunctional core-shell particles allow magnetic separation, enrichment, label-free detection and selective photothermal destruction of *Salmonella*. Due to anti-*Salmonella* antibodies on the specific isolation of the pathogen is enabled. The aggregation of the nanoparticles and the bacterial cells provides a high SERS intensity, facilitating a detection limit of 10^2 CFU/ml. Further Fan et al. demonstrate that targeted destruction of the bacterial cells using the same laser source as for the SERS measurements is possible. The complete assay requires 30 min [77].

Drake et al. demonstrate how magnetic nanoparticles can not only be used for targeted isolation of bacteria from samples, but also for aggregating SERS active nanoparticles with the analyte in order to achieve a higher enhancement. For this purpose iron oxide magnetic nanoparticles and gold nanoparticles with a Raman reporter were both modified with single domain antibodies specific for *S. aureus* protein A. Samples with *S. aureus* were incubated with both types of nanoparticles, which will bind to the bacterial surface and allow magnetic trapping and SERS based detection. For SERS measurements the nanoparticle modified *S. aureus* cells are gathered using a small magnet. Using this approach a SERS signal of the Raman reporter was still detectable for cell concentrations lower than 100 CFU/ml in buffer solution. With this high sensitivity a concentration range relevant for food analytics and medical applications becomes accessible. So far, however, this detection limit was only demonstrated for buffer solution and not for more demanding matrices like actual food samples or body fluids [78].

Temur et al. developed an immunosensor with SERS based detection using two different types of gold nanoparticles for analyzing water samples. Gold coated slides were modified with *E. coli* antibodies, allowing specific capture of the target microorganisms. After capturing the bacteria, the gold surface was incubated either with spherical or rod shaped gold nanoparticles, which were previously modified with a Raman reporter and *E. coli* specific antibodies. It was found that the gold nanorods enable more sensitive detection than the spherical nanoparticles. Concentrations lower than 10 CFU/ml were detectable. The assay was further challenged by analyzing tap and lake water samples for contamination with *E. coli*. Results were obtained within 70 min and in agreement with classical counting methods [79].

Huang et al. employed aggregates of Au nanoparticles, encapsulated with a silica shell for a higher stability, for SERS based detection of *S. aureus*. The nanoaggregates were further modified with rhodamine 6G (R6G) as Raman reporter and *E. coli* specific antibodies. It was found that the *S. aureus* was detected via SERS mapping using characteristic R6G bands and also SERS spectra of single cells were obtained [80].

2.9. Antibiotics

Next to antibodies, antibiotics specifically interacting with the bacterial cell walls can be used as capture probes for bacteria as well. Liu et al. functionalized arrays with SERS active Ag nanoparticles with vancomycin (Van) for detecting bacteria in human blood. They found that the Van coating of the SERS substrates significantly improves the detection of bacteria from whole blood, because the affinity to bind red blood cells and other components of the sample matrix is dramatically reduced. It is demonstrated that differentiating between Van resistant and Van susceptible strains of *Enterococcus* is possible. Being able to capture and detect bacteria from whole blood samples is an important step towards clinical applications [18].

**Fig. 5.** Immobilization of bacteria via immunocapture can either be achieved by using a chip based approach (a) or with micro- or nanoparticles (b).
Wu et al. employed silver nanorod arrays functionalized with vancomycin for SERS based detection of pathogens in mung bean sprout samples. Sample preparation involved two filtration steps. In the first step, larger particles were removed using a filter with 40 μm pore size. In the second step, the bacteria were recovered from the previous filtrate using a filter with 0.22 μm pore size. After removing the bacteria from the filter membrane with sterile water, the bacteria samples were incubated with the Van modified SERS substrates for two hours. For E. coli, S. epidermidis and four Salmonella spp., the limit of detection was determined to be 10^3 CFU/g mung bean sprouts. It can be differentiated between the six species using the principal component analysis (PCA). The whole assay requires four hours and SERS spectra were acquired using a portable Raman setup in order to demonstrate the potential for in-situ applications [81].

Sivanesan et al. modified a bimetallic silver-gold SERS substrate with vancomycin in order to capture bacteria from sample blood. Like Liu et al., they found that the blood components do not bind to the Van coated SERS substrates. Blood samples spiked with S. epidermidis, E. coli and S. enterica were investigated. The SERS spectra of the isolated bacteria cells were in agreement with reference spectra. The concentrations of the bacteria in the blood samples were not specified [82].

2.10. Targeting cell wall structures

In addition to antibodies and antibiotics, which target very specific moieties of the bacterial cell wall, it is also possible to exploit general characteristics of the cell membranes for capturing bacteria. Mircescu et al. demonstrated how bacteria can be immobilized by using chemically modified glass slides instead of specific capture molecules. Terminal amine groups are introduced via silanization and PEGylation. A treatment of the slides with HCl will result in the protonation of the amine groups. The positive charge can be used to immobilize E. coli cells, which exhibit a negative charge on their cell wall due to phosphate and carboxylate groups of lipopolysaccharides, which are typical for Gram-negative microorganisms. This approach is very advantageous because no species-specific capture probes are required [83].

Tamer et al. modified gold-coated magnetic nanoparticles with self-assembled monolayers (SAMs) of 3-mercaptophenylboronic acid (3-MBA) and 1-decanethiol (1-DT) for capturing bacteria. The authors propose that the affinity of the magnetic nanoparticles to the bacteria can be ascribed to the interaction of the alkanethiolate moieties of the 1-DT with the outer phospholipid membrane of the bacterial cell wall. Furthermore, the phenyl boronic acid group might target the carbohydrate groups of the exposed lipopolysaccharides. It was found that a mixture of both molecules provides the best capturing efficiency for E. coli bacteria. The isolated bacteria cells were investigated via SERS [84].

2.11. Summary

In the previous section, different methods for a Raman compatible isolation of bacterial cells have been discussed. The investigated matrices include water, various food samples and body fluids like blood and urine. For many samples even several possible isolation strategies are available. Unfortunately the detection limit is sometimes far from the requirements of the proposed application. Nevertheless, it has to be noted that in most of these cases the samples had a very complex composition and further optimization or combination of different enrichment procedures might be expedient. Quite often the reported methods and assays are still in a proof of concept state and not fully optimized, yet. On the other hand, some approaches are capable of achieving the necessary sensitivity or very low detection limits. When it comes to the diagnosis of urinary tract infections centrifugation and the combination of filtration [59] and dielectrophoresis [85] are good choices. In both cases, sensitivities below 10^5 cells/ml can be detected, which is already in the range of clinical relevant concentrations [86,87]. With SERS tags a detection limit of 10^3 CFU/ml was achieved in complex food matrices like spinach and peanut butter [76]. Regarding the doses necessary for causing an infection, this sensitivity is quite satisfying [88]. Furthermore, it can be considered as a great success, that already actual clinical samples were successfully analyzed via Raman spectroscopy and the results were found to be in perfect agreement with the findings of the microbiologists. Remarkably, the time needed for sample preparation and Raman measurements is 2 h or below for the vast majority of the proposed assays. This is a very promising result, since this time range is already sufficient for most applications.

A compact overview is given in Table 1 to allow a direct comparison of the introduced approaches. The diversity of the available methods is quite pleasing and noteworthy. This might be due to the fact that over the last years it has been recognized that sample preparation is a crucial part of the process chain, which has a tremendous effect on the sensitivity.

3. Identification

Once the isolation procedure is completed, the bacteria are available in an adequate state for Raman measurements. With Raman microspectroscopy, a valuable tool for acquiring information regarding the chemical composition of bacterial cells is given. In order to exploit the full potential of the recorded spectra, statistical methods have to be employed. By means of multivariate data analysis, minor phenotypic differences between bacterial species can be analyzed and utilized for the specific and reliable identification of pathogens. A further advantage of this approach is that even large amounts of data can be processed automatically, since the statistical analysis is a computer-assisted approach.

However, various aspects concerning the pre-treatment of the sample, the physiological state of the microbial cells or the measurement parameters can affect the identification process. Additionally, it has to be made a precise distinction, whether the identification of bacteria rests on single cell measurements or on bulk analysis. In some cases it is also possible to perform Raman measurements on microorganisms directly in the matrix instead of employing an isolation strategy. This chapter addresses these points and additionally gives an overview on studies, where Raman spectroscopy was successfully applied to identify bacteria out of complex matrices. Firstly, the requirements and challenges for a reliable Raman spectroscopic identification are carried out, before secondly, examples of application-oriented identification of bacteria are specified. Thereby, the attention is concentrated on clinical applications, food and water contamination, warfare agents and environmental samples, and also biofilm detection.

3.1. Requirements for a reliable Raman spectroscopic identification

The success of Raman spectroscopic identification is based on the quality of the underlying database and the statistical algorithms, which are applied to the Raman spectra. If a new database for a specific application is supposed to be established, several considerations have to be made. Of course Raman spectra of all the relevant species as well as related strains, species, and genera have to be included. A huge challenge regarding the investigation of ‘real-world-samples’ is the fact, that the growth conditions of the bacteria, like matrix composition, temperature, time and others, will affect the features of the cell spectra. Ideally, the cultivation conditions of the bacterial cells for the database should be as close as possible to the environment in the actual samples. In practice this is only realizable to a certain extent, because the sample composition, especially in the medical field, might differ significantly in each case. In order to make allowance for these variations, more than one batch of each class (strain or species) has to be used for collecting the spectra for the database. A batch describes an independent sample that differs from other batches at best in the way it was prepared, e.g.
age, cultivation medium, cultivation temperature, cultivation time, preparation date.

The influence of parameters like growth phase [89], microbial intrinsic storage material [90] or heavy metal contamination [91] on Raman spectra of microbial cells are frequently discussed. Furthermore also the preparation of the samples might affect the whole analysis [92], but this is also true for other methods. Conductively, the cultivation and preparation of bacterial cells should be performed under reproducible and stable conditions [93,94]. Even if all external conditions are taken into account, a suitable pre-processing of the measured raw data is obligatory to render the Raman spectra comparable [95].

When a sufficient amount of spectra for the database has been acquired, the pre-processed Raman data are chemometrically analyzed and a classification model is created. This classification model illustrates the discriminatory power of Raman spectroscopy in combination with statistics, but it does not revalidate it. It can be further challenged with the identification of ‘real-world-samples’ like patients’ samples or artificially contaminated matrices, e.g. soil or food stuff. Within this context it is important to differentiate between the terms ‘classification’ and ‘identification’. The latter one should exclusively be used when independent samples have been analyzed. The scheme in Fig. 6 depicts the whole analytical process.

Usually, it is assumed, that only one microbial species is present in the sample. However, there are some exceptions from this case. Especially, when it comes to analyzing patients’ samples, it is of great importance to find all relevant pathogens. This becomes even more of a challenge, when the concentration of the different species is divergent. Per se the investigation of bacterial mixtures is possible via Raman microspectroscopy, since single cell spectra can be recorded and each spectrum is individually assigned by the classifier.

<table>
<thead>
<tr>
<th>Isolation strategy</th>
<th>Matrix</th>
<th>Investigated species</th>
<th>Cell concentration</th>
<th>Time (time for isolation)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>N.A.</td>
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<td>[48]</td>
</tr>
<tr>
<td></td>
<td>Buffer solution</td>
<td>P. aeruginosa</td>
<td>N.A.</td>
<td>N.A.</td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td>Isolates from clinical samples</td>
<td>S. aureus (MRSA)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>[50]</td>
</tr>
<tr>
<td>Filtration</td>
<td>Buffer solution</td>
<td>S. typhimurium, E. coli, S. aureus</td>
<td>&lt;10⁵ CFU/ml</td>
<td>45 min</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>High purity water</td>
<td>E. coli</td>
<td>10⁶ cells/ml</td>
<td>10 – 20 min</td>
<td>[52]</td>
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</tr>
<tr>
<td>Centrifugation</td>
<td>Feedstuff</td>
<td>B. malti, B. pseudomallei</td>
<td>N.A.</td>
<td>a few hours</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Water</td>
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<td>&gt;10⁵ CFU/ml</td>
<td>10 min</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>Milk</td>
<td>Brucella spp., Escherichia spp., Octrobacterium spp., Pseudomonas spp., Yersinia spp.</td>
<td>10⁷ CFU/ml</td>
<td>2 h</td>
<td>[55,56]</td>
</tr>
<tr>
<td></td>
<td>Powder samples</td>
<td>endospores of B. anthracis, B. megaterium, B. mycoides, B. subtilis, B. thuringiensis</td>
<td>10⁵ CFU/g</td>
<td>3 h</td>
<td>[57,58]</td>
</tr>
<tr>
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<td>E. coli, E. faecalis</td>
<td>&gt;10⁵ CFU/g</td>
<td>2 h</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>Meat</td>
<td>E. coli, L. monocytogenes, P. aeruginosa, Salmonella spp., S. aureus, Y. enterocolitica</td>
<td>10⁶ cells/ml</td>
<td>2 h</td>
<td>[60]</td>
</tr>
<tr>
<td>Enhanced evaporation</td>
<td>Red blood cells</td>
<td>S. aureus</td>
<td>10⁵ CFU/ml</td>
<td>(15 min)</td>
<td>[61]</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>S. aureus, E. coli, P. aeruginosa</td>
<td>10⁵ CFU/ml</td>
<td>(3 min)</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>S. aureus, P. aeruginosa</td>
<td>10⁵ CFU/ml</td>
<td>12 h</td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>E. coli, E. faecalis</td>
<td>&gt;10⁵ CFU/ml</td>
<td>35 min</td>
<td>[64]</td>
</tr>
<tr>
<td>Optical trapping</td>
<td>Mixture of cells</td>
<td>S. cerevisiae, E. coli, P. fluorescens</td>
<td>&gt;10⁵ CFU/ml</td>
<td>(30 s)</td>
<td>[65]</td>
</tr>
<tr>
<td>LIFT</td>
<td>Ground water</td>
<td>E. coli</td>
<td>10⁵ cells/ml</td>
<td>N.A.</td>
<td>[70]</td>
</tr>
<tr>
<td>Immunoapture</td>
<td>Buffer solution</td>
<td>Salmonella enterica paratyphi</td>
<td>N.A.</td>
<td>2 h</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td>Buffer solution</td>
<td>P. aeruginosa, K. pneumoniae, E. coli, E. faecalis, E. faecium, S. aureus</td>
<td>&gt;10⁴ cells/ml</td>
<td>(20 min)</td>
<td>[72]</td>
</tr>
<tr>
<td></td>
<td>Buffer solution</td>
<td>S. typhimurium, L. pneumophila</td>
<td>&gt;10⁵ CFU/ml</td>
<td>N.A.</td>
<td>[73,74]</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Buffer solution</td>
<td>E. coli</td>
<td>&gt;10⁵ CFU/ml</td>
<td>2 h</td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td>Spinach, peanut butter</td>
<td>S. enterica, S. aureus</td>
<td>&gt;10⁵ CFU/ml</td>
<td>N.A.</td>
<td>[76]</td>
</tr>
<tr>
<td></td>
<td>Buffer solution</td>
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<tr>
<td></td>
<td>Buffer solution</td>
<td>S. aureus</td>
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<td>[78]</td>
</tr>
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<td></td>
<td>Water</td>
<td>E. coli</td>
<td>&gt;10⁵ CFU/ml</td>
<td>70 min</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>S. aureus</td>
<td>single cells</td>
<td>N.A.</td>
<td>[80]</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>E. coli, L. plantarum, E. faecalis</td>
<td>10⁵ CFU/ml</td>
<td>(1 h)</td>
<td>[81]</td>
</tr>
<tr>
<td></td>
<td>Mung bean sprouts</td>
<td>E. coli, S. epidermidis, Salmonella spp.</td>
<td>&lt;10⁴ CFU/g</td>
<td>&lt;4 h</td>
<td>[82]</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>S. epidermidis, E. coli, S. enterica</td>
<td>N.A.</td>
<td>N.A.</td>
<td>[83]</td>
</tr>
<tr>
<td>Targeting cell wall structures</td>
<td>Buffer solution</td>
<td>E. coli, P. mirabilis</td>
<td>N.A.</td>
<td>&lt;2 h</td>
<td>[84]</td>
</tr>
<tr>
<td></td>
<td>Buffer solution</td>
<td>E. coli</td>
<td>10⁵ CFU/ml</td>
<td>(30 min)</td>
<td>[85]</td>
</tr>
</tbody>
</table>

The in

Table 1
Comparison of Raman-compatible isolation strategies regarding matrix, analyzed microbial species, cell concentration and time.

<table>
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</table>
it was assigned as ‘unknown’. This concept was also successfully applied analyzing organic and inorganic components using Raman spectroscopy in combination with chemometrics by Schumacher et al. [98]. Another approach for novelty detection was revealed by Kemmler et al. [99] as well as Schmid et al. [100] applying a Gaussian process to the data.

Another approach, which has to be considered in the context of the identification process, comprises database independent identification of bacterial samples via SERS tags. The specificity of this method is based on the use of capture probes (mostly antibodies) for different bacterial species. By coupling these capture probes with distinct Raman labels and metallic nanoparticles a sensitive and convenient detection method, which does not require a database, is given. Within this context, the assay developed by Xiao et al. is of interest, which offers an alternative method of detection using SERS tags. While commonly only the signal of the Raman label is detected, Xiao et al. measured the superimposed SERS signal of the bacteria and the Raman tag. Since the binding event between bacterial cells and the SERS tag can be observed via the Raman spectrum, this assay does not require washing steps for ensuring the specificity. Here, the effect is exploited that the Raman signal of the bacteria will be only enhanced when the metallic nanoparticles bind to the bacterial cell wall via specific antibodies. With this innovative system a detection limit of $10^5$ CFU / ml for *E. coli* was achieved [101]. However, often the availability of different markers for different species is restricted. Accordingly, just a limited number of different bacteria can be analyzed in one step, which results in a loss of time.

3.1.1. Examples of application-oriented Raman-based identification of bacteria

In the following section, the utilization of Raman spectroscopic techniques to identify microorganisms is discussed for different scenarios: An overview for the usage of Raman spectroscopy in clinical areas
given and the application of this method in the food section is as well taken into account. Additionally, the possibilities, which are offered when Raman spectroscopy is applied to homeland security problems or when dealing with biofilms, are highlighted.

3.1.2. Raman spectroscopy for clinical applications

The benefits of an early and rapid detection of microbial-caused diseases are obvious: Therapeutic strategies can be chosen more individually, and therefore, the complete recovery of single patients can be promoted. For this reason, even a reduction of economic resources is accomplished, since the pharmaceuticals, especially the antibiotics as well as the medical care, can be better adapted to the problem at hand.

Microbial contaminations are the main reason for a high number of infectious diseases, like urinary tract infection, meningitis or sepsis and were often detected in body fluids, like urine, sputa, blood, ascites or liquor. Although the bacterial load in samples functions as a good marker for microbial contaminations, for medical purposes more information is required. Here, the exact identification of bacteria is necessary in order to provide an appropriate therapy and to prevent antibiotic resistance.

A range of Raman studies concerning the identification of bacteria in body fluids were already performed. For example, Kloß et al. reported on a cultivation-independent Raman microspectroscopy-based identification of bacteria from patient urine samples [59,85]. A wide-ranging database including eleven different species, which were all urinary tract infection (UTI)-relevant and which were directly cultivated in sterile urine samples, enabled a correct identification of bacterial contaminations from eleven patient samples, which were contaminated with Escherichia coli or Enterococcus faecalis. Also other publications deal with the discrimination of patho-gens causing urinary tract infections. Thereby, only pre-cultivated samples were in focus of the analysis [102,103]. Also the influence of antibiotics to the UTI pathogens was addressed [103].

The effect of antibiotics to bacteria is an important point of interest, too. Next to Münchberg et al. [104], Moritz et al. [105] focused on this problem, whereas these approaches could not differ more. Münchberg et al. investigated Gram-negative Escherichia coli and Pseudomonas spp. by treating them with four different kinds of antibiotics (ampicillin, ciprofloxacin, gentamicin and sulfamethoxazole), which all target different sites of the microbial cells. After performing Raman microspectroscopic measurements on single-cell level, the Raman data were compared to those, which were measured from untreated cells to investigate the impact of the antibiotic treatment of bacteria on the performance of a classification model. The identification of antibiotic-treated cells was more sufficient, when spectra of bacteria with antibiotic stress were integrated in the training data. In more detail, the general representation of the stressed microorganisms in the model improves species identification performance, while a representation of a specific antibiotic improves strain distinction capability [104].

Moritz et al. performed laser tweezers Raman spectroscopy on E. coli to analyze their metabolic states and spectral changes associated with the cellular response to the antibiotic cefazolin. As a result they claimed that potential spectral features exist that can be used to identify the response of E. coli to antibiotic drug treatment [105].

Raman spectroscopy has additionally been used to analyze bacteria in patients with cystic fibrosis. In this particular field, Rusciano et al. detected Pseudomonas aeruginosa and Staphylococcus aureus directly in sputum by performing photo-bleaching to the sample prior to the actual measurement [106]. The sputum samples, which contained a high amount of cellular debris, were investigated in bulk phase and a PCA allowed differentiating between patients infected with P. aeruginosa or S. aureus and patients without infection.

In the context with sputum samples, even the detection of Mycobacterium tuberculosis, the causative agent of tuberculosis, is of high interest. The applicability of Raman spectroscopy for discriminating mycobacteria species has already been shown in diverse publications. But almost exclusively, the identification rested on cultured bacteria, isolation from real-world samples is still pending [107–109].

Another critical respiratory disease is caused by Mycoplasma pneumoniae accounting for 20% of all community-acquired pneumonia. Hennigan et al. dedicated themselves to this diagnostic challenge using nanorod array-SERS (NA-SERS) to differentiate M. pneumoniae strains. Furthermore they could demonstrate that by applying NA-SERS to true clinical throat swab samples a discrimination between un- and infected specimens is possible [110].

Contamination of cerebrospinal fluid (CSF) with N. meningitides causes meningitis, which is - despite the availability of modern treatment strategies - a life-threatening disease that causes significant morbidity and mortality. The challenge of an in-time discrimination of specific bacterial pathogens in CSF was addressed by Harz et al. [111] using Raman microspectroscopy. It was found that the matrix components like proteins and salts did increase the background signal, but the single-cell Raman spectra were not masked. Next to a discrimination of different bacterial species via a hierarchical cluster analysis, the proof-of-principle study showed the potential of Raman microspectroscopy to identify N. meningitidis cells in CSF from patients with bacterial meningitis, too. However, the investigation of much more samples would be necessary in order to validate the method in this field of action [111]. Gonchukov et al. investigated dried CSF samples via Raman spectroscopy in order to spatially localize bacteria, leukocytes and erythrocytes and to work out characteristic spectral features to point out the presence of pathogenic cells [112].

3.1.3. Microbial contamination in food and water

Many reports and current processes, e.g. the E. coli outbreak in 2011, clarify that food contamination is an increasing problem with an ascending interest of the publicity. To ensure food safety and public health, the demand for advanced diagnostic tools is continuously rising.

Next to toxins and metals, bacteria are one of the main causes of foodborne illnesses. Among others Salmonella spp., Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes, Legionella spp. and Staphylococcus aureus belong to the most prominent troublemakers in food stuff. Therefore, a fast and sensitive detection of these microorganisms is desirable. But, also the detection of bacteria, like Pseudomonas spp., that cause spoilage in food, is of high importance to guarantee the freshness of the processed food.

The applicability of Raman microspectroscopy to identify foodborne pathogens from milk and meat on single-cell level was shown by Meisel et al. Bacterial contaminations of spiked milk samples, as well as of artificially contaminated chicken breast and minced meat samples, were correctly identified on a species-level [55,56,60].

Assaf et al. tried different paths of using Raman spectroscopy in an ISO standard to identify Salmonella spp [94]. It was pointed out that especially for the food industry a standardized protocol is essential to safely identify food-borne bacteria. They suggested that a pre-enrichment step is necessary to do so, for then other crucial parameters, e.g. physiological state of the cells, are comparable. By doing so, the identification of Salmonella spp. from rice, oat, wheat, maize, chicken and pork was performed successfully.

Some SERS investigations on food-borne pathogens were summarized by Craig et al. [113]. Although, plenty of techniques were presented, an approach to identify the bacteria directly after isolation from food samples is shown in only a few publications. For example, Wang et al. detected S. aureus and S. enterica from spinach and peanut butter by monitoring the spectral features of SERS tags [76].

Other SERS-based studies, also dealing with the identification of food-borne pathogens, showed again the discriminatory power of this technique, but unfortunately an identification step of spiked or real-world samples is not considered [114,115]. Instead, Wu et al. formulated the idea to apply a Vancomycin-functionalized silver nanorod array substrate in combination with a Raman setup to identify Salmonella.
spp., *Escherichia coli* and *Staphylococcus epidermidis* from mung bean sprouts [81].

Next to the compliance with food safety rules, safeguarding the quality of water is of high importance, too. Silge et al. demonstrated the identification of *Pseudomonas aeruginosa* in bottled natural mineral water [116]. Additionally, the study aimed to investigate of the influence of different aquatic environmental conditions, like pH or mineral content, and growth phase on the cultivation-free differentiation between water-conditioned *Pseudomonas* spp. *via* Raman microspectroscopy. Also, Kusić et al. and van de Vossenberg et al. demonstrated the potential of Raman spectroscopy to identify water pathogens with focus on *Legionella* spp. [61,117]. The detection of living bacteria in drinking water was also performed by Zhou et al. [31], who used an *in situ* coating of bacteria with silver nanoparticles for a SERS-based discrimination of *Escherichia coli* and *Staphylococcus epidermidis*.

### 3.1.4. Warfare agents and environmental samples

Biological warfare is the intentional use of living organisms or their toxic products, to cause death, disability or damage in man, animals, or plants. Bioterroristic attacks may be employed in various ways, like spreading through the air, water or in food, to gain enormous harm in public and economy.

The best known scenario of a bio-terroristic attack can be given by the anthrax letters. In 2001, unsuspicious-looking envelopes, which include white powders probably contaminated with *Bacillus anthracis* endospores, were sent among others to different persons of public interest, like politicians, or TV stations. While common microbiological practices provide improper results due to the interaction with the white powders, Stöckel et al. succeeded in the identification of single *B. anthracis* endospores isolated from “white powders”, e.g. baking powder [57,58]. By combining an appropriate inactivation, which additionally ensures the working safety [97], and an isolation step, the Raman microspectroscopic measurement and chemometric analyses allow an identification of *Bacillus anthracis* within three hours. The equal strategies were pursued to identify *Brucella* spp. from milk [55] and *Burkholderia mallei* from feeding stuff [54]. Cowcher et al. achieved appropriate detection levels of dipicolinate as marker for *Bacillus* endospores by means of SERS [118]. However, dipicolinate is only a generic marker for the presence of endospores, but cannot be utilized to distinguish different *Bacillus* species.

Rygula et al. performed Raman measurements on the surface of textiles. Cotton and polyester fabric were investigated for contamination with *E. coli*. Furthermore the effect of washing with a laundry detergent was a subject in this study. Discrimination of infected and non-infected textiles can be achieved via cluster analysis and PCA. A limit of detection was not determined. The fabrics were incubated with *E. coli* samples, having a concentration of $6 \times 10^6$ cells/ ml [119].

### 3.1.5. Identification of biofilms

Biofilms are communities of microbial cells, which are embedded in a matrix. The matrix is mainly formed from water and extracellular polymeric substances (EPS), which are biopolymers of microbial origin such as polysaccharides, nucleic acids, lipids and proteins. Next to the bacteria, EPS forms the structure of a biofilm, ensuring cell-cell contact, protection against environmental stress like antibiotics and prevention of biofilm desiccation. But the microbial species, their physiological states, the nutrient sources and, among others, also the age of the biofilm plays an important role in biofilm formation. To optimize biocides, antifouling strategies and biological wastewater treatment it is necessary to seek for techniques, which lead to a better understanding of structure and chemical composition.

In this context, Ileva et al. and Wagner et al. investigated the microbial constituents and EPS matrix of multispecies biofilm formation as well as different stages of biofilm development via a combination of confocal laser scanning microscopy (CLSM) and Raman microscopy [120,121]. The identification of different types of EPS was in the focus of studies from Chen et al., whereas a microbial analysis was not carried out [122]. Liu et al. investigated EPS extracted from *Bacillus subtilis* by Raman spectroscopy to gain comprehension in the formation of mineral-organic associations in soil [123].

The characterization of the EPS matrix in biofilms is the emphasis of many studies. So, gold nanoslands were used as SERS substrate to detect chromate, sulfate and nitrate localization sites in remediating bacteria biofilms by Ravindranath et al. [124].

A SERS study from Chao et al. demonstrated that the collected information from the matrix like carbohydrates, proteins or lipids are strongly connected to the bacterial growth behavior and they therefore showed that even the chemical variations during the biofilm formation give information about the involvement of e.g. Gram-positive or Gram-negative bacteria [125]. The possibility to discriminate Gram-positive and Gram-negative bacteria in biofilm formations is additionally shown by Efeoglu et al., they analyzed *Escherichia coli* and *Staphylococcus cohnii* during the growth of biofilm by placing silver nanoparticle substrates on the biofilm. This application enabled the monitoring of molecular changes in the biofilm during its formation [126].

In contrast, Kniggendorf et al. reported about the applicability of resonance Raman microspectroscopy as a tool for analyzing biofilms. Different *Nitrosomonas* species as well as different strains of *Rhodobacter sphaeroides* were investigated and characterized by their resonance Raman spectra. Additionally, the bacterial response to the variant toxicity of the mineral phase was under investigation, since purple bacteria were observed to overgrow polymorphic TiO2 microparticles [127].

*Streptococcus sanguinis* and *Streptococcus mutans*, which are major microbial components of oral plaque, were studied by Beier et al. In a first attempt each species was analyzed as pure biofilm by means of confocal Raman microscopy. Afterwards the biofilms of both species were mixed up. A prediction model based on principal component analysis was applied to the biofilm mixture and allowed a discrimination of both species with an accuracy of 97% [128]. Later on, the species classification algorithm was successfully used to investigate dried biofilms, which were transferred from their original substrates. The presence of both species could thus be shown and spatial maps within the biofilms could be created [129].

Biofilm formation can be monitored even on paintings. Rosado et al. studied the color alteration of green areas in mural paintings due to microorganisms by means of Raman spectroscopy. The metabolic activity of the microorganisms present in the paintings results in a promotion of calcium oxalate formations over the malachite paint layers [130].

### 3.2. Summary

The previously discussed paragraph summarizes the problems and strategies around the identification of bacteria by means of Raman spectroscopy. It was shown that the construction of databases is a crucial point especially regarding single-cell analyses. Furthermore the terms classification/ model, validation and identification were explained to highlight the differences in analyzing strategies. Moreover issues like bacterial mixtures, other identification strategies and unknown samples were addressed. Ongoing, a variety of application examples concerning the usage of Raman spectroscopy for the identification of bacteria was given.

Thereby the literature of sundry fields of interest was carried together. Clinical aspects, food and water safety, environmental issues as well as the emerging topic biofilm formation were introduced.

The development in the last five years definitely showed a trend in direction of identifying real-world-samples.

### 4. Conclusion and future prospects

Within this article the recent advancement of Raman spectroscopy as promising technique for a fast and reliable identification of bacteria has been reviewed. Because most applications require some kind of...
sample preparation before the Raman measurements can be conducted, this aspect has also been carefully discussed.

Since the consequences of bacterial contamination, for example in the food chain or the water supply system, can be severe, the demands regarding the detection system are accordingly high. A crucial factor is the time needed for detection of the contaminant. Obviously, the sooner the source of infection can be identified, the better is the chance that greater damage can be prevented. Concerning this aspect, Raman spectroscopy is an excellent choice for the detection of bacteria, since the spectra can be recorded within seconds. Even though the exact identification of the species requires the employment of statistical models and a database, the results still can be obtained within a few minutes. However, it has to be taken into account that the most time consuming step in the process chain is the inevitable sample preparation. In order to be able to conduct the Raman measurements the cells usually need to be isolated from their surrounding matrix first. Depending on the sample composition this task can be quite challenging. Nevertheless, within the last years numerous approaches, which enable the investigation of various matrices via Raman spectroscopy, have been developed. Next to mechanic strategies like filtration and centrifugation, also microfluidic devices, chip based systems, magnetic beads, optical trapping and dielectrophoresis have all been proven to be Raman compatible. Including sample preparation the identification of the bacterial cells can often be achieved in only 2 – 3 h. This is a sufficient time frame for most applications and clearly advantageous in comparison to culturing, which can require even days.

Another key point in the field of bacterial detection is the sensitivity. While Raman microspectroscopy allows the investigation of single cells, the detection limit is mainly determined by the isolation strategy for the cells. For samples with low complexity detection limits of 10^9 cells/ml have already been achieved. For more demanding samples like blood the detection limit is still much higher and needs to be further improved in order to enable the analysis of clinical relevant concentrations. For urinary tract infections it has already been shown, that with an appropriate sample preparation protocol, Raman spectroscopy can be a valuable tool for diagnostics [59].

For most applications a high specificity is needed. With the Raman spectrum of a bacterial cell valuable information about the phenotype can be obtained. In combination with chemometrics this enables the identification of bacteria on species or even strain level. Since the assignment of the single cell Raman spectra happens individually, the investigation of samples, which contain more than one species can be accomplished as well. As already pointed out, the reliability of the identification strongly depends on the quality of the database. Accordingly, a lot of effort should be invested in establishing a database for a new application. A further advantage concerning Raman based detection of bacteria is the non-invasive character of the technology. The cells remain intact and are available for further analysis.

In order to exploit the full capability of Raman spectroscopic identification of bacteria, it would be worthwhile to address the following issues in near future:

For promoting the use of Raman based detection systems in point-of-care diagnostics or for on-site applications, further miniaturization and automation is desirable. However, in the last years the technological development already made a clear advancement in this direction, since the devices became smaller and more robust. Also, first instruments which enable the automated analysis of bacteria cells via Raman spectroscopy are currently under development (BioParticle Explorer, raplD, Germany) or even available on the market (SpectraCell RA, RiverD, the Netherlands).

The implementation of a standard operating procedure would be a useful step to reproduce the whole process chain beginning with sampling, over isolation procedures right up to the Raman measurement parameters and the chemometric-based identification of the bacteria. In a first attempt, Assaf et al. implemented a Raman-based standard protocol for Salmonella detection, which actually fundamentally rests on cultivation [94]. However, the great number of various isolation and identification strategies, shown in the review, highlighted that it is possible to find an individual solution for any specific question. This aspect was also addressed by Ashton et al. describing the pros and cons of the phenotypic Raman identification procedure [39].

Another crucial point is the transferability of Raman databases: In contrast to other methods, like mass spectrometry, the existing Raman databases are mainly device-specific. Initial steps were performed by Dörfer et al., who examined and improved the calibration of Raman spectra [131]. Another challenge concerning the databases is the variability of microbial Raman spectra due to their physiological state or different growth media. Therefore, most often sample-specific databases were created. It is still debatable how many species, samples or batches have to be involved in a Raman database to ensure that all issues were addressed. To overcome this problem, a possible solution is the integration of a classifier, which is able to recognize samples as ‘unknown’.

When the above mentioned points are overcome, Raman spectroscopy can be established in routine analytics for pathogen detection. The great diversity of the investigated samples shows the broad applicability of Raman spectroscopy. Generally, it can be concluded that Raman spectroscopy has an enormous potential in the field of bacterial detection, especially in combination with efficient isolation strategies for microorganisms.

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