Rapid Analysis of Microbiological Systems Using SERS

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1 Introduction

Research in the biological sciences field is now moving forward at an unprecedented rate. Modern technological advances, particularly computational processing power, but also modern analytical methods, have been embraced by the life sciences. In combination these have developed to such an extent that it is now possible to begin to investigate complex systems using powerful techniques that in the past would most likely have been the preserve of the analytical chemist. There is still a wealth of highly challenging problems in biology that need to be solved, and this will necessitate the continual evolution of novel analytical strategies.

Genotyping technology revolutionized the study of biological systems; by using the polymerase chain reaction to amplify genetic material extracted from a cell, gel electrophoresis and more recently multiplex genotyping could be performed to map genetic sequences, and observe genetic differences between organisms. However, even this highly qualitative approach has yielded many more questions than it actually answers, since the function of genes of interest cannot normally be directly inferred from the "static" sequence blueprint. Thus, within the areas of functional genomics and systems biology many studies are actually aimed at investigating the organism's phenotype directly. The phenotype is defined as the result of the expressed genotype of the organism and is influenced by the environment which it inhabits. Whilst genetic analysis can be enlightening, it is often not as informative as phenotypic information, and because of this, in microbial research biochemical analyses have been adopted to measure quantitatively cellular components such as proteins and metabolites. However, more recently great steps have been made to introduce rapid spectroscopic approaches to study the phenotype.

So, exactly what are biologists interested in understanding about microbial systems? Broad areas of interest include: microbial classification and identification; mRNA, protein and metabolic profiling; functional genomics; systems biology; optimization, regulation and understanding of microbial bioprocesses; and more specifically with increased incidence of antibiotic resis-

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tance, the mode of action of hopefully antimicrobial drugs. To extract the maximum information from these experiments the best approach is to employ global analysis tools; which often indicates spectroscopic measurements, from which relevant knowledge can be extracted. This data-mining process uses mathematical tools to ask questions of the data that have much lower dimensionality than the spectral data and typically involve quantitative or categorical modeling. For example, a question might be; "based on the Raman fingerprint I have generated from this group of bacteria, which bands are discriminatory and allow objective identification?" In addition, many spectroscopic methods provide a real insight into the biochemistry of an organism by conventional interpretation of spectral bands, and these data are far more easily and rapidly obtained through this route as opposed to that of the traditional biochemical approach.

There are a wide range of spectroscopic methods encompassing the vibrational, nuclear magnetic resonance and hyphenated mass spectrometries that have been used to great effect on a range of biological problems. The purpose of this Chapter is to introduce some (micro-)biological applications for which surface-enhanced Raman scattering (SERS) has been recently employed. SERS is emerging as a very powerful tool in the biological sciences due to its excellent sensitivity and ability to quench fluorescence, which can plague Raman measurements of biological material excited in the visible to the near-infrared. Specifically we shall discuss, SERS for the characterization and identification of micro-organisms, the monitoring of industrial bioprocesses and finally, gene-function analysis.

2 Spectroscopic Characterization of Micro-Organisms

Traditionally, the task of classifying microbes has been performed by a comparison of macro- and micromorphological characteristics or by biochemical tests. In more recent times genomic analysis has been used as a means of identification or classification and 16S ribosomal RNA sequencing is now the "gold" standard used for this task [1]. However, there are drawbacks associated with all of these approaches. Naturally, comparison of morphology is not a wholly reliable means of classification; this particular phenotype of an organism can be repeatedly expressed amongst biochemically diverse species and therefore is not on its own a suitable means of differentiating between bacteria [2]. The API system (`http://www.biomerieux.com/`) is a popular biochemical method used in routine laboratory analysis, approximately 2000 research publications (since the early 1990s) refer to the use of API. With API, a series of biochemical tests is applied to an organism cultured in the laboratory, the response of the organism to these tests is then matched against a database of possible results to provide identification. Whilst biochemical detection methods are reliable, they do not always provide conclusive decisions at the species level, whilst strain-level characterization is generally

impossible and analysis of environmental isolates uncertain. The process itself is also very time consuming; cell culturing, running the test and analysis of results often require several days. The analysis of rDNA also has the same issues since many steps are involved including cell culturing, DNA extraction, sequencing and results analysis. In addition, as the 16S rRNA is highly conserved it is only useful at the species level.

In contrast to these biochemical and molecular techniques another route has been taken through the use of vibrational spectroscopy to generate "fingerprints" of intact bacterial samples. As far back as 1911, Coblentz suggested that biological samples could be analyzed by infrared (IR) absorption spectroscopy [3]. IR spectroscopy is a vibrational technique that measures the absorbance of radiation by a sample. The two vibrational techniques of IR and Raman spectroscopy are useful complementary techniques, since they can be used to probe a broad range of molecular symmetries [4]. It was IR spectroscopy that was first applied to the identification and characterization of *Eubacteriales* and *Lactobacillus* isolates, with data published as early as the 1950s [3, 5]. Despite these early successes the application of IR spectroscopy to the microbial taxonomy field did not gain popularity. Unfortunately at this time, the engineering behind spectrometers was still not advanced enough to provide rapid, sensitive, reproducible and low-cost instrumentation. It was not until the last quarter of the 20th century that the development of the interferometer, the microprocessor and powerful wave-transformation algorithms led to a resurgence of literature reporting whole-organism fingerprinting studies using IR technology [6, 7].

Raman spectroscopy is a recent addition to the physicochemical spectroscopic technologies that have been applied to the problem of rapid characterization and identification of micro-organisms. Although, in 1974 *Spiro* first suggested that resonance Raman spectroscopy could have potential for biological analysis [8], the earliest literature suggesting that Raman had the capability to be used in categorical analysis of microbes did not come until the early 1980s, and even then the reports did not go so far as to demonstrate that this was possible in practice [9, 10, 11]. In the 1990s the first reports of NIR FT-Raman were published, which examined the chemical nature of both bacterial and fungal cells, but again did not go so far as to use those data for discrimination [12, 13]. It was not until the start of this century that significant work showing the ability of Raman spectroscopy to be used as a microbial characterization and discrimination tool was reported [14,15,16,17]. These studies showed that Raman spectroscopy at near-infrared frequencies could be used to characterize bacterial cells at the earliest stages in colony development. Using Raman spectroscopy, single bacterial cells have also been analyzed, both conventionally using Raman microscopy [18], and with more complex laser tweezers systems that can aid the reduction of fluorescence at NIR wavelengths [19, 20].

The major shortcoming of Raman spectroscopy is that given the weak Raman scattering achieved by many biological samples, the spectral acquisition

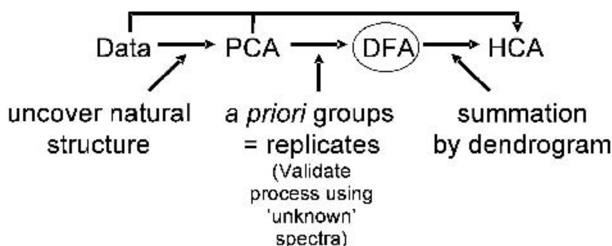

Fig. 1. The typical multivariate analysis methodology applied to the problem of biological characterization using SERS spectral fingerprints. The underlying theme is simplification or dimensionality reduction

time can be many minutes. However, enhancement methods such as SERS will provide a solution to this problem. Several investigations into SERS of bacteria have been undertaken [21, 22, 23, 24, 25], and more recently it has been shown that SERS can reliably differentiate between different bacteria [26, 27]. There is also much interest in detecting aerosols of spore-forming pathogens, with the obvious target being the identification of *Bacillus anthracis*. Preliminary work has already been carried out using SERS for this purpose and it has been shown that the dipicolinic acid biomarker, common to all spore-forming pathogens, can be detected [28, 29, 30, 31].

3 Introduction to Multivariate Cluster Analysis

By their very nature SERS spectra are multivariate. That is to say, each Raman wave number shift measured can be plotted against each other such that if we measured a meager 100 shifts then a sample can be said to reside somewhere in 100-dimensional space. Obviously this abstract space is very hard to visualize (!) and thus the underlying theme of multivariate cluster analysis is dimensionality reduction of the SERS spectra to a few new components that can be easily plotted and the relationship between the spectra visualized.

In this Chapter we are discussing the application of SERS to the problem of classification in highly complex biological systems. The major step that needs to be taken with large sets of multivariate data to achieve this objective, is a transformation of the original spectral domain into a reduced form that is both robust and interpretable in terms of the problem being studied.

Whilst there are many chemometric methods that can be applied to multivariate data (the reader is referred to [32, 33, 34]), the strategy that we have adopted for cluster analysis as reported in [35] is depicted in Fig. 1.

Initially, principal components analysis (PCA [36]) is used, which is an unsupervised method of data reduction where the original data matrix is projected onto a smaller variable subspace, and the resultant principal-com-

ponent scores represent a majority of the variance in the data. This can be represented as:

$$T = XL\,, \tag{1}$$

where T is an $n \times d$ matrix of principal-component scores with a magnitude of d (dependent on the number of PCs to be extracted); X is an $n \times p$ matrix of independent variables (e.g., mean-centered spectra); and L is a $p \times d$ loadings matrix. In the situation where groups of SERS spectra from different bacteria are separated in the first PC, the loadings matrix from PC1 can be inspected to ascertain which SERS bands are most important for this separation.

However, often an unsupervised approach is insufficient to separate closely related bacterial classes based on their complex spectral profiles, and therefore one must use supervised analysis that can be used as predictive models. This involves proposing an a-priori class structure from which a determinative model is derived. One popular supervised method is discriminant function analysis (DFA [37]), which maximizes the within-group to between-group ratio (Fisher ratio) to differentiate between classes (groups). This approach has been used with great success for classification problems involving spectral fingerprinting [38, 39, 40]. DFA calculates a number of linear discriminant functions for separating groups by finding the eigenvalues and eigenvectors of the expression:

$$W^{-1}B\,, \tag{2}$$

where W is the within-sample matrix of sums of squares and crossproducts, and B is the between-sample matrix of sums of squares and crossproducts. As DFA is a supervised algorithm that optimizes the Fisher ratio, so as to separate different classes, it is necessary to avoid overfitting. In overfitting, the model has learnt the (training) data perfectly but is no longer able to predict the identity of new (test) data. That is to say it can not generalize. To avoid this we project test SERS data obtained from fresh cultures of bacteria of which we know the identity into a previously generated DFA cluster space. If the test data cocluster with the corresponding training data we are convinced that our model is valid.

For the identification of large numbers of different bacteria one often needs to inspect more than just the first 2 or 3 discriminant functions. In our strategy depicted above (Fig. 1) we reduce this problem by constructing a dendrogram based on DFA output using hierarchical cluster analysis (HCA). In this process, we can further summarize the multivariate outputs from DFA by taking the Euclidean distance between the a-priori group centers in PC-DFA space to construct a similarity matrix. These distance measures can then be processed by an agglomerative clustering algorithm to produce a dendrogram [37]. This can provide more lucid results than those obtained from plotting discriminant function scores as ordination plots.

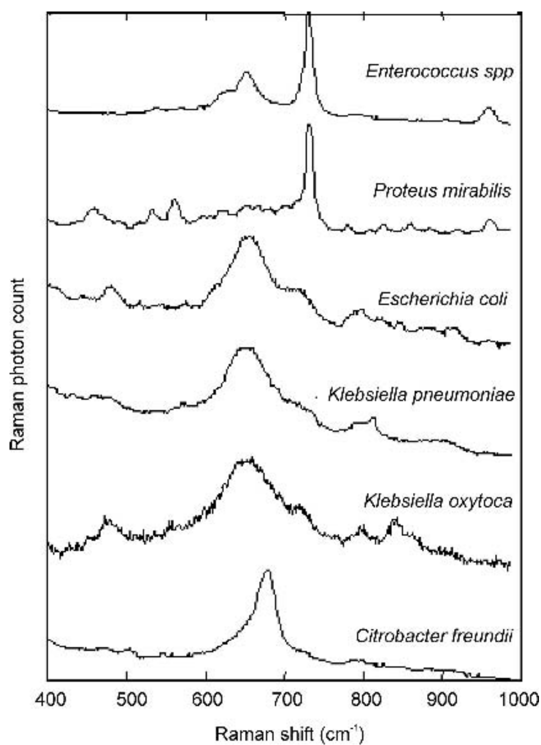

Fig. 2. Typical unprocessed SERS spectra showing an example from each species of UTI isolates studied. Each spectrum took 10 s to collect and the counts were in the thousands

4 Identification of Micro-Organisms Using SERS

It is possible to acquire extremely good SERS data from bacterial cells using minimal preparation. In the examples shown in Fig. 2, which are SERS spectra of urinary tract infection (UTI) isolates, using aggregated citrate-reduced colloid [41] strong spectral signals were obtained in only 10 s with 785 nm excitation and $\sim$ 2 mW laser power at the sample. Importantly, these spectral fingerprints also have clear characteristic differences, which show great promise for application to the problem of bacterial characterization. In fact, using SERS fingerprints from a large number of UTI clinical isolates (courtesy of Bronglais Hospital, Aberystwyth), it has been possible to define a categorical model to discriminate between the major causative organisms of UTI [26].

One of the major benefits of taking a spectroscopic approach to bacterial characterization is that there is the potential to classify the microbial isolate to the subspecies or strain level. This is not possible using conventional biochemical methods; however, with the sensitivity of SERS it is possible

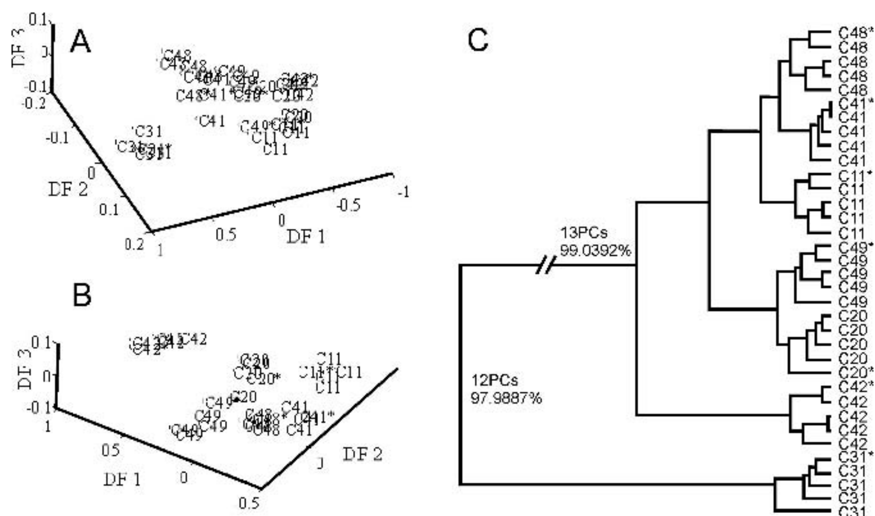

Fig. 3. (**a**) An ordination plot characterizing 7 clinical isolates of *Escherichia coli* from urinary tract infections. The items highlighted in *bold* with an asterisk are the validation samples. Isolate C31 is massively different from the other isolates, therefore C31 was extracted and the remaining data analyzed again. This result (**b**) clearly demonstrates that SERS can be applied to discrimination of microorganisms at the strain level. (**c**) A composite dendrogram generated by HCA using the combined PC-DFA space from the training and validation replicates used to generate the ordination plots in (**a**) and (**b**). This representation of the results shows how the validation replicates fall tightly within the clusters formed from the training data

to distinguish such closely related strains based on the organism phenotype. For isolates of *Escherichia coli*, obtained from patients with UTI, subspecies classification is shown in Fig. 3. This takes a stepwise approach to classification modeling, that is detailed fully in [26], and essentially involves removing groups from the analysis that are so clearly different that they prevent clear separation of other classes.

5 Monitoring Industrial Bioprocesses

The ability to control a bioprocess is paramount for product yield optimization, and it is imperative that the *concentration* of the fermentation product is assessed accurately [42]. Raman spectroscopy has historically been used to monitor the reaction of chemical processes and it was only a matter of time before this approach was used to analyze bioprocesses. Whilst chemical reactions involve few chemical species and vibrational modes more readily attributable to substrates and products, biological processes are more complex and rarely are vibrations directly related to bioproduct formation. In

addition, for low-concentration products in a complex milieu the signal from the analyte of interest is often masked because of the weakness of the Raman effect, and this has perhaps discouraged analyzts from taking this approach. However, SERS provides an opportunity for off-line and at-line monitoring of bioprocesses that is both sensitive for the detection and quantification of low yield primary and secondary metabolites. Clearly SERS would not be performed on-line due to the poisonous nature of the silver colloid on the microbial process.

Our initial work has concentrated on the production of penicillin. In this industrially important bioprocess, *Penicillium chrysogenum* fermentations produce penicillin G as the major secondary metabolite of commercial interest. In conventional Raman spectroscopy using 785 nm excitation, the Raman spectra of penicillin G at high concentrations are dominated by the resonance enhancement of the aromatic ring vibration at $1005\,\mathrm{cm}^{-1}$. By contrast, SERS penicillin G spectra, also collected at 785 nm, contained a greater number of peaks, with a much-improved signal-to-noise ratio and with significantly reduced fluorescence. With respect to the quantification of penicillin G it was shown that Raman spectroscopy could be used to quantify the amount of penicillin present in broths when relatively high levels of penicillin were analyzed ($> 50\,\mathrm{mM}$). By contrast, using simple integration under SERS-enhanced peaks excellent quantification of penicillin G from considerably lower concentrations of the antibiotic were achieved.

6 Gene-Function Analysis

Whole genome sequencing has shown that there are many genes for which the function is unknown. There is thus a requirement to assign functions to these orphan genes and one approach to this is through "guilt by association" [43, 44]. By analyzing knockouts of a known function together with those of an *un*known function, cluster analysis on spectroscopic measurements can be used to infer metabolism classes based on the distances between groups [45]. The approach to this problem in terms of data analysis is the same as for bacterial characterization; the main differences are in the type of sample under analysis and the way in which the cluster analyzes are interpreted.

The "metabolic footprint" is a measure of the metabolites in the extracellular material, such as spent culture media, urine or blood [43, 44]. Spectroscopic fingerprints of such samples are seen as the best method for determining gene function by an inductive approach (i.e., mining data for knowledge, rather than testing a hypothesis) [46, 47, 48, 49]. However, detecting small quantitative or qualitative changes in growth media requires sensitive instrumentation, and therefore mass-spectral techniques have primarily been the method of choice. Consequently, sample preparation and spectral collection times are many minutes, which can be limiting when large libraries of knockout mutants need to be profiled. SERS is potentially a more

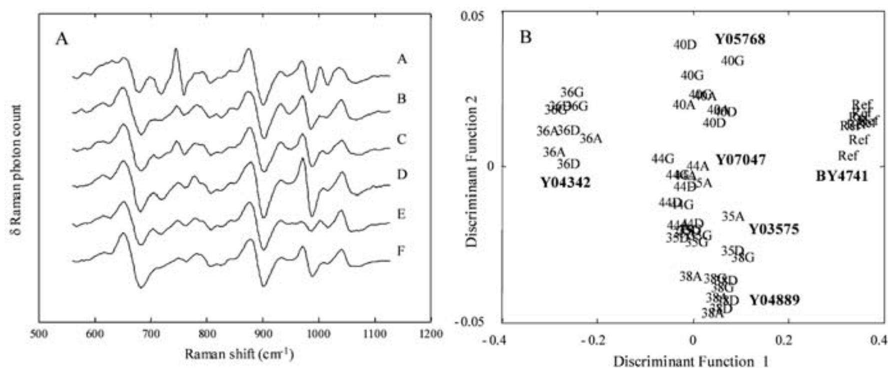

Fig. 4. (**A**) First-derivative SERS spectra (785 nm, ∼ 8 mW laser power at the sample) of depleted haploid yeast-cell culture media from (**A**) BY4741 Wild type; (**B**) Y07047; (**C**) Y03575; (**D**) YO5768; (**E**) Y04889; (**F**) Y04342. (**B**) PC-DFA model using SERS spectra, showing the haploid yeast reference strain and 5 haploid mutant strains resolved into separate clusters

rapid "holistic" fingerprinting method than MS. The low limit of detection for SERS measurements also makes the method an ideal candidate for detecting small differences in extracellular metabolites between gene knockout mutants.

Table 1. Haploid yeast knockout mutant strains used in a SERS metabolic footprinting study

Experiment Ref.	ID	Metabolism descriptor
Reference	BY4741	Wild type
35 A, 35D, 35G	Y03575	Zinc-finger transcription factor, controls expression of ADH2, peroxisomal, ethanol, glycerol and fatty acid genes
36 A, 36D, 36G	Y04342	Broad-specificity amino-acid permease, high-affinity glutamine permease
38 A, 38D, 38G	Y04889	Iron homeostasis
40 A, 40D, 40G	Y05768	Involved in manganese homeostasis
44 A, 44D, 44G	Y07047	A transcriptional repressor for allantoin and GABA catabolic genes, a negative regulator of multiple nitrogen catabolic genes

As a preliminary example, a small subset of the 7000 eukaryotic *Saccharomyces cerevesiae* single-gene knockout mutants (courtesy of Prof. Stephen G. Oliver, The University of Manchester) are listed in Table 1 and these were analyzed by SERS. The putative metabolism classes for these mutants suggest that the deleted genes relate to a broad range of biochemical pathways

within the cell. In Fig. 4a examples of first-derivative SERS spectra acquired from the metabolic footprint of these samples are shown, with a total spectral integration time of only 1 min for each sample.

Whilst some obvious quantitative differences can be seen between these spectral fingerprints, for the discovery of the relationships between the wild type and the various gene knockouts analyzed, it is necessary to take a multivariate approach using cluster analysis. PC-DFA clearly separates these mutants from wild type and separate groups were recovered for each of the knockouts (Fig. 4b). This preliminary result demonstrates the potential for SERS to be used as a rapid screening technique in gene-function analysis and will be explored more fully in the future.

7 Concluding Remarks

Raman and SERS spectroscopy clearly presents itself as a highly versatile tool that provides complex chemical fingerprints from a wide range of biological materials. For microbial investigations, these generally require multivariate cluster analysis, or more advanced machine-learning techniques [50], for clear microbial characterization, in terms of elucidating the relationship between bacteria, and for robust unequivocal identification of infectious agents. We have recently demonstrated that SERS has the exquisite sensitivity required for the classification of micro-organisms and is reproducible enough for the identification of a wide variety of bacteria [26, 27, 31]. It is noteworthy that in our studies we have not just used the "stare and compare" analysis adopted by others, but have employed multivariate methods that show reproducibility across the full spectral range collected. Finally, we have also demonstrated single bacterial cell analysis using SERS [27] and that this approach is reproducible enough to be used for the quantification of microbial fermentations [51].

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