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Differentiation and classification of bacteria using vancomycin functionalized silver nanorods array based surface-enhanced Raman spectroscopy and chemometric analysis

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ABSTRACT

Twenty seven different bacteria isolates from 12 species were analyzed using intrinsic surface-enhanced Raman scattering (SERS) spectra with recently developed vancomycin coated silver nanorod (VAN AgNR) substrates. The VAN AgNR substrates could generate reproducible SERS spectra of the bacteria with little to no interference from the environment or bacterial by-products as compared to the pristine substrates. By taking advantage of the structural composition of the cellular wall which varies from species to species, the differentiation of bacterial species is demonstrated by using chemometric analyses on those spectra. A second chemometric analysis step within the species cluster is able to differentiate serotypes and strains. The spectral features used for serotype differentiation arises from the surface proteins, while Raman peaks from adenine dominate the differentiation of strains. In addition, due to the intrinsic structural differences in the cell walls, the SERS spectra can distinguish Gram-positive from Gram-negative bacteria with high sensitivity and specificity, as well as 100% accuracy on predicting test samples. Our results provide important insights for using SERS as a bacterial diagnostic tool and further guide the design of a SERS-based detection platform.

1. Introduction

Over the past decade, surface-enhanced Raman scattering (SERS) has been considered a powerful platform for the rapid and sensitive detection of bacteria [1–14]. SERS brings the analyte molecules into close proximity with the appropriate metallic nanostructures, thus significantly increasing the Raman vibrational signals of the analyte. These molecular vibrational modes represent a unique "fingerprint" spectrum consisting of Raman peaks that can be used to identify the particular molecule(s) being probed [15]. This method of enhancement is often employed to facilitate sensitivity so as to allow for the trace detection of molecules, even with single molecular sensitivity [16–20].

For SERS based bacterial detection, two of the most important tasks are (1) to detect bacteria with high sensitivity from complex media such as food matrices or clinical samples, and (2) to distinguish between bacterial species using their intrinsic SERS spectra. In fact, researchers have put forth great efforts to answer two critical questions which include whether or not the intrinsic SERS spectra arising from the bacteria whole cell can be used for differentiation and classification of bacteria, and what would be the most effective strategy to utilize chemometric analysis to achieve such a differentiation. Although the origin of the SERS signal which arises from the bacteria is still not clearly understood, the majority of researchers agree that it primarily originates from the external structure of the bacterial cell, i.e. cell wall and proteins [12]. Hence, it is hypothesized that the spectral differences in SERS that occurs between bacterial isolates reflect these external structural differences between bacteria. However, the chemical makeup of the bacterial cell wall is very similar between species, so it is difficult to differentiate between bacterial species through visual inspection of the SERS spectra alone. Since the SERS spectra can be viewed as multi-variant data, chemometric analysis is often used to differentiate bacteria by reducing the dimensionality of the data set and maximizing the variance among spectral fingerprints.

Various chemometric methods have been adapted to analyze the bacterial SERS spectra, including principle component analysis (PCA),

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hierarchical cluster analysis (HCA), partial least square discriminant analysis (PLS-DA), partial least square regression (PLS), discriminant function analysis (DFA), linear discriminant analysis (LDA), and support vector machine (SVM). PCA is a primary mathematical method that reduces the data dimensions by identifying correlations amongst a set of variables and then projects the original set of variables into a new set of uncorrelated variables called principal components (PCs). PCA is an unsupervised chemometric method often used for the purpose of pattern recognition [1–10]. To further improve the grouping of the SERS spectra from different bacteria, HCA is often employed. HCA assigns samples to the individual cluster according to the similarity between them, based either on PCA or Mahalanobis distance, and generates a dendrogram [2–4,7,9]. If the classes of which the bacterial species being analyzed are already known, supervised methods such as PLS-DA can be used in order to yield more robust discrimination [3,11]. PLS-DA uses a linear combination of the predictor variables to project the original data into a new set of coordinates to generate a positive/negative prediction. It is often used to classify bacteria based on known characteristics, such as Gram stain results, i.e. Gram positive (G+) versus Gram negative (G−). Choosing the appropriate chemometric method is based on the nature of the data and the objectives of the research.

The literature on the differentiation of bacteria based on their intrinsic SERS spectra is summarized in Table S1 in Supplementary information. The discrimination between bacterial species has been achieved by several authors using PCA, HCA, LDA, etc., with sample sizes of up to eight individual bacterial species. Such differentiation is easy to achieve since the structural differences between species is significant [1,2,4,5,9,10,21]. Researchers have also explored the ability to discriminate between bacteria at the sub-species level using up to four individual serotypes of *Salmonella* [11] or 14 individual strains of *Arthrobacter* [7]. Unfortunately, all those results were analyzed by chemometric methods on the individually measured spectra of bacterial isolates, and the bacterial mixtures were not yet well studied. We still lack a comprehensive understanding on what molecular structures or components contribute to the differences observed between bacterial spectra. In addition, the reproducibility of the spectra obtained has not been able to support any specific claims, and the molecular principle behind such differentiation was not well comprehended.

In order to reliably combine chemometrics with SERS as a bacterial detection platform, the SERS spectra generated needs to be reproducible and reliable. Thus, three important factors should be carefully considered: (1) reproducibility of the SERS-active nanostructures, (2) contamination from the environment, and (3) interference from the bacterial metabolic by-products. SERS-active nanostructures with high reproducibility can generate spectra with less variance from experiment to experiment, and from sample to sample. Researchers have used different types of nanostructures to study the SERS of bacteria such as silver metal deposits [22,23], silver colloid solutions [2,24,25], gold colloid solutions [26,27], electrochemically roughened metal surfaces [28], Ag nanocrystal assembled Ag nanospheres (AgNSs) [29,30], silver film over nanospheres (AgFON) [31], and silver nanorod array substrates [11,13,32]. Gold or silver colloids are the most widely used SERS active substrates due to the easy manufacturing process and low cost. However, they often lack reproducibility due to large variations in cluster size and shape. The nanostructure involved in the other “substrate” types tends to have more reproducibility due to their highly uniform structures. Among them, silver nanorod (AgNR) array substrates fabricated by the oblique angle deposition (OAD) method have been shown to have a SERS enhancement factor $> 10^8$ and a batch-to-batch variability $< 15\%$ [33,34]. The detection of bacteria using AgNR substrates has been demonstrated with high sensitivity and specificity [11]. However, the use of silver nanostructures, such as AgNR, AgNSs, or AgFON for bacterial SERS detection poses some disadvantages, including cytotoxicity, so the binding between silver and bacteria is not favored. In addition, silver is relatively chemically active, so it can react with the sulfur that is present in the environment. The reaction with sulfur will interfere with the SERS signal, thus preventing one from obtaining clear and reproducible SERS spectra.

Finally, it is possible that the by-products synthesized by the bacteria may be detected by the highly sensitive SERS substrates thus interfering with the bacterial SERS spectra. To overcome these disadvantages, the surface of AgNR substrates can be modified by a coating that can actively capture the bacteria, yet protect the surface from reacting with unwanted substances. The use of a vancomycin (VAN) coating on the silver nanostructure has been explored by different research groups to illustrate its ability to capture bacteria and subsequently improve sensitivity [11,35]. However, the ability of VAN coated substrates to protect the silver nanostructure surface and improve the reproducibility of the spectra has not yet been explored. A VAN coating on the AgNR substrate may protect the silver surface from reacting with environmental contaminants, thus resulting in a low to completely absent background signal. Furthermore, because VAN selectively binds to the bacterial cell wall, other biomolecules such as bacterial by-products will have a lower chance of binding to the SERS substrate surface [35]. Thus, the vancomycin coated silver nanorod (VAN AgNR) substrates will ensure that the SERS signal obtained are from the outer structures of the bacteria, thus providing insight into the molecular principles involved in differentiating between bacteria using SERS. Overall, the high reproducibility and sensitivity of the AgNR substrates in combination with the protection and selectivity given by the VAN coating will generate reliable SERS measurements when analyzing bacterial cells.

In order to design a better differentiation strategy and validate the ability of chemometric methods to differentiate between bacteria in a large bacterial sample, this study used three conventional multivariate analysis methods, PCA, HCA, and PLS-DA, to differentiate and classify the SERS spectra of 27 bacterial isolates from 12 species with various strains and serotypes. The results reveal the advantages of using the VAN AgNR SERS substrates to (1) protect the substrate's surface from environmental contamination and bacterial metabolic products, (2) generate reproducible SERS spectra of bacteria, and (3) to consequently improve the differentiation of bacteria. PCA and HCA methods are employed to illustrate the feasibility of using simple multivariate analysis methods to differentiate bacteria between species, serotypes, and strains. The discrimination between G+ and G− bacteria are tested by PLS-DA methods. These results provide insights for the future application of SERS as a bacterial diagnostic platform.

2. Materials and methods

2.1. AgNR SERS substrate fabrication

The bacterial SERS spectra were acquired using AgNR array substrates fabricated by the oblique angle deposition (OAD) technique in a custom-designed electron beam evaporation (e-beam) system [33,34,36]. Briefly, glass microscopic slides (Gold Seal® Catalog No.3010, Becton, Dickinson and Company, Portsmouth, NH) were cleaned with piranha solution (80% sulfuric acid, 20% hydrogen peroxide in volume) and rinsed with deionized (DI) water. The substrates were then dried with a stream of nitrogen gas before being loaded into the deposition system. In the deposition system, the substrate surface was held perpendicular to the incident vapor direction while a 20-nm titanium film and then a 200-nm silver film were evaporated onto the glass slides at a rate of ~0.2 nm/s and 0.3 nm/s, respectively. Monitoring of this process was performed *in situ* by a quartz crystal microbalance (QCM). The substrates were