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**Chemistry**

**Detection of Bioaerosols by Surface-Enhanced Raman Spectroscopy**

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**Declaration of Authorship**

I, Adam Lister, declare that this thesis and the work presented in it are my own, and has been generated by me as a result of my own original research.

**Detection of Bioaerosols by Surface-Enhanced Raman Spectroscopy**

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this university.
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this university, this has been clearly stated.
3. Where I have consulted the published work of others, this is always clearly attributed.
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
5. I have acknowledged all main sources of help.
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I contributed myself.

Signed:

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**Definitions and Abbreviations**

|  |  |
| --- | --- |
| (M/B)CARS | (Multiplex/Broadband) Coherent Anti-Stokes Raman Spectroscopy |
| (4-)MBA | 4-mercaptobenzoic acid |
| (Au)NPs | (Gold) Nanoparticles |
| (D)R/XNA | (Deoxy)ribo/xenonucleic acid |
| BTX | Botulinum Toxin |
| BWA | Biological Warfare Agent |
| CDC | Centre for Disease Control |
| CWA | Chemical Warfare Agent |
| DPA | 2,6-pyridinedicarboxylic acid / dipicolinic acid |
| GA | Tabun |
| GB | Sarin |
| GD | Soman |
| GF | Cyclosarin |
| HCA | Hierarchical Cluster Analysis |
| HD | Sulfur mustard gas |
| LDA | Linear Discriminant Analysis |
| LoD/Q | Limit of Detection/Quantification |
| NIR | Near Infrared |
| PA | Anthrax Protective Antigen |
| PBS | Phosphate buffered solution |
| PCA | Principle component analysis |
| PhSH | Thiophenol |
| RA/BC | Ricin A/B Chain |
| RDX | Research Department Explosive/Royal Demolition Explosive |
| RS | Spontaneous Raman Spectroscopy |
| SERS | Surface-Enhanced Raman Spectroscopy |
| TNT | Trinitrotoluene |
| UV | Ultra-violet |
| VX | Venemous Agent X |

**1 Introduction**

* 1. **Aims**

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* 1. **Enhancement of Raman Scattering**

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* + 1. **SERS Enhancement**

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* 1. **Raman Scattering Techniques for Homeland Security Applications**

# Introduction

**1.4.1.1 Introduction to threat agent detection.**

In the last twenty years, the world has seen a reminder that the way we think about war and threats to our nations can take many forms. Incidents like the 1995 sarin gas attack on the Tokyo subway, or the ricin-containing letters from April 2003 have shown that individuals and groups are capable of securing these agents and are willing to deploy them against civilians and important figures to further their agendas. It is perhaps fair to say that the attack on the World Trade Centre in 2001 catalysed a renewed global vigilance against terrorism on the homeland, whilst the use of Sarin gas in Syria (2013) once again provided a stark reminder that explosives are not the only substances against which nations must remain vigilant.

In response to these threats, there has been a surge of interest from governments, universities and private sector defence companies into ways of detecting these threats before they can be deployed or in recognising the nature of a threat agent after it has been released. This has included techniques such as vibrational spectroscopy, fluorescence and UV-Visible absorption spectroscopy, turn off and turn on detectors, as well as techniques such as ion mobility spectrometry and gas chromatography. Many of these techniques suffer from issues, however. For example, low specificity can be a problem, leading to false positive results for threat agents, whilst other techniques can require lengthy analysis times, which is clearly undesirable when dealing with potential exposure to chemicals and biological materials which are toxic in minute quantities. Examples of the kinds of agents with which research groups are particularly interested in detecting at included in Table **1**.

In the following sections, this article will discuss the application of Raman spectroscopy to the detection of biological and chemical threat agents, including a description of the science that underpins the technique, and a discussion of the strengths and weaknesses of the techniques. Additionally, techniques that have been developed from spontaneous Raman spectroscopy are discussed, including discussion of the applications of those techniques in the literature.

|  |  |  |  |
| --- | --- | --- | --- |
| ***Table 1:*** List of the CDC’s ’Dirty Dozen’ biological agents, and chemical agents of importance[1]. | | | |
| **Chemical** | **Biological toxin** | **Bacteria** | **Virus** |
| Nerve agents (Sarin, VX) | Botulinum toxin | *Bacillus anthracis* | Variola Major (Smallpox) |
| Blood agents (Cyanide) | Staphylococcus enterotoxin B | *Francisella tularensis* | Haemhorrhagic fevers (Ebola, Marburg) |
| Choking agents (Chlorine gas, phosgene) | Ricin | *Yersinia Pestis* | Viral encephalitis (VEE) |
| Vesicants (Sulphur mustard) |  | *Burkholderia mallei* |  |
|  |  | *Burkholderia pseudomallei* |  |
|  |  | *Brucella melitensis* |  |
|  |  | *Brucella abortus* |  |
|  |  | *Coxiella burnetii* |  |

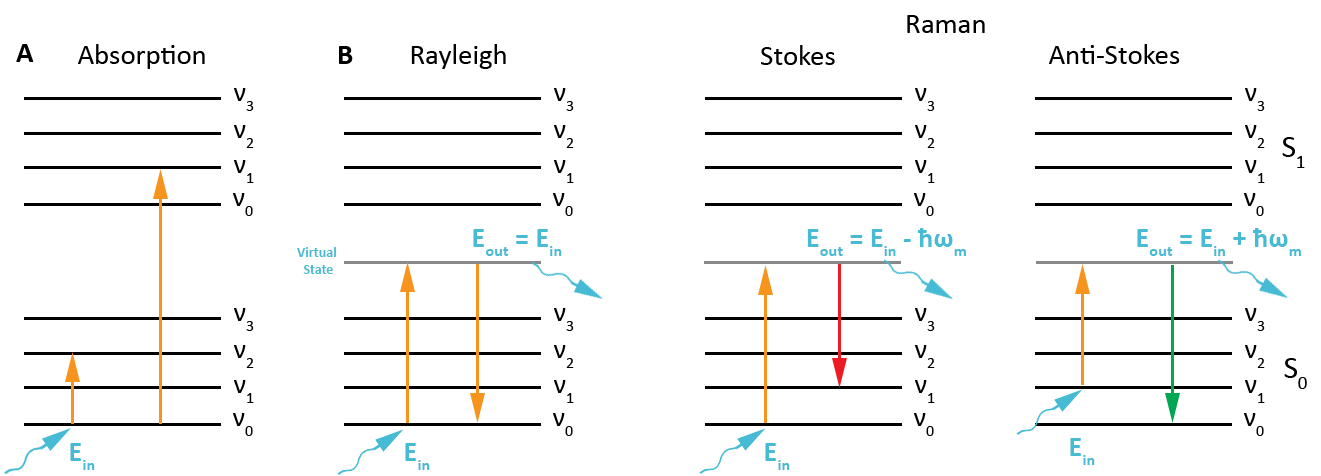
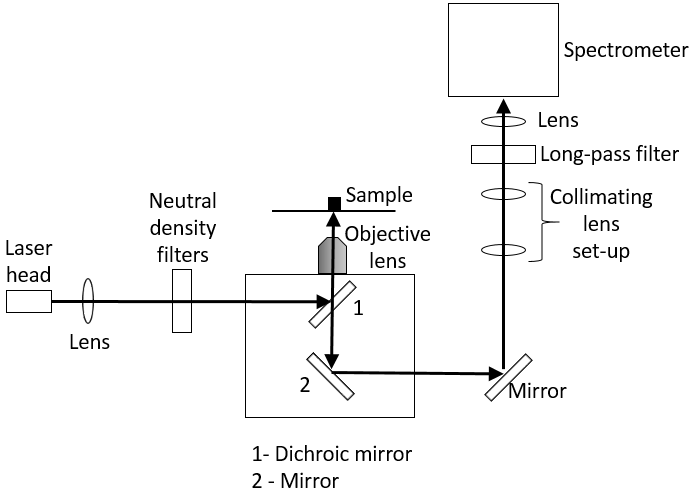
# 1.4.2 Theory of Raman spectroscopy

# 1.4.2.1 Raman spectroscopy

Raman spectroscopy (RS) is a technique that relies on the scattering of light from the molecules of a sample. In this article, spontaneous RS is regarded as being the collection of laser light that has been inelastically scattered from a sample without any modification to the sample, incident beam of laser light, or the signal from the scattered light. When light interacts with matter, the photons can either be absorbed or scattered back from the sample. For absorption to occur, the energy of the incident photon must match the difference in energy levels between its current state, which is often the ground state, *S0*, and an excited electronic level (*S1*, *S2*, …) or excited vibrational level (v*1*, v*2*, …). Should the photon match the energy of one of these transitions, it will transfer its energy to the molecule. This energy can later be lost as the molecule relaxes to a lower energy state (possibly involving multiple intermediate states). The process of energy loss that results in the generation of a photon is called emission. Absorption of light is used as the underpinning principle of infrared (IR) spectroscopy, and is represented in a Jablonski diagram below (Figure **1A**) but is dealt with no further in this article.

Scattering is a different process, and is the near-simultaneous absorption and emission of a photon of light. There are two distinct types of scattering that may occur: elastic and inelastic. These types of scattering are depicted as a Jablonski diagram below (Figure **1B**).

Rayleigh, also known as elastic, scattering is the scattering process by which a photon of light is scattered from the molecule, leaving with the same energy as the incident photon, and the molecule remains in the same energetic state. By contrast, Raman spectroscopy is one of many processes by which the emitted photon leaves the molecule with an energy that is measurably different from the incident photon.

Raman scattering can then be divided into two types: Stokes and anti-Stokes scattering. These types correspond to scattered photon leaving the molecule with lower energy than the incident photon (Stokes scattering), or a higher energy than the incident photon (anti-Stokes scattering). This effect was first theorised by the Austrian physicist Smekal in 1923[2], but it was not until the work of C. V. Raman in 1928 that the first experimental observations of the phenomenon were made[3]. In his early work, Raman had to generate his monochromatic light source from sunlight using a mirror, filters and lenses, but the invention of the laser has allowed scientists to generate highly monochromatic light in between the near ultraviolet (UV) and near infrared (NIR) regions. Since then, the field has expanded rapidly into a powerful analytical technique for probing the vibrational states of a molecule. As such, it can act as a “fingerprint” for the molecule, and has been used in the detection and identification of materials in a variety of applications. A typical setup for spontaneous Raman spectroscopy is shown in Figure **2**.

**Figure 1** Simplified Jablonski diagram showing the electronic states (S0 and S1) and vibrational energy levels (v0-3) of a molecule. Blue arrows represent incident light (EIn) and scattered light (Eout). Orange arrows represent excitations of the molecule, and red and green lines represent relaxations of excited states resulting in loss and gain of energy, respectively. (A) Depicts the absorption process, wherein the incident photon excites vibrational or electronic changes as might be seen from IR and UV/Visible light, respectively. (B) Depicts the Rayleigh and Raman scattering processes, wherein a photon is simultaneously absorbed and emitted from the molecule. In Rayleigh scattering, the photon excites the molecule to a temporary virtual state, and the molecule relaxes back into the same energy level from which it came. In Raman scattering, the photon excites the molecule into a temporary virtual state, but the molecule then relaxes back into either a higher (Stokes) or lower (Anti-Stokes) energetic state than it started.

**Figure 2** Simplified diagram of a conventional Raman spectrometer, configured in the 180° geometry. This backscattered geometry is especially common in microprobe experiments, whilst a 90-degree configuration is used frequently for macromode work.

Spontaneous RS has many advantages as a technique. One of these advantages that is particular useful in many applications is that Raman spectroscopy requires little-to-no sample preparation, whereas techniques will often require the labelling of biological or non-fluorescent samples with a fluorophore.

The technique goes so far as to permit many samples to be analysed within glass or plastic containers, which is a considerable boon in forensics or hazardous material detection[4]. Further, RS can be used on samples in all states of matter (solid, liquid, and gas) through the use of specialised vessels that are designed to contain the analyte. Another considerable boon of Raman spectroscopy is that, unlike IR spectroscopy, it is water insensitive[5, 6]. This property makes Raman spectroscopy very useful for biological detection applications and in water monitoring, where part per million levels of detection for Raman-active salts have been demonstrated[7].

Despite the positive attributes mentioned above, Raman is not without weaknesses. The primary downfall of spontaneous RS is that the Raman scattering process is very weak. It is estimated that Raman scattering has a quantum yield of around 1 in every 106 of the incident photons[8]. This poor cross section for spontaneous RS leads to an inherent insensitivity unless modifications are made to the technique to enhance the degree of scattering. That said, modern optics, lasers and detectors have improved the detecting power of Raman considerably, allowing the technique to move into the domain of portable applications instead of being confined to the laboratory bench[4].

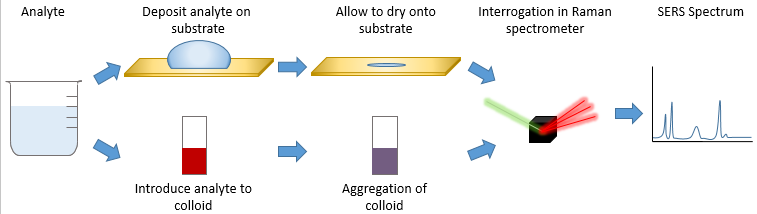
Another key problem with RS is fluorescence that may arise from unintentional electronic excitation of the molecule caused by the laser. It should be noted that this fluorescence may not arise from the target analyte, and may arise from sources like impurities or the environment[9]. Fluorescence can be mitigated or eliminated by experimental choices, however. For example, the choice to analyse the anti-Stokes spectrum as opposed to the Stokes spectrum will avoid the problem of fluorescence entirely, as fluorescence must be emitted at a lower energy than the incident light. However, it should be noted that the anti-Stokes spectrum is many times weaker than the Stokes spectrum at room temperature. Selection of a laser with a longer wavelength, such as a NIR beam, will also reduce fluorescence in the spectrum but result in a drop in the intensity of scattered light, as Raman scattering is dependent on the wavelength of the incident light (λ-4). Despite this drop in signal intensity, it is often possible to see the features of the spectrum thanks to the reduction or elimination of fluorescence from the collected spectrum.

Given the relationship between energy of the incident photon and the intensity of Raman scattering, it may be tempting to use near UV lasers for all analyses, but these beams can cause photodegradation of some samples. While this can be mitigated by methods like spinning the sample, UV lasers remain a poor choice for some samples[10].

**1.4.2.2 Surface-enhanced Raman spectroscopy**

Surface-enhanced Raman spectroscopy (SERS) is a modification of the spontaneous RS technique that was first observed at the University of Southampton in 1973 by Fleischmann et al[11]. SERS is a surface-sensitive technique in which the Raman scattering from the sample is enhanced by adsorption of the analyte onto nanostructured surfaces such as electrochemically roughened noble metal, or nanoparticles. The enhancement factor observed in SERS can be up to 1010 to 1011[12,13], which has been demonstrated to be sufficient for single molecule detection[12,14]. The precise mechanism by which the SERS enhancement occurs is a topic of ongoing debate, but two key components are believed to contribute. One of these theories is a theory of electromagnetic enhancement, first proposed by Jeanmaire and VanDuyne in 1977[15]. The theory states that the laser induces the electrons on the surface of the nanostructured substrate to resonate together in a surface plasmon, which generates an oscillating electric field that couples into the Raman scattering process analyte molecules that are confined to the surface by adsorption. This increases the quantum yield of the process, providing the observed enhancement of Raman scattering. This particular mechanism is believed to account for the majority of the observed enhancement. A charge transfer mechanism has also been put forward, which requires that the analyte form a chemical bond to the substrate. Continuing research supports the electromagnetic theory[16, 17], though it is probable that both theories contribute to the overall effect to some degree. Figure **3** shows a schematic representation of a typical SERS experiment.

SERS signal enhancement from an individual nanoparticle is not particularly significant. The most significant enhancements to the signal come from so-called “hot spots”. A hot spot is a region of intense local field enhancement that occurs in nanostructured metals, such as between two nanoparticles that are within close proximity to one another.

As mentioned before, the major advantage of SERS is the considerable boost to the efficiency of Raman scattering events that occurs, making the technique far more sensitive than spontaneous RS. Another advantage over spontaneous RS is that the interaction of the analyte with the substrate can provide an alternative path through which the molecule can relax from its excited state, quenching fluorescence[18].

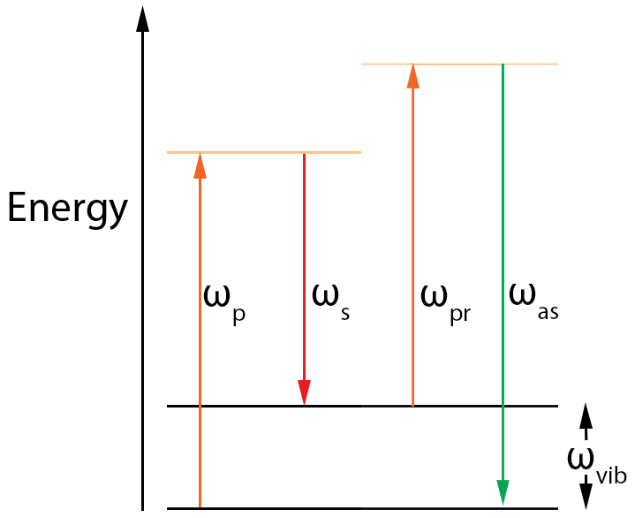
**Figure 3** Diagrammatic representation of two primary means of undertaking a SERS experiment. Top – deposition of analyte onto a substrate such as Klarite. Bottom – Mixing with colloidal nanoparticles and aggregation with a material such as salt.

SERS is, however, not without disadvantages. Reproducibility can be problematic owing to the difficulties in making sure that substrates are the same between batches and analyses. This issue is particularly notable in substrates such as solutions of bare nanoparticles, where aggregation between particles and interaction with the analyte cannot be properly controlled. Similarly, stability of substrates can also be a source of irreproducibility, as aggregation and oxidation of substrates can lead to variability between analyses that is difficult to account for. Research into novel substrates fabricated by methods such as nanosphere and electron-beam lithography[19], silver or gold coated microspheres[20], metal-polymer nanocomposites[21], has helped mitigate these problems to some degree.

The other major issue with SERS is that the signal enhancement requires being in close proximity to the local surface plasmon. The enhancement drops off rapidly as a function of distance, and requires the analyte to be within around 10nm of the surface to be effective.

**1.4.2.3 Coherent Anti-Stokes Raman Spectroscopy**

Coherent Anti-Stokes Raman Spectroscopy (CARS) is a four-wave technique that utilises multiple photons to produce a coherent input that probes the vibrations of a molecule and produces a signal. The technique is a third order non-linear optical process, and uses three beams to produce a signal that is several orders of magnitude stronger than would arise from conventional Raman spectroscopy[22].

The beams used in CARS are the pump beam (*ωp*), the Stokes beam (*ωs*), and the probe beam (*ωpr*). The output is the anti-Stokes beam (*ωas*). In CARS, the pump beam impacts the sample, exciting the sample to a virtual state and is brought back down to a real higher vibrational energy state by the Stokes beam. When the difference in energy between the two energy levels approaches the energy of a Raman active vibration, *ωvib* (when *ωp* – *ωs* = *ωvib*), the vibration is much more active. This vigorous vibration is then probed by the probe beam and excited to a higher energy level, and scatters with an energy of *ωas* = *ωpr* + *ωp* – *ωs*. The probe beam is typically at the same energy as the pump beam, thus *ωas* = 2*ωpr* – *ωs*[22, 23].

**Figure 4** Energy level diagram for the CARS process.

Given that CARS relies on the energy difference, *ωvib*, which is specific to particular Raman active modes in a molecule, this technique typically only probes one Raman active mode at a time. This can be advantageous for applications such as mapping of cells to image protein or lipid distributions. It should also be noted that multiplex/broadband CARS (M/BCARS) is also possible, and allows CARS imaging of multiple vibrational modes simultaneously[24-28]. Additionally, the frequency of the Stokes beam can be swept across a range to obtain a spectrum of intensity of *ωas* against *ωpr* – *ωs*[23]. An energy level diagram for the CARS process is depicted in Figure **4**.

As with SERS, CARS has the advantage that the signals generated are far stronger than conventional Raman spectroscopy. It also inherently bypasses issues with fluorescence, as the anti-Stokes beam is blue shifted from the incident light whilst the fluorescent light is red-shifted.

As with all techniques, CARS also has disadvantages. One such disadvantage is that CARS setups are expensive to put together, requiring more than one laser, and an array of other optical components to phase match the incident beams, and to spatially and temporally overlap them on the sample. One-laser setups have been proposed[29]. Additionally, CARS in solution-based analyses can display a significant background that can be difficult to suppress[22].

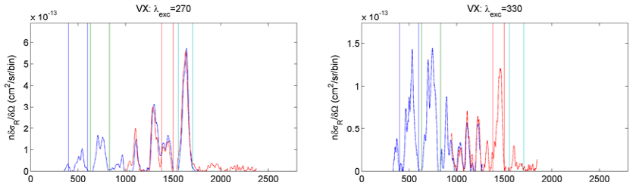
Table **2** summarises the various advantages and disadvantages of the three techniques discussed above.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Table 2:** Summary of basic advantages and disadvantages of three common Raman spectroscopy techniques. | Disadvantages | Fluorescence can dominate spectra.  Scattering events are rare, causing weak signals. | Signal enhancement relies on close proximity to metal surface.  Reproducibility of results between batches of substrate or different kinds of substrate can be problematic  Sensor degradation over time can alter signals. | Suppression of non-resonant background can be non-trivial.  CARS set-ups are expensive to implement and require precise alignments. |
| Advantages | Little or no sample preparation.  Provides a molecular fingerprint of the analyte.  Water insensitive.  Can be used in conjunction with resonance Raman for greater enhancements. | Enhanced scattering provides much greater signal intensity.  Can be used in conjunction with resonance Raman for greater enhancements.  Adsorption to noble metal quenches fluorescence. | Good for mapping/imaging single vibrational modes.  Signal enhancement over conventional Raman spectroscopy.  Can use in conjunction with resonance and/or surface-enhanced Raman for even greater signal.  CARS signal is blue shifted, which avoids fluorescence. |
| Technique | Conventional Raman Spectroscopy | Surface-Enhanced Raman Spectroscopy | Coherent Anti-Stokes Raman Spectroscopy |

* + 1. **Applications of Raman scattering-based techniques to homeland security**
       1. **Non-biological molecule detection**

Small molecules of non-biological origin are of intense interest in homeland security. Explosive chemicals such as TNT and RDX, as well as chemical warfare agents (CWAs) such as cyanide, phosgene, mustard gas, and the famous V and G series nerve agents pose a considerable potential threat to both civilians and military personnel across the globe. As a result, considerable effort has been put into detecting them to help minimise risk of exposure and subsequent harm to individuals. Raman spectroscopy has been used extensively to investigate the detection of these molecules in both air and water through their characteristic vibrational modes. Owing to their extreme toxicity, much of the work on this field has been conducted on simulant chemicals with similar molecular features, such as organophosphates, or on the hydrolysis products of these materials[27, 28, 30-41]. It is worth noting that many of simulants used are the same as the hydrolysis products. As such, they remain valuable detection targets in their own right, serving as markers of the release of chemical agents. Despite the hazards, work on live agents has also been conducted[30-33, 42-47].

One of the primary issues with the detection of these agents is the need to reach extremely low limits of detection, often in the low ppb range. Whilst some agents, such as cyanide, are easily detectable, many have extremely poor Raman scattering cross-sections associated with their vibrational modes. As an example, the G series agents, GA, GB, GD, and GF have been experimentally determined to have cross sections ranging between 1x10-30 and 1.0251x10-27 cm2/sr/molecule associated with their various vibrational modes when excited in the UV region[42]. In a bid to help mitigate this poor scattering efficiency, several studies have attempted to exploit the relationship between shorter wavelengths and increased Raman scattering by working with excitation sources in the UV range[31, 33, 39, 42].

It has also been found that for V series agents, moving deeper into the UV can reveal additional spectral features. In particular, Kullander *et al*. found that excitation at 270nm causes a large peak to appear at 1650cm-1 and becomes the strongest spectral peak. This feature is absent in the spectrum taken at 330nm (Figure **6**). No such changes were found for sulphur mustard (HD) and Tabun (GA)[33].

**Figure 5** Raman spectra of VX at 270nm and 330nm, respectively. The appearance of a dominant peak at 1650cm-1 can clearly be seen. Taken from Kullander et al. 2016.

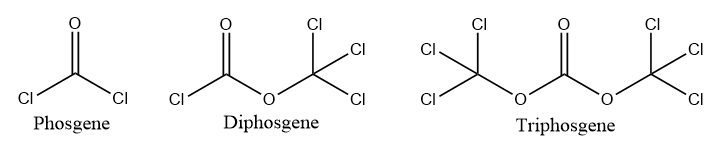
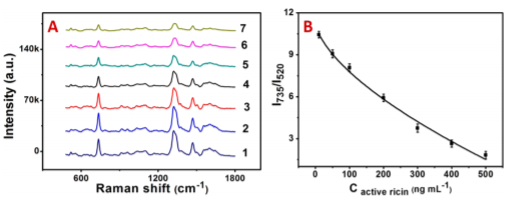
To help overcome the issue of poor Raman scattering cross-sections of many of these analytes, SERS and CARS have been investigated as detection methodologies, owing to their large degree of signal enhancement when compared to spontaneous Raman spectroscopy. This has included several studies on live agents[30, 32, 45, 47, 48] and simulants and/or hydrolysis products[30, 32, 34-37, 40, 41, 45, 48]. It has been noted that pH can play an important role in SERS measurements, due to the protonation or deprotonation of the analyte affecting interaction with the SERS substrate. This effect can hinder quantitation efforts, or prevent detection all together[32].

Recently, Gao *et al* developed a methodology for the detection of phosgene and its related compounds, di- and triphosgene. Phosgene, a choking agent, is an important chemical to industry, as are the related chemicals diphosgene and triphosgene. Phosgene and diphosgene are extremely toxic. Triphosgene offers good stability and similar reactivity to phosgene, but can decompose to phosgene. Detection of these chemicals is, therefore, important for industrial monitoring and homeland security. However, phosgene has no strong Raman scattering, and decomposes readily in aqueous solution[47]. The structures of these compounds are shown in Figure **7**. To circumvent the issue of poor Raman scattering, the group developed a solution based on utilising SERS to indirectly monitor the presence of phosgene via a chemical transformation method. They utilised the stoichiometric conversion of phosgene and diphosgene into iodine in the presence of potassium iodide according to the following reactions:

**COCl2 + 2 KI → 2 KCl + I2 + CO**

**C2O2Cl4 + 4 KI → 4 KCl + 2 I2 + CO**

The resulting I2 was then detected in solution by SERS with limits of LoDs in the low micrograms per litre range. The method was validated on samples of diphosgene in air[47].

Brady *et al* used CARS for studies into the detection of explosives precursors and simulants of chemical warfare agents. In these works, MCARS was used to obtain spectra of agents within millisecond sampling times using uncooled USB spectrometers. This represents a significant improvement over spontaneous Raman, which requires seconds of acquisition to acquire workable spectra when using cooled and intensified spectrometers. Their set-up used a femtosecond pulsed Ti:Sapphire laser that was split into two portions. One of these was used to generate a super-continuum that could drive all the vibrational modes of the analytes simultaneously, allowing them to generate the entire spectrum of the sample at once[27, 28]. Additionally, one of these studies used PCA to classify the spectra of their analytes, showing that they could be clustered together with a good degree of fidelity, which is important for automatic identification of unknowns[28].

**Figure 6** Structures of all three phosgene agents.

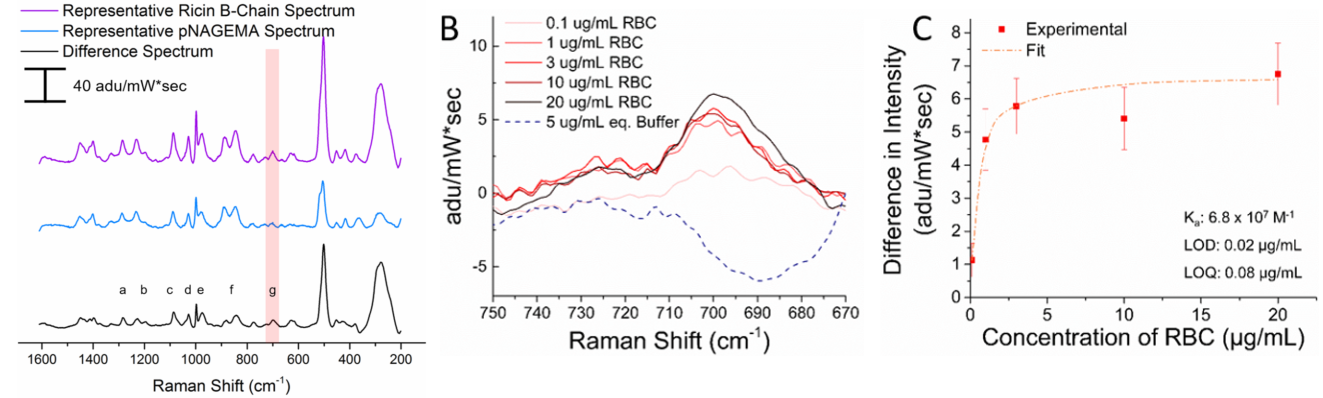
**Figure 7** (A) Signal attenuation of adenine at differing concentrations of intact ricin. (B) Calibration curve for the determination of ricin concentration. Taken from Tang et al. 2016

* + - 1. **Biomarkers and biomolecules**

Table 1 shows that biological warfare agents (BWAs) can be either whole pathogens, or toxins such as the toxic proteins, ricin and botulinum toxin (BTX). Raman spectroscopy has been able to detect proteins for some time[41, 49, 50], and its application to the detection of ricin has been demonstrated in numerous studies that have used the harmless A chain (RAC)[51] or B chain (RBC)[1, 51-54], as well as whole ricin[51, 55, 56]. Detection in complex media such as might be found in diagnostic, or in food that had been maliciously contaminated[52, 55, 57, 58], has also been demonstrated by multiple groups.

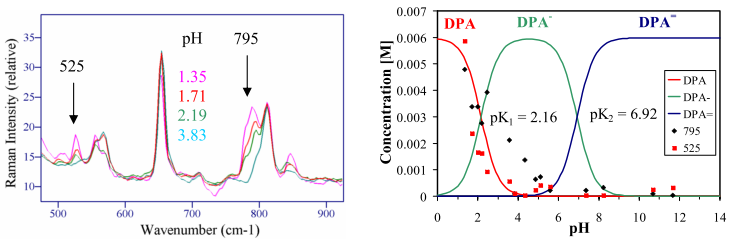
When detecting such biological samples in complex media, it is often useful to be able to selectively bind the target molecule to the sensor substrate, or otherwise isolate it from its environment to remove spectral contributions from other chemicals in the mixture. One method of achieving this is using aptamers. An aptamer is a molecule that binds to a specific target molecule. There are two types of aptamer: peptide aptamers, made of one or more peptide domains; oligonucleotide aptamers, which consist of short strands of DNA, RNA, or XNA. They are similar in concept to antibodies, but offer several advantages. First, peptide aptamer structures are more stable under variations in temperature, pH and ionic strength. Second, they are easily synthesised in bulk, which makes them better suited for high throughput systems. Lastly, their smaller size can potentially allow several peptides to bind to different epitopes on a single protein[59]. Aptamers have been studied for detection of bioweapons in numerous studies. As examples, aptamers have been tested on RBC[54, 57], Anthrax Protective Antigen (PA)[60], Anthrax Lethal Factor[61], and *bacillus* bacteria[62, 63].

Tang *et al*. developed a SERS chip for the detection of ricin. The chip was comprised of a single-stranded oligonucleotide functionalised gold nanoparticles on a silicon wafer. On mixing with whole ricin, the protein selectively depurinated the nucleotide by hydrolysing the adenine from its structure. The group were able to detect and quantity the amount of ricin present by measuring the signal attenuation of peaks associated with adenine (Figure **7**). The chip was then tested to on ricin mixed into foods and biological samples, and found to follow the calibration curse with relative errors of less than 7.6%. Additionally, the sensors showed little change in spectra after being stored for three months at 4°C[55].

The biological activity of ricin was also successfully exploited for its detection by Szlag *et al*. RBC is a lectin that binds to extracellular glycoproteins to perform its function. To exploit this interaction, the group anchored glycopolymer oligomers to a gold film-over-nanosphere SERS substrate. The oligomer coating acted as a capture layer to bring RBC into the enhancing field. Binding of ricin caused signal enhancement of peaks, visible from the difference spectrum. This data is shown in Figure **8**. Using this method, detection of ricin in fruit juices was achieved with a quantitative range and a LoD of 20ng/mL, which is comparable to other studies[52].

**Figure 8** (A) Difference spectrum showing changes to the oligomer spectrum due to RBC binding. (B) Difference spectra at varying concentrations of RBC in the 670-750cm-1 region. (c) Quantification of the increased amplitude at 700cm-1 with increasing concentrations. Taken from Szlag et al. 2016.

SERS Extraction and detection of ricin from paper has also been achieved53. Zheng *et al* developed a protocol for the screening of papers for ricin contamination, investigating two extraction methods for RBC on three types of paper. The first method was non-destructive, and involved pipetting 1mL of phosphate buffered solution (PBS) onto the area between 3 and 30 times, then mixing this liquid with silver dendrites. The second method was more destructive, and involved cutting the region out of the paper and mixing this directly with PBS and silver dendrites. In both cases, after the mixing, the resulting solution was centrifuged and 10µL of the precipitate from the bottom of the tube was dried onto glass and analysed via SERS. It was found that the extraction efficiencies for method 1 and method 2 were 20.5%/28.0%, 42.5%/60.5%, and 80.0%/90.0% for hydrophilic, envelope, and hydrophobic papers, respectively. Additionally, principal component analysis allowed the group to discriminate the deposit of RBC from the spectra of liquids that might be found on papers, such as coffee, juice, or tea. They also established a limit of detection of 0.044g, which is substantially beneath the toxic dose. This method, therefore, offers good potential as a screening method for ricin on papers, at least in small-scale applications.

Pathogens such as bacteria need not be detected simply as entire organisms, but can also be detected by the presence of a compound that indicates their presence. These compounds, known as biomarkers allow for the detection and/or identification of pathogenic species, and have been studied extensively to design new modalities to help protect people from these harmful materials. A good example of such a system is the detection of spore-forming bacterial species by detection of dipicolinic acid (DPA), which compromises roughly 5-15% of the dry weight of *bacillus* spores[64, 65]. An important consideration for the detection of DPA is that is spectral features depend on pH, due to its diprotic nature (Figure **9**)32. Various studies have reported low levels of detection for pure DPA, including detection in the low micromolar range[66, 67].

**Figure 9** Left: Raman spectra of DPA species at different pH values. Right: Concentrations of different DPA species as a function of pH. Black and red dots represent the intensities of the peaks at 795cm-1 and 525cm-1, respectively. It can be seen that the peak a t 525 cm-1 corresponds strongly to DPA. The peak at 795 cm-1 is less clear, which is attributed to an overlap with a neighbouring band that is not pH dependent. Taken from Farquharson et al. 2004.

SERS has been commonly employed for the detection of DPA in several studies[66-69]. In one such study, Zhang *et al* extracted the DPA from *b. subtilis* spores by sonication and then analysed by SERS. The group found that with a calculated extraction efficiency of around 34%, their limit of detection was 2.6x103 spores within one minute, which is below the infectious dose of *b. anthracis*[68].

Recently, Cheung *et al* also used SERS to achieve a limit of detection of 10-6mol dm-3, which corresponds to approximately 18 spores. This value is two orders of magnitude lower than previous measurements, and substantially below the infectious dose of bacillus anthracis (104 spores)[69]. This low level of detection was achieved by the preparation of micro pillars of copper wire, coated with a superhydrophobic perfluorinated thiol. The top of the wire was cut to reveal bare copper, which then acted as a hydrophilic region onto which meso-droplets of DPA solution and silver colloid could be placed, using a GC syringe with a superhydrophobic needle. This droplet was then probed with a 633nm laser and the SER spectra were used to build a calibration model and calculate the limit of detection. The group concluded that their method could detect DPA at less than an infectious dose of spores, even if the extraction method to liberate the DPA was only around 0.2% effective.

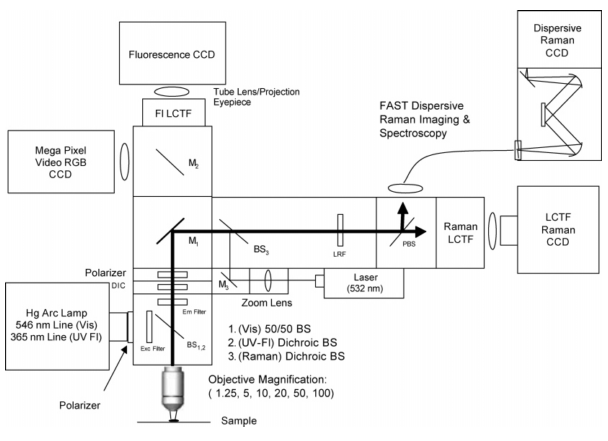
* + - 1. **Pathogen detection**

In addition to the detection of biomarkers and biological toxins, detection of intact pathogens by Raman spectroscopy has also been investigated. Bacterial detection has received a great deal of attention amongst researchers concerned with detecting possible BWAs. Using Raman-based techniques, researchers have studied a variety of biothreat pathogens and their simulants, including *b. anthracis* and related *bacillus* bacteria[1, 6, 34, 41, 56, 62, 63, 65, 68, 70-76], Yersinia pestis and other *yersinia* bacteria[1, 36, 56, 73], *burkholderia mallei*[1, 56], *francisella tularensis*[1, 56, 77], and *brucella abortus and related bacteria*[1, 56, 73]. As with CWAs, work on closely related bacterial species is important outside of just the scope of hazard reduction for researchers. Many of these bacteria are sufficiently close to actual threat agents that, unless a method is sufficiently capable, false positives and false negatives can pose a considerable problem.

Raman spectroscopy allows for the discrimination of different bacterial species, including, differentiation between Gram-positive and Gram-negative bacteria[36, 73], between spores and vegetative cells[73, 78], and potentially between living and dead bacteria[36]. Various studies have also demonstrated the ability of Raman techniques to discriminate between bacteria at the sub-species and strain levels[77-80].

Though conventional RS is a very weak phenomenon, it is possible to obtain whole-organism spectra from single spores or cells[56, 71, 81]. For applications where speed of acquisition may be required, the signal enhancement from SERS offers the possibility of reduced detection times, and has also been shown to yield good signals for single cells and spores[71].

In addition to decreasing acquisition times, SERS offers other advantages over spontaneous RS for biological samples. First, it avoids the innate fluorescence associated with biological samples by offering a fluorescence-quenching pathway. Additionally, SERS spectra are often less congested and more distinctive than spontaneous RS spectra[74]. This is because of the distance dependence of SERS enhancement causing a selective enhancement of only peaks associated with surface features of the bacteria. The resulting distinctiveness suggests that closely related species different more in their surface components than in their cytoplasmic contents.

Raman spectroscopy has been combined with other techniques to help circumvent weaknesses. For example, the weak signal of conventional RS requires longer acquisition times. This can be a detriment to high-throughput analysis, or to real time detection. To mitigate this, two groups have designed systems in which samples are interrogated by white light optical imaging and fluorescence to help locate regions of interest on a sample and determine whether the particulate matter in that region is biotic or abiotic. This particle screening drastically reduces the number of particles that would have to be interrogated, by ensuring that only the signals of biotic particles are measured[56, 82]. An example of such a setup that exploits a common aperture for all the optical components is shown in Figure **10**.

**Figure 10** Schematic diagram of the Raman Chemical Imaging System (RCIS) by Kalasinsky et al. BS – Beam splitter, M – mirror, PBS – polarisation beam splitter, LRF – laser rejection filter, LCTF – liquid crystal tuneable filter. Image taken from Kalasinsky et al. 2007.

Similarly, it has been noted that one of the primary issues concerning the application of SERS to biothreat detection is the reproducibility of spectra. Part of this reproducibility can arrive from arise from an inability to visualise regions in which some SERS substrates, such as colloids, are co-localised with the target analyte. To help combat this problem, SERS was combined with electron microscopy. The use of secondary electron detection on a scanning electron microscope allowed for the detection of regions of good co-localisation, which were subsequently targeted with the probe laser for spectral acquisition. The spectra acquired in this manner showed excellent reproducibility, and the authors could clearly discriminate *b. anthracis* and *e. coli* from one another using chemometrics.

An important aspect of biological agent detection is the capability to detect the release of these agents into the atmosphere, fuelled by incidents such as the Tokyo Sarin attacks, the use of chemical weapons on Syrian civilians, and the reported testing of aerosolised ricin toxin on animals by Ansar al-Islam in 2002. Agents such as ricin and *b. anthracis* are particularly dangerous if aerosolised, and so it is vital that methods be developed to reflect this threat. Studies have reported on the detection of bioaerosols by Raman scattering techniques, showing promise for these techniques to fulfil this need[82-85]. Additionally, the Resource Effective Bioidentification System (REBS) from Batelle is a robust system designed for this role.

Viruses are also potential candidates for use as BWAs. By contrast to bacteria and biotoxins, there is little work published on the most important agents in this category. This may be because of their comparative rarity, the extreme complexity of working with these agents, or the difficulty of obtaining and fielding these pathogens as weapons. Despite this, viruses such as influenza or SARS could be deployed as incapacitating agents, causing a potentially massive economic impact if not controlled. To this end, virus detection remains important.

For the most part, work on viruses seems to centre of on the use of SERS[86-96], but detection of phages by spontaneous Raman coupled with electrokinetic capture of virions has been demonstrated[97]. This body of work on viruses shows that Raman scattering techniques have potential to be applied to the problem of viral BWA detection. Beyond the simple detection of viruses, extraction and detection of viruses in cell media in a SERS-based immunoassay has been demonstrated, offering promise that SERS could be deployed on complex real world samples of these important threat agents[91]. Discrimination between three types of avian influenza has also been demonstrated by Song *et al.* using a handheld Raman system[77]. Similarly, other groups have also demonstrated species and strain level discrimination of viruses[89, 90, 93].

One of the significant problems of Raman spectroscopy for biothreat analysis is the large degree of qualitative spectral similarity between many biothreat agents and close related species. This can make the visual determination of the identity of an unknown sample difficult or impossible. The use of chemometric methods such as multivariate statistical analysis are powerful tools that can help resolve this issue. This will be discussed later in the next section.

* + - 1. **Chemometrics as applied to threat agent detection**

Many of the studies and methods discussed in this review employ the use of powerful statistical techniques to reduce the dimensionality of the data in a spectrum and to determine the identity of the sample by comparison to training models of known reference spectra. Perhaps the most common statistical techniques for this task are principal component analysis (PCA), hierarchical cluster analysis (HCA), and linear discriminant analysis (LDA).

PCA and LDA are closely related statistical techniques for pattern recognition in data analysis that look for combinations of variables that explain the variance in the data. As a technique, LDA attempts to model the difference between classes of data, which PCA does not endeavour to do. HCA is, as its name suggests, a clustering algorithm that groups observations into clusters based on a chosen metric of distance, such as Euclidean or Mahalanobis distance. It is common to reduce the dimensionality of data via a technique such as PCA prior to clustering. The output of HCA is often diagrammatically represented as a dendrogram.

The use of chemometrics has been applied to examination of both chemical and biological threats, allowing for the detection and identification of specific agents, as well as biological toxins and organisms.

Perhaps the most common chemometric technique used in threat agent detection, with numerous studies having investigated its utility in threat agent identification, including chemical[28], protein[52, 53, 57], bacterial[56, 70, 78-80, 98], and viral threats[92]. In their multiplex CARS study on CWAs, Brady *et al.* subjected their CARS spectra of four common simulants to PCA. Plotting the first three principal components graphically revealed clear separation of the molecules, allowing easy identification of unknowns when compared to the results of a training set[28]. Additionally, PCA has proven to be a valuable tool in detecting threat agents in complex media, like food and paper[52, 53, 57]. HCA has frequently been paired with PCA as a means of classifying spectra[56, 79, 80, 90, 95]. In these cases, PCA is often used to decrease the dimensionality of the spectral dataset to a few PCs, and then clustering is performed on the model set. Unknowns can then be assigned to the clusters the basis of K-nearest neighbour calculations, or the Mahalanobis distance.

LDA has also been used to study bacterial threat detection[75, 76, 79]. Stöckel *et al* published a study in 2012, showing their work on detecting *bacillus* spores in powder samples by linear discriminant analysis. The group cultivated several species of *bacillus* bacteria and spiked them into a variety of common powders, such as powdered milk and baking powder. These spectra were used to define a model that could discriminate between the various species. As a test to ensure that the model did not overfit the data, unknown samples of five of the bacterial species were tested against it. The test yielded an overall accuracy of 96.8%. A test of *b. anthracis* in table salt (a matrix not included in the model) classified these spectra with the other *b. anthracis* spectra, showing the capacity of the model to handle contamination with unknown matrix particles[76].

In addition to these two techniques, a range of other techniques have been explored, including the use of support vector machines[75], partial least squares (and partial least squares discriminant analysis)[41, 89, 92, 93], multivariate adaptive embedding[1], and soft independent modelling of class analogues[41] have also been explored within the scope of threat agent detection and classification. With such a range of techniques, and a clear track-record of success, chemometric techniques are a powerful tool in the detection of these chemicals and organisms.

* + - 1. **Stand-off and robotic detection**

To best avoid the risk of accidental contamination or harm to first responders or military personnel, detection and identification of highly toxic, dangerous, or unknown chemical/biological hazards is best performed from a distance. This detection-at-distance is often referred to as stand-off detection. It should be noted that NATO define true stand-off detection as being detection at a range greater than 200m. Most applications require detection at ranges smaller than this, however. Detection in the range of 10cm to 200m is called proximal detection. As such, most studies on ‘stand-off’ detection are in fact proximal detection.

Raman spectroscopy is a good candidate for applications in detecting at a distance, thanks to its ability to be able to collect signals over potentially very long distances. Additionally, technologies such as portable Raman spectrometers permit the design of compact devices that can be mounted on unmanned ground vehicles (UGVs) such as the military use[38, 50]. Raman is not without drawbacks for such applications, however. The greatest issue for fielding Raman as a stand-off or proximal detection system is the collection of the signal from the sample. The returning light is reduced as the square of the distance to the detector, with further signal loss from absorption and scattering of light by air. Another issue for stand-off Raman is background. Collection of samples in daylight, for instance, can lead to an enormous amount of ambient light reaching the detector if it is operated constantly, such as it would when the operator is using a continuous wave laser source. Issues with this can be alleviated via the use of pulsed lasers and time-gated detectors that are synchronised to the laser pulses, so that they are operating only when the laser is emitting[99, 100]. Another possible solution to operation in daylight is to choose wavelengths that are in the so-called solar blind region in the UV region at wavelengths shorter than 260nm[42]. Using UV lasers with a wavelength shorter than 250nm also has the added benefit of avoiding the fluorescence associated with biological samples[42].

Despite the difficulties of collecting the Raman signal at distance, systems have been designed that can detect Raman signals at distances of over 100m[101], with one recent study claiming detection at an distance of 400m using random Raman lasing to collect a stimulated Raman signal. The authors of this study claim that, once corrected for clipping losses and imperfect reflections, their setup corresponded to an effective distance of greater than a kilometre[102], indicating that true stand-off detection by Raman is indeed possible.

A number of studies have examined the use of Raman for detecting chemical hazards, such as might be found in a chemical spill or possible explosive material, including the use of conventional RS to detect 60µL nitrogen mustard deposited on concrete at a distance of 10m using a commercially-available system from DeltaNu[99]. The same system has also been used to detect explosive materials 25m[100].

CARS has also been explored for stand-off detection, with early demonstrations of this technique working effectively at 12m arriving in 2008[103, 104]. Subsequent work as demonstrated CARS imaging and low LoDs of explosive materials[105, 106]. These results indicate that CARS is a valuable tool for proximal detection for threat materials. Given the extensive use of CARS in analysing biological samples such as cells, it seems promising that the technique may be applicable to the proximal detection of BWAs.

**1.4.3 Conclusions**

Rapid and accurate detection and identification of chemical and biological threat agents is possible through Raman spectroscopy techniques. The ability to detect potentially dangerous materials at proximal distances, or remotely via robots, is an invaluable tool for reducing the risk potential for people who may be exposed to these hazards. Raman spectroscopy also offers far more timely analysis of biohazards than existing techniques, such as PCR and pulsed-field gel electrophoresis, making it viable as a technique for detect-to-warn applications. This can help contain the spread of - and limit exposure to - released agents. Coupled with the discriminating power of chemometrics, Raman spectroscopy can unambiguously classify even closely related samples, and suspected samples in matrixes that have previously not been encountered.

In future work, it would be beneficial to see further development of techniques for biological toxins and whole pathogens that do not rely on lengthy extraction steps. This would further expand the limits of Raman scattering techniques as a candidate for a detect-to-warn platform that can be used against a wide range of threats. Further, additional work to develop autonomous, stand-alone systems for this application would a valuable contribution to homeland chemical and biological defence, as well as a potent tool for protection of soldiers deployed abroad.

1. **Methodologies**
   1. **SERS**

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* 1. **SERS Spectral processing and chemometric analysis**

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1. **Development of a SERS-based detection system for bioaerosols**
   1. **Introduction**

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* 1. **Methods**

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* 1. **Results and discussion**

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* 1. **Conclusions**

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1. **Outlooks and further research**

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1. **Acknowledgements**

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1. **References**

1. S. R. Talbot and G. Sartorius, *Engineering in Life Sciences*, 2011, **11**, 468-475.

2. A. Smekal, *Naturwissenschaften*, 1923, **11**, 873-875.

3. C. V. Raman and K. S. Krishnan, *Curr. Sci.*, 1998, **74**, 381-381.

4. K. Carron and R. Cox, *Analytical Chemistry*, 2010, **82**, 3419-3425.

5. C. Matthäus, B. Bird, M. Miljković, T. Chernenko, M. Romeo and M. Diem, *Methods Cell Biol*, 2008, **89**, 275-308.

6. J. W. Chan, A. P. Esposito, C. E. Talley, C. W. Hollars, S. M. Lane and T. Huser, *Analytical Chemistry*, 2004, **76**, 599-603.

7. B. K. Dable, B. A. Love, T. M. Battaglia, K. S. Booksh, M. D. Lilley and B. J. Marquardt, *Applied spectroscopy*, 2006, **60**, 773-780.

8. C. Krafft, B. Dietzek, M. Schmitt and J. Popp, *BIOMEDO*, 2012, **17**, 0408011-04080115.

9. T. G. Matthews and F. E. Lytle, *Analytical Chemistry*, 1979, **51**, 583-585.

10. M. O. Scully, G. W. Kattawar, R. P. Lucht, T. Opatrný, H. Pilloff, A. Rebane, A. V. Sokolov and M. S. Zubairy, *Proceedings of the National Academy of Sciences*, 2002, **99**, 10994-11001.

11. P. H. M. Fleischmann, A. McQuillan, *Chemical Physical Letters*, 1974, **26**, 163-166.

12. E. J. Blackie, E. C. L. Ru and P. G. Etchegoin, *Journal of the American Chemical Society*, 2009, **131**, 14466-14472.

13. E. C. Le Ru, E. Blackie, M. Meyer and P. G. Etchegoin, *The Journal of Physical Chemistry C*, 2007, **111**, 13794-13803.

14. E. C. Le Ru, M. Meyer and P. G. Etchegoin, *The Journal of Physical Chemistry B*, 2006, **110**, 1944-1948.

15. D. L. Jeanmaire and R. P. Vanduyne, *J. Electroanal. Chem.*, 1977, **84**, 1-20.

16. V. I. Kukushkin, A. B. Van’kov and I. V. Kukushkin, *JETP Letters*, 2013, **98**, 64-69.

17. S. Yang, X. Dai, B. B. Stogin and T.-S. Wong, *Proceedings of the National Academy of Sciences*, 2016, **113**, 268-273.

18. C. L. Haynes, A. D. McFarland and R. P. V. Duyne, *Analytical Chemistry*, 2005, **77**, 338 A-346 A.

19. N. A. Abu Hatab, J. M. Oran and M. J. Sepaniak, *ACS Nano*, 2008, **2**, 377-385.

20. W. Cai, W. Wang, L. Lu and T. Chen, *Colloid and Polymer Science*, 2013, **291**, 2023-2029.

21. K. S. Giesfeldt, R. M. Connatser, M. A. De Jesús, P. Dutta and M. J. Sepaniak, *Journal of Raman Spectroscopy*, 2005, **36**, 1134-1142.

22. E. Smith and G. Dent, in *Modern Raman Spectroscopy – A Practical Approach*, John Wiley & Sons, Ltd, 2005, DOI: 10.1002/0470011831.ch7, pp. 181-202.

23. W. M. Tolles, J. W. Nibler, J. R. McDonald and A. B. Harvey, *Applied Spectroscopy*, 1977, **31**, 253-271.

24. S. Bowman Pilkington, S. D. Roberson and P. M. Pellegrino, Baltimore, Maryland, United States, 2016.

25. K. Hashimoto, M. Takahashi, T. Ideguchi and K. Goda, *Scientific Reports*, 2016, **6**, 21036.

26. S. H. Parekh, Y. J. Lee, K. A. Aamer and M. T. Cicerone, *Biophysical Journal*, 2010, **99**, 2695-2704.

27. J. J. Brady and P. M. Pellegrino, Baltimore, Maryland, United States, 2013.

28. J. J. Brady, M. E. Farrell and P. M. Pellegrino, *OPTICE*, 2013, **53**, 021105-021105.

29. T. W. Kee and M. T. Cicerone, *Optics letters*, 2004, **29**, 2701-2703.

30. S. Farquharson, P. Gift A Fau - Maksymiuk, F. Maksymiuk P Fau - Inscore and F. Inscore, *Applied Spectroscopy*, 2005, **59**, 654-660.

31. F. Kullander, L. Landström, H. Lundén, A. Mohammed, G. Olofsson and P. Wästerby, Baltimore, Maryland, United States, 2014.

32. S. Farquharson, A. Gift, P. Maksymiuk, F. E. Inscore and W. W. Smith, Providence, Rhode Island, United States, 2004.

33. F. Kullander, P. Wästerby and L. Landström, Baltimore, Maryland, United States, 2016.

34. F. Yan and T. Vo-Dinh, *Sensors and Actuators B: Chemical*, 2007, **121**, 61-66.

35. K. M. Spencer, J. M. Sylvia, S. L. Clauson, J. F. Bertone and S. D. Christesen, Providence, Rhode Island, United States, 2004.

36. K. M. Spencer, J. M. Sylvia, S. L. Clauson and J. A. Janni, Boston, MA, United States, 2002.

37. J. A. Hoffmann, J. A. Miragliotta, J. Wang, P. Tyagi, T. Maddanimath, D. H. Gracias and S. J. Papadakis, Baltimore, Maryland, United States, 2012.

38. N. R. Gomer, C. W. Gardner and M. P. Nelson, Baltimore, Maryland, United States, 2016.

39. F. Kullander, L. Landström, H. Lundén and P. Wästerby, Baltimore, Maryland, United States, 2015.

40. J. Wang, G. Duan, G. Liu, Y. Li, Z. Chen, L. Xu and W. Cai, *Journal of Hazardous Materials*, 2016, **303**, 94-100.

41. W. F. Pearman and A. W. Fountain, *Applied Spectroscopy*, 2006, **60**, 356-365.

42. S. D. Christesen, J. P. Jones, J. M. Lochner and A. M. Hyre, *Applied Spectroscopy*, 2008, **62**, 1078-1083.

43. E. Roy, P. G. Wilcox, S. Hoffland and I. Pardoe, Toulouse, France, 2015.

44. A. Hakonen, T. Rindzevicius, M. S. Schmidt, P. O. Andersson, L. Juhlin, M. Svedendahl, A. Boisen and M. Kall, *Nanoscale*, 2016, **8**, 1305-1308.

45. S. Farquharson, A. Gift, P. Maksymiuk, F. E. Inscore, W. W. Smith, K. Morrisey and S. D. Christesen, Providence, RI, United States, 2004.

46. R. L. Aggarwal, L. W. Farrar, S. Di Cecca and T. H. Jeys, *AIP Advances*, 2016, **6**, 025310.

47. H. Gao, J. Wu, Y. Zhu, L. Guo and J. Xie, *Journal of Raman Spectroscopy*, 2016, **47**, 233-239.

48. F. Inscore and S. Farquharson, *Proceedings of SPIE - The International Society for Optical Engineering*, 2006, **6378**, 63780X-63788.

49. X. X. Han, G. G. Huang, B. Zhao and Y. Ozaki, *Analytical Chemistry*, 2009, **81**, 3329-3333.

50. C. W. Gardner, R. Wentworth, P. J. Treado, P. Batavia and G. Gilbert, Orlando, Florida, United States, 2008.

51. T. L. Paxon, R. S. Duthie, C. Renko, A. A. Burns, M. L. Lesaicherre and F. J. Mondello, Orlando, Florida, United States, 2011.

52. V. M. Szlag, M. J. Styles, L. R. Madison, A. R. Campos, B. Wagh, D. Sprouse, G. C. Schatz, T. M. Reineke and C. L. Haynes, *ACS Sensors*, 2016, **1**, 842-846.

53. J. Zheng, C. Zhao, G. Tian and L. He, *Talanta*, 2017, **162**, 552-557.

54. A. Zengin, U. Tamer and T. Caykara, *Journal of Materials Chemistry B*, 2015, **3**, 306-315.

55. J.-j. Tang, J.-f. Sun, R. Lui, Z.-m. Zhang, J.-f. Liu and J.-w. Xie, *ACS Applied Materials & Interfaces*, 2016, **8**, 2449-2455.

56. K. S. Kalasinsky, T. Hadfield, A. A. Shea, V. F. Kalasinsky, M. P. Nelson, J. Neiss, A. J. Drauch, G. S. Vanni and P. J. Treado, *Analytical Chemistry*, 2007, **79**, 2658-2673.

57. L. He, E. Lamont, B. Veeregowda, S. Sreevatsan, C. L. Haynes, F. Diez-Gonzalez and T. P. Labuza, *Chemical Science*, 2011, **2**, 1579-1582.

58. L. He, B. Deen, T. Rodda, I. Ronningen, T. Blasius, C. Haynes, F. Diez‐Gonzalez and T. P. Labuza, *Journal of food science*, 2011, **76**, N49-N53.

59. K. Ryu, A. J. Haes, H.-Y. Park, S. Nah, J. Kim, H. Chung, M.-Y. Yoon and S.-H. Han, *Journal of Raman Spectroscopy*, 2010, **41**, 121-124.

60. M. E. Farrell and P. M. Pellegrino, San Diego, California, 2012.

61. A. E. Boyer, C. P. Quinn, A. R. Woolfitt, J. L. Pirkle, L. G. McWilliams, K. L. Stamey, D. A. Bagarozzi, J. C. Hart and J. R. Barr, *Analytical Chemistry*, 2007, **79**, 8463-8470.

62. C. Shende, F. Inscore, H. Huang, S. Farquharson and A. Sengupta, Baltimore, Maryland, United States, 2012.

63. L. He, B. D. Deen, A. H. Pagel, F. Diez-Gonzalez and T. P. Labuza, *Analyst*, 2013, **138**, 1657-1659.

64. T. A. Slieman and W. L. Nicholson, *Applied and Environmental Microbiology*, 2001, **67**, 1274-1279.

65. G. F. Bailey, S. Karp and L. Sacks, *Journal of bacteriology*, 1965, **89**, 984-987.

66. S. E. J. Bell, J. N. Mackle and N. M. S. Sirimuthu, *Analyst*, 2005, **130**, 545-549.

67. X. Zhang, J. Zhao, A. V. Whitney, J. W. Elam and R. P. Van Duyne, *Journal of the American Chemical Society*, 2006, **128**, 10304-10309.

68. X. Zhang, M. A. Young, O. Lyandres and R. P. Van Duyne, *Journal of the American Chemical Society*, 2005, **127**, 4484-4489.

69. M. Cheung, W. W. Y. Lee, D. P. Cowcher, R. Goodacre and S. E. J. Bell, *Chemical Communications*, 2016, **52**, 9925-9928.

70. A. Lai, S. Almaviva, V. Spizzichino, A. Palucci, L. Addari, D. Luciani, S. Mengali, C. Marquette, O. Berthuy, B. Jankiewicz and L. Pierno, Amsterdam, Netherlands, 2014.

71. W. R. Premasiri, D. T. Moir, M. S. Klempner, N. Krieger, G. Jones and L. D. Ziegler, *The Journal of Physical Chemistry B*, 2005, **109**, 312-320.

72. S. Farquharson, L. Grigely, V. Khitrov, W. Smith, J. F. Sperry and G. Fenerty, *Journal of Raman Spectroscopy*, 2004, **35**, 82-86.

73. J. Guicheteau and S. D. Christesen, *Proceedings of SPIE - The International Society for Optical Engineering*, 2006, **6218**, 62180G.

74. W. R. Premasiri, D. T. Moir and L. D. Ziegler, Orlando, Florida, United States, 2005.

75. S. Stöckel, S. Meisel, M. Elschner, P. Rösch and J. Popp, *Analytical Chemistry*, 2012, **84**, 9873-9880.

76. S. Stöckel, S. Meisel, M. Elschner, P. Rösch and J. Popp, *Angewandte Chemie International Edition*, 2012, **51**, 5339-5342.

77. P. D. Fey, M. M. P. Dempsey, M. E. Olson, M. S. Chrustowski, J. L. Engle, J. J. Jay, M. E. Dobson, K. S. Kalasinsky, A. A. Shea, P. C. Iwen, R. C. Wickert, S. C. Francesconi, R. M. Crawford and S. H. Hinrichs, *American Journal of Clinical Pathology*, 2007, **128**, 926.

78. R. M. Jarvis, R. Brooker A Fau - Goodacre and R. Goodacre, *Faraday Discussions*, 2006, **132**, 281-292.

79. R. M. Jarvis and R. Goodacre, *Analytical Chemistry*, 2004, **76**, 40-47.

80. I. S. Patel, W. R. Premasiri, D. T. Moir and L. D. Ziegler, *Journal of Raman spectroscopy : JRS*, 2008, **39**, 1660-1672.

81. A. P. Esposito, C. E. Talley, T. Huser, C. W. Hollars, C. M. Schaldach and S. M. Lane, *Applied Spectroscopy*, 2003, **57**, 868-871.

82. P. Rösch, M. Harz, K.-D. Peschke, O. Ronneberger, H. Burkhardt, A. Schüle, G. Schmauz, M. Lankers, S. Hofer, H. Thiele, H.-W. Motzkus and J. Popp, *Analytical Chemistry*, 2006, **78**, 2163-2170.

83. A. Tripathi, R. E. Jabbour, J. A. Guicheteau, S. D. Christesen, D. K. Emge, A. W. Fountain, J. R. Bottiger, E. D. Emmons and A. P. Snyder, *Analytical Chemistry*, 2009, **81**, 6981-6990.

84. A. Sengupta, M. L. Laucks, N. Dildine, E. Drapala and E. J. Davis, *Journal of Aerosol Science*, 2005, **36**, 651-664.

85. A. Sengupta, N. Brar and E. J. Davis, *Journal of Colloid and Interface Science*, 2007, **309**, 36-43.

86. P. Bao, X. Liu, T. Huang, B. Jiang and G. Wu, Denver, CO, United States, 1999.

87. P. D. Bao, T. Q. Huang, X. M. Liu and T. Q. Wu, *Journal of Raman Spectroscopy*, 2001, **32**, 227-230.

88. J.-y. Lim, J.-s. Nam, S.-e. Yang, H. Shin, Y.-h. Jang, G.-U. Bae, T. Kang, K.-i. Lim and Y. Choi, *Analytical Chemistry*, 2015, **87**, 11652-11659.

89. J. D. Driskell, Y. Zhu, C. D. Kirkwood, Y. Zhao, R. A. Dluhy and R. A. Tripp, *PLOS ONE*, 2010, **5**, e10222.

90. J. D. Driskell, S. Shanmukh, Y. J. Liu, S. Hennigan, L. Jones, Y. P. Zhao, R. A. Dluhy, D. C. Krause and R. A. Tripp, *IEEE Sensors Journal*, 2008, **8**, 863-870.

91. J. D. Driskell, K. M. Kwarta, R. J. Lipert, M. D. Porter, J. D. Neill and J. F. Ridpath, *Analytical Chemistry*, 2005, **77**, 6147-6154.

92. C. Song, J. D. Driskell, R. A. Tripp, Y. Cui and Y. Zhao, Baltimore, Maryland, United States, 2012.

93. T. A. Alexander, *Analytical Chemistry*, 2008, **80**, 2817-2825.

94. K. Olschewski, E. Kammer, S. Stockel, T. Bocklitz, T. Deckert-Gaudig, R. Zell, D. Cialla-May, K. Weber, V. Deckert and J. Popp, *Nanoscale*, 2015, **7**, 4545-4552.

95. S. Shanmukh, L. Jones, Y. P. Zhao, J. D. Driskell, R. A. Tripp and R. A. Dluhy, *Analytical and Bioanalytical Chemistry*, 2008, **390**, 1551-1555.

96. S. Shanmukh, L. Jones, J. Driskell, Y. Zhao, R. Dluhy and R. A. Tripp, *Nano Letters*, 2006, **6**, 2630-2636.

97. M. R. Tomkins, D. S. Liao and A. Docoslis, *Sensors (Basel, Switzerland)*, 2015, **15**, 1047-1059.

98. R. M. Jarvis, A. Brooker and R. Goodacre, *Analytical Chemistry*, 2004, **76**, 5198-5202.

99. R. J. Clewes, C. R. Howle, D. J. M. Stothard, M. H. Dunn, G. Robertson, W. Miller, G. Malcolm, G. Maker, R. Cox, B. Williams and M. Russell, Edinburgh, United Kingdom, 2012.

100. R. Cox, B. Williams and M. H. Harpster, Baltimore, Maryland, United States, 2012.

101. A. K. Misra, S. K. Sharma, C. H. Chio, P. G. Lucey and B. Lienert, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2005, **61**, 2281-2287.

102. B. H. Hokr, J. N. Bixler, G. D. Noojin, R. J. Thomas, B. A. Rockwell, V. V. Yakovlev and M. O. Scully, *Proceedings of the National Academy of Sciences*, 2014, **111**, 12320-12324.

103. H. Li, D. A. Harris, B. Xu, P. J. Wrzesinski, V. V. Lozovoy and M. Dantus, *Optics express*, 2008, **16**, 5499-5504.

104. M. Dantus, H. Li, D. A. Harris, B. Xu, P. J. Wrzesinski and V. V. Lozovoy, Orlando, Florida, United States, 2008.

105. M. T. Bremer, P. J. Wrzesinski, N. Butcher, V. V. Lozovoy and M. Dantus, *Applied Physics Letters*, 2011, **99**, 101109.

106. O. Katz, A. Natan, Y. Silberberg and S. Rosenwaks, *Applied Physics Letters*, 2008, **92**, 171116.