Raman Scattering Techniques for Defence and Security Applications

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**Abstract:** In this review we discuss the recent advances in the application of Raman scattering and related techniques to the detection of chemical and biological threat agents. One of the main aims of this review is to provide a new perspective on the application of advanced and emerging Raman techniques such as surface-enhanced Raman, spatially-offset Raman, waveguide-enhanced and coherent Raman spectroscopies, respectively, to the detection of threat agents such as explosives, toxins, viruses and bacteria. Combination with multivariate and computational analysis to augment the analytical abilities of Raman techniques as well as hyphenation and integration with various field deployment strategies such as robotic and stand-off detection are discussed. Importantly this interplay between the detection technique, analysis and engineering technology will be essential for developing powerful solutions for field applications in defence and security.

# 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 to further their agendas. It is perhaps fair to say that the attack on the World Trade Center 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 hazards, there has been a surge of interest from governments, universities and private sector defence companies into ways of detecting them, either before they can be deployed or after it has been released. This has included techniques such as vibrational spectroscopy1-3, fluorogenic and chromogenic tests4, 5, detectors based on the activation6 or deactivation7 of luminescence in lanthanide complexes by endospore biomarkers, as well as techniques such as ion mobility spectrometry8, 9 and mass spectrometry1, 10, 11. Many of these techniques suffer from issues, however. For example, low specificity can be a problem, leading to false positive results, whilst some techniques can require specialist laboratory facilities and highly trained personnel to achieve the best results. Examples of the kinds of agents that are of particular interest for detection are included in Table **1**.

An alternate to the techniques mentioned above for threat and hazard detection is to use the optical phenomenon of Raman scattering that has resulted in a host of analytical techniques that have evolved over the years. Raman scattering involves vibrational fingerprinting of molecules and materials. While out of the Raman-based techniques, Raman spectroscopy (or, more correctly, spontaneous Raman spectroscopy) has been by far the most prevalent analytical technique, many advances have led to developments that overcome some limitations. The limitations of sensitivity (or speed) are overcome in techniques such as surface-enhanced Raman, waveguide-enhanced and coherent Raman spectroscopy, while the depth penetration is improved in spatial-offset Raman spectroscopy. Similarly, other innovations, such as stand-off Raman, have been developed for detection at a distance. However, in this review we do not describe the techniques themselves in detail and the interested reader is referred to textbooks and recent reviews on them12-17.

In the following sections, this article will discuss the application of Raman scattering to the detection of biological and chemical threat agents. We describe the applications in the area of threat detection of advanced and emerging techniques based on the Raman scattering phenomena such as surface-enhanced, spatially-offset, waveguide-enhanced and coherent Raman spectroscopy. Importantly, an overview on field analysis and perspective on their combination with numerical and computational methods to enhance their analytical abilities and widespread adoption is provided.

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| ***Table 1:*** List of the CDC’s ’Dirty Dozen’ biological agents, and chemical agents of importance18. | | | |
| **Chemical** | **Biological toxin** | **Bacteria** | **Virus** |
| Nerve agents (Sarin, VX)  Blood agents (Cyanide)  Choking agents (Chlorine gas, phosgene)  Vesicants (Sulphur mustard) | Botulinum toxin  Staphylococcus enterotoxin B  Ricin | *Bacillus anthracis*  *Francisella tularensis*  *Yersinia Pestis*  *Burkholderia mallei*  *Burkholderia pseudomallei*  *Brucella melitensis*  *Brucella abortus*  *Coxiella burnetii* | Variola Major (Smallpox)  Haemhorrhagic fevers (Ebola, Marburg)  Viral encephalitis (VEE) |

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# Applications of Raman scattering-based techniques to defence and security

## Chemical and explosive hazard detection

Small molecules of non-biological origin are of interest for a range of defence and security applications. Explosive chemicals, such as TNT and RDX, as well as chemical warfare agents (CWAs) such as cyanide, phosgene, mustard gas, and the 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.

Explosive materials are an ongoing threat to civilians and military personnel. These materials are concealable in seemingly benign containers and locations to escape detection before detonation can occur. As such, sensitive detection of explosive materials in a timely manner is of paramount importance. In recent years, a number of studies have been published that highlight the potential of Raman-based techniques for this role. Much of this work has centred on time-gated Raman for stand-off detection19-21, which is discussed in a later section, and Spatially-Offset Raman spectroscopy (SORS)22-26; however, surface-enhanced Raman spectroscopy (SERS) has also been investigated27-35, alongside other techniques36-38.

Raman spectroscopy is often said to differ from other analytical techniques in that it allows analysis to be performed on a sample without the need to remove it from its container. Whilst this is true, it is often only viable for clear glass, or very thin plastics. Coloured glasses generate problematic fluorescence, and opaque or thick plastics cause attenuation of the incident beam, plus these materials contribute to the fluorescent background and exhibit a strong Raman signal as well. SORS is a variant of Raman spectroscopy that allows the collection of Raman signals through a wider range of containers than would be feasible with conventional Raman systems. Spatially offsetting the detection and excitation paths enables the reduction of signal (both fluorescence and Raman scattering) collected from the container, as these signals drop off faster than that of the analyte as a function of the increasing separation. This produces advantages in terms of safety, as personnel don’t need to contact the potentially hazardous contents, and permits the efficient use of time and PPE, as well as preserving the integrity of evidence in investigations.

Recently, Frisby *et al* utilised SORS to detect explosive materials, as well as CWA simulants and albumin (used as a surrogate for ricin toxin), from within a range of containers, including those with a thickness of up to 6mm, at a distance of 20cm24. The group found that the type and thickness of material, as well as colour, appeared to play a role in the effectiveness of SORS. Transparent coloured plastics, and lighter coloured glasses, provided better SORS signals, but the signal-to-noise ratio (SNR) was still around 5 through 3mm of opaque white plastic. A study of the effect of barrier thickness mapped SNRs in SORS spectra, and found that the signal decay rate is exponential, and dependent on the material and colour. Even so, spectra could still be recovered through 6mm of yellow HDPE with 10 accumulations of 1s integration times24. This rapid analysis and extreme versatility, coupled with the availability of portable SORS22, 39, paves the way for the use of SORS in applications such as cargo and luggage screening for transport hubs like airports, and its use by first responders and military personnel in the field.

Owing to their extreme toxicity, much of the work on CWAs has been conducted on simulant chemicals with similar molecular features, such as organophosphates, or on the hydrolysis products of these materials24, 25, 38, 40-61. 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, with the potential to act as markers of the release of CWAs. In addition, work using CWAs continues to be conducted to assess the performance of sensors and protective measures40-43, 62-67.One of the primary issues with the detection of CWAs is the need to reach extremely low limits of detection, often in the low parts per billion (ppb) range. Whilst some agents, such as cyanide, are easily detectable due to the intense, distinct C≡N band, 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 spectral region62. 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 range41, 43, 51, 62.

It has also been found that for V series agents, excitation using deep UV photons 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; no such changes were found for sulphur mustard (HD) or Tabun (GA)43. These data highlight that the precise choice of laser wavelength can impact upon the detection of these materials. Further illustration of this is found in the work of Kondo *et al*, where it was demonstrated that detection of many chemical warfare agents yielded very clear Raman spectra at 785nm excitation, but agents such as adamsite, and HN3 nitrogen mustard yielded only broad fluorescence backgrounds68. Changing excitation wavelength to 1064nm resulted in clear spectra that could enable identification of these materials. Given these considerations, it is important that standard methods of analyses normalised or independent to the laser excitation for these materials be developed, to avoid the risk of misidentification. It is important to note that the Raman cross-section is wavelength dependent and hence, while it is no surprise that peak intensities are different with different excitation wavelengths, this must be taken into account to make data comparable.

To help overcome the issue of poor Raman scattering cross-sections of many potential analytes, SERS and coherent anti-Stokes Raman scattering (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 SERS studies on live CWAs40, 42, 65, 67, 69-71 and simulants and/or hydrolysis products40, 42, 44-46, 49, 52, 53, 65, 69. 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 altogether42. As with the selection of wavelengths, it is important that standard analyses be developed for SERS based methodologies if Raman-derived techniques are to gain prominence in the detection of CWAs as the enhancement observed for different peaks can also be wavelength dependent, especially when operating with near infrared excitation sources.

Gao *et al* developed a methodology for the detection of phosgene and its related compounds, di- and triphosgene67. 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. Despite being electron rich and having a small number of possible vibrational modes, the low density of scattering molecules in the gas phase means the overall signal of these molecules is low. Additionally, these molecules decompose readily in water, making analysis in aqueous solution nonviable. To circumvent the issue of poor Raman scattering, a method based on utilising SERS to indirectly monitor the presence of phosgene via a chemical transformation method via the following reactions was utilised:

**COCl2 + 2 KI → 2 KCl + I2 + CO**

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

The resulting I2 was detected in solution by SERS with limits of detection (LoDs) in the low micrograms per litre range. The method was validated on samples of diphosgene in air, and achieved an LoD of 30μg/L67.

Detection of CWA vapours is an important ability, as these toxic chemicals exhibit a range of volatilities. Given the extreme toxicity of many CWAs, sensitive detection of vapours is an enormously beneficial capability for any detection modality. Recently, a number of studies have shown the capability to detect gaseous CWAs using Raman methods57, 58, 72. Lafuente *et al* have presented the detection of DMMP carried on a stream of nitrogen gas57. The material was captured on a self-assembled layer of citrate capped gold nanoparticles inside a gas cell. The authors detected 130ppb of DMMP, with detection stabilising in around 100s of flow, and 20s integration times for the SERS spectra. This rapid process, and the simplicity of the analytical methodology, suggests great promise for similar techniques, as long as the results are similarly promising for an extended range of chemical hazards. Lafuente *et al* also reported on the use of portable Raman for gas phase detection of CWAs58. In this second study, SERS spectra of DMMP were obtained from a substrate of silver plates on graphite. In this case, the graphite helped with fluorescence quenching, which is important for acquisition of high-quality (high SNR) Raman spectra, and also to help mitigate thermal decomposition of the sample. As with the prior study, DMMP was carried on a stream of nitrogen gas, and detected on a substrate inside a microfluidic gas chamber. They reported a limit of detection of 14mg/m3, further illustrating the potential of SERS devices as a potential tool for first responders in situations where chemical hazards are thought to be present58.

Waveguide-enhanced Raman spectroscopy (WERS) provides an alternative means of enhancing Raman signal, and has been shown as a possible tool for detecting chemical hazards in the vapour phase. Emmons *et al* fabricated waveguides coated with a sorbent polymer to capture vapours from DMMP, a CWA simulant, with a device that had a footprint of 10x10cm, and a height of 5cm56. The researchers monitored the 713cm-1 band in the WERS spectrum as an indicator of the presence of DMMP in the sorbent coating. Such a device would be readily portable, or easily deployed in a location; however, further work is required to improve the sensitivity of the device, to improve the kinetics of DMMP adsorption, and to reduce the fluorescence background from the waveguide itself.

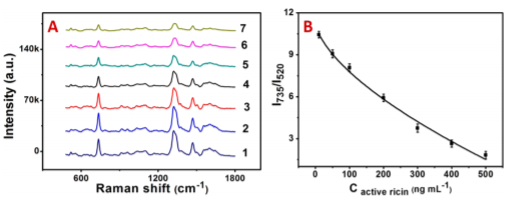
Brady *et al* used CARS for studies into the detection of explosives precursors and simulants of chemical warfare agents[41](#_ENREF_41), [42](#_ENREF_42). In these works, MCARS was used to obtain spectra of agents within millisecond sampling times using uncooled USB spectrometers. This represents a significant improvement over spontaneous Raman, which requires seconds of acquisition to acquire workable spectra using cooled and intensified focal plane arrays. Their set-up used a femtosecond pulsed Ti:Sapphire laser that was split into two portions. One of these beams was used to generate a broadband super-continuum that could drive all the vibrational modes of the analytes simultaneously, allowing them to generate the entire spectrum of the sample at once47, 48. Additionally, one of these studies used PCA to demonstrate the clustering of the spectra of their analytes, showing that they could be grouped together with a good degree of fidelity, which is important for automatic detection of unknowns48. These studies offer faster analytical times than spontaneous Raman, as well as greater sensitivity as a result of the signal enhancement that is offered by CARS, which is beneficial for CWAs with low Raman scattering cross-sections. However, CARS equipment is complex and expensive, and more cumbersome to transport than spontaneous Raman hardware, making it unsuitable as of yet for portable field-detection scenarios.

## Biological hazard detection

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 used to detect proteins for some time24, 53, 73, 74, and its application to the detection of ricin has been demonstrated in numerous studies that have used the harmless A chain (RAC)75 or B chain (RBC)18, 75-78, as well as whole ricin75, 79, 80. Detection in complex media such as in diagnostic samples, or in food that had been maliciously contaminated76, 79, 81, 82, has also been demonstrated by multiple groups at levels below the LD50 of the toxin.

When detecting such biological samples in complex media, it is often useful 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 aptamers: peptide aptamers, made of one or more peptide domains; and 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 protein83. Aptamers have been combined with SERS for detection of bioweapons in numerous studies. SERS based aptamer based sensors, for example, have been tested on Ricin B Chain (RBC)78, 81, Anthrax Protective Antigen (PA)84, Anthrax Lethal Factor85, and *bacillus* bacteria86, 87. These aptamer-based methods require that aptamers be developed for each target of interest, which would involve a considerable effort to optimise and produce. Further, the deployment of aptamers requires some a priori knowledge of the hazard. Given that they would also have to be supplied to a detection device as a reagent, they can also create undesirable logistical complexity for remote-sensing applications, and present an additional burden to personnel if deployed as part of a portable detection platform.

Non-aptamer based approaches have also been developed. Tang *et al*. developed a SERS chip for the detection of ricin using single-stranded oligonucleotide functionalised gold nanoparticles79. 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 quantify the amount of ricin present by measuring the signal attenuation of peaks associated with adenine (Figure 1). The chip was then tested on ricin mixed into foods and biological samples, and found to follow the calibration curve with relative errors of less than 7.6%. Additionally, the sensors showed little change in acquired spectral signatures after being stored for three months at 4°C79. The group established an LoD of 8.9 ng mL-1, which is far below the median lethal dose (LD50) by both ingestion and injection in mice (20mg/kg88 and 5μg/kg89, respectively). There is no confirmed LD50 for humans, but estimates suggest values of 1mg/kg88 and 1-10μg/kg90 for ingestion and injection, respectively.

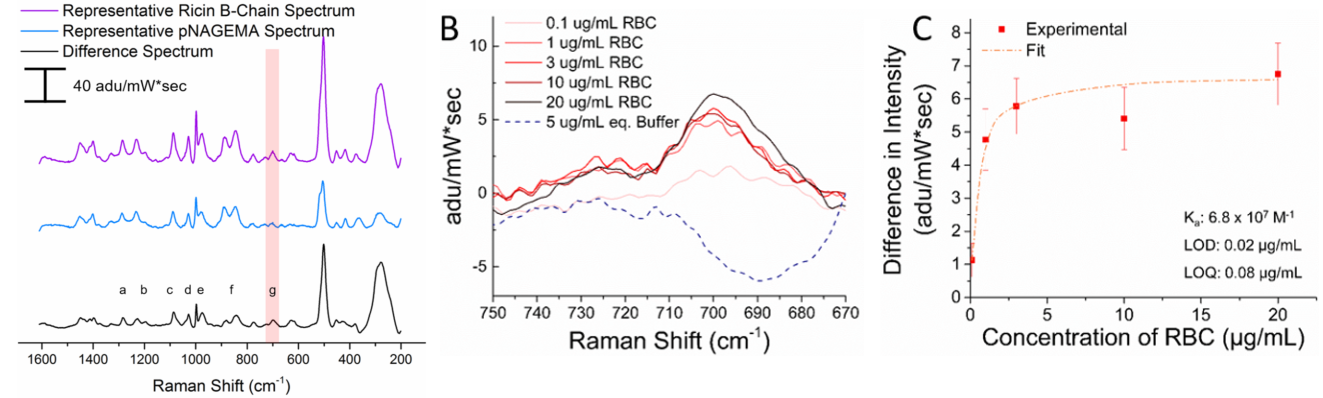


**Figure 1** (A) Signal attenuation of adenine at differing concentrations of intact ricin. (B) Calibration curve for the determination of ricin concentration. Reprinted with permission from J.-j. Tang, J.-f. Sun, R. Lui, Z.-m. Zhang, J.-f. Liu and J.-w. Xie, ACS Applied Materials & Interfaces, 2016, 8, 2449-2455. Copyright 2016. American Chemical Society.

The biological activity of ricin was also successfully exploited for its detection by Szlag *et al*76. 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. Using this method, detection of ricin in fruit juices was achieved with a quantitative range and a LoD of 20ng/mL (Figure 2), which is comparable to other studies using SERS76.

Hazardous threat agents are often dispersed through paper via postal mail. 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.77 Based on extraction and detection of ricin from paper their first method was non-destructive, and involved pipetting 1 mL 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 10 µL 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 (PCA) 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 0.044μg. This method, therefore offers good potential as a screening method for ricin on papers, at least in small-scale applications.

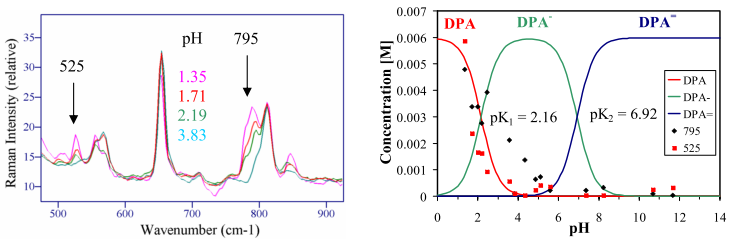
**Figure 2** (A) Difference spectrum showing changes to the oligomer spectrum due to RBC binding. (B) Difference spectra at varying concentrations of RBC in the 670-750cm-1 spectral region. (c) Quantification of the increased amplitude at 700cm-1 with increasing concentrations. Adapted with permission 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. Copyright 2016. American Chemical Society.



Contaminated surfaces or matrices can be extracted and the analyte toxins can be captured by efficient materials such as porous polymers. Szlag *et al* have recently developed a series of poly(N-acryloyl glucanamide) (pNAgA) polymers for use as capture agents for SERS detection of a fungal toxin, aflatoxin B191. They assessed four molecular weights of pNAGA and studied the dependence of their affinity for the toxin on chain length. The polymers were mounted on to a gold film-over-nanosphere SERS substrate. The substrates were excited via a fibre optic probe, with 10s integration times. The authors found that the length of the polymer chain and the manner by which the analyte and capture agent were introduced, as well as the PNAGA anchoring chemistry, all played a role in determining the sensitivity; however, their optimal setup of pNAGA22, anchored by a thiol group, and in-solution mixing of the toxin and capture agent, achieved a limit of detection of 10ppb. This result is a hundred-fold improvement on SERS of aflatoxin on gold film over nanosphere (FON) substrates that do not have the affinity agent. This study highlights the role of seamless integration of pre-concentrating matrices to enhance detection abilities.

Pathogens, such as bacteria, need not be detected simply as entire organisms, but can also be detected indirectly by the presence of a biomarker compound. These compounds 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* spores92, 93. An important consideration for the detection of DPA is that is spectral features depend on pH, due to its diprotic nature (Figure 3)42. Various studies have reported low levels of detection for pure DPA, including detection in the low micromolar range94, 95.

SERS has been employed for the detection of DPA in several studies35, 94-98. 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*96. If the sonication and sample preparation is included, this detection time extends to 11 minutes, which is a comparatively short window and is highly desirable in terms of potentially helping to minimising exposure to pathogenic spores in the event of an attack. The use of a battery powered spectrometer, coupled with the 40-day temporal stability of the SERS substrate, in this study is an encouraging demonstration of the feasibility of SERS for field-deployable applications, though it suffers from the requirement to replace the substrates over time. This could perhaps be overcome by the development of, for example, regenerable SERS substrates to prolong the life of each substrate.

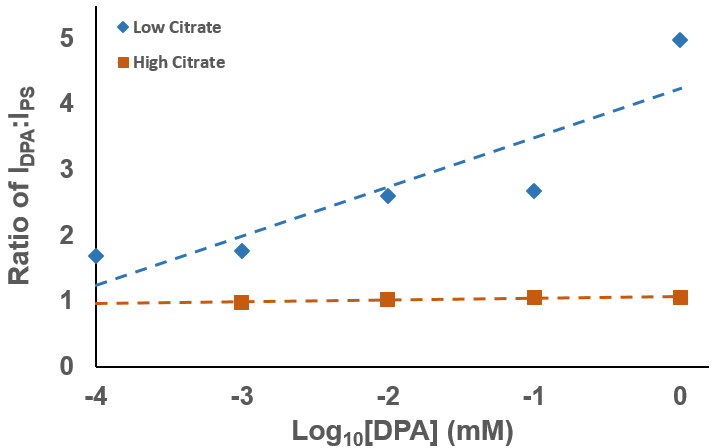
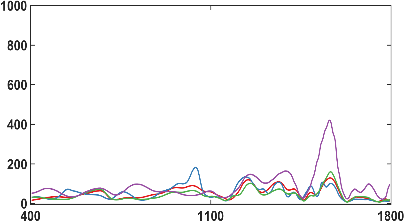
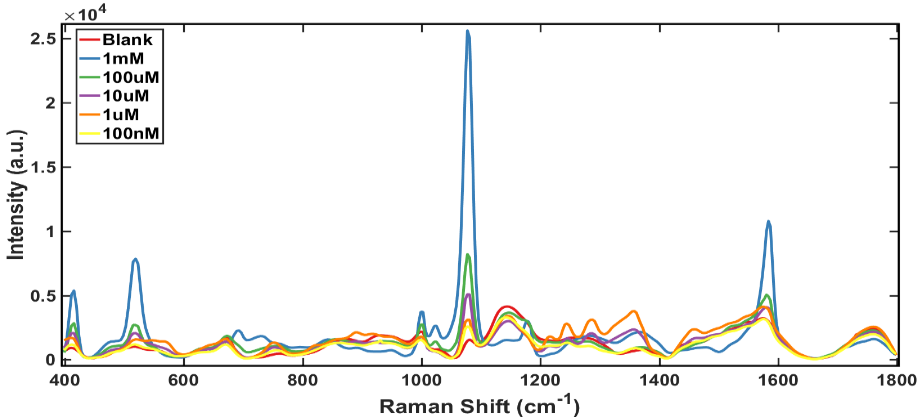


**Figure 3** Left: Raman spectra of DPA species at different pH values. Right: Concentrations of different DPA species as a function of pH. Black and red dots represent the intensities of the peaks at 795cm-1 and 525cm-1, respectively. It can be seen that the peak at 525 cm-1 corresponds strongly to 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. Adapted with permission from “Ultraviolet Raman scattering from persistent chemical warfare agents” by F. Kullander, P. Wästerby and L. Landström, in SPIE Security + Defence (Volume 9824), Baltimore, Maryland, United States, 2016.

Cheung *et al* also used SERS to achieve a LoD of 10-6mol dm-3, which corresponds to approximately 18 spores of anthrax97. This value is two orders of magnitude lower than previous measurements, and substantially below the infectious dose of bacillus anthracis (104 spores)97. This low level of detection was achieved by the preparation of micropillars 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. This method requires special hydrophobic gas chromatography syringes. This, and highly precise positioning of the sample, making it unsuitable for field-deployment. However, it offers exquisite sensitivity as a lab-based methodology for analysis of trace levels of unknown material, and would likely still be significantly faster than culture-based methods.

Naqvi *et al* have recently published the use of a reusable SERS substrate produced by the texturing of a surface by a femtosecond laser, and subsequent decoration of the resulting nanostructures with gold nanoparticles35. This substrate was used to detect DPA, as well as several explosive materials, and could easily be regenerated for reuse with four fifteen minute cycles of washing with ethanol. Limits of detection for the materials used in this study were 0.83 pg/L, 3.6 pg/L and 2.3 pg/L for DPA, DNT, and PA, respectively. The substrates required expensive equipment to fabricate, but re-use of substrates, as well as modifications to the production methods can be utilised to reduce the cost significantly by increasing the number of chips produced in each fabrication by a factor of around ten. It is advantageous that this substrate is demonstrably capable of extremely sensitive detection of both DPA and explosive materials, as substrates with extensive generalizability offer a considerable simplification in the logistics of deploying SERS for security and defence applications.

Recent work within our own group suggests that it is possible to improve the response of SERS sensors to DPA by modulating the surface charge by reducing the amount of citrate anions in the environment surrounding the gold or silver surface. To test this, two batches of layer-by-layer SERS sensors based on polystyrene microsphere of the type used by Anderson *et al* were prepared99. In one batch, the gold nanoparticles were used as-sold, with the level of citrate provided by the manufacturer; in the other, the gold was pelleted and the citrate-contained supernatant was removed and replaced with deionised water prior to resuspension and use in sensor fabrication. These sensors were soaked in varying concentrations of DPA, and the ratio of the intensity of the ring stretching peak of DPA to that of the ring stretching peak of polystyrene within the sensor was measured. High-citrate sensors showed a lower response than sensors that had been produced from low citrate gold nanoparticles, as shown in Figure 4. This data suggests that citrate concentration (and the electrostatic charge it induces on a surface) may be a significant factor to consider in the design of DPA-sensing systems.



**Figure 4** Top – Class mean SERS spectra of dipicolinic acid (DPA) at varying concentrations for low citrate layer-by-layer SERS-active microbeads. The inset shows SER spectra for the same layer-by-layer SERS-active microbeads made with high citrate gold. Bottom – Plots of the ratios of intensities for DPA to polystyrene in low and high citrate layer-by-layer SERS sensors, showing that low-citrate sensors are more responsive than high-citrate ones.

## Pathogen detection - bacteria

In addition to the detection of biomarkers and biological toxins, detection of intact pathogens by Raman spectroscopy has been investigated a lot especially in recent years. Bacterial detection, in particular, 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* bacteria18, 28, 44, 53, 80, 86, 87, 93, 96, 100-110, *Yersinia pestis* and other *Yersinia* bacteria18, 46, 80, 104, 110, *Burkholderia mallei*18, 80, *Francisella tularensis*18, 80, 110, 111, and *Brucella abortus* and related bacteria18, 80, 104. 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 biological hazards 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 bacteria46, 104, between spores and vegetative cells104, 112, and potentially between living and dead bacteria46. Various studies have also demonstrated the ability of Raman techniques to discriminate between bacteria at the sub-species and strain levels111-114.

Though conventional RS is a very weak technique, it is possible to obtain whole-organism spectra from single spores or cells80, 102, 115, 116. 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 spores102, 116.

In addition to decreasing acquisition times, SERS offers other advantages over spontaneous RS for biological samples. Firstly, it avoids the innate fluorescence associated with biological samples by offering a fluorescence-quenching pathway. Secondly, SERS spectra are often less congested and more distinctive than spontaneous RS spectra105. 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 exhibit greater differences in their surface components than in their cytoplasmic contents. However, it should be noted that SERS spectra may not completely match the spontaneous Raman spectrum of the same species, which means that standard RS spectral libraries often cannot be used for SERS spectra. As such, new libraries would need to be developed.

The most common method for SERS based pathogen detection is to drop cast the analyte suspension on the enhancing substrate. Recently, Prakash *et al* deployed PCA and canonical discriminant analysis for the differentiation of *E. coli*, *S. typhimurium*, and *B. subtilis*, based on their SERS spectra108. The group used positively charged, bimetallic nanoparticles, comprised of silver and gold, believing that the positive surface charge would be of benefit in probing negatively charged analytes, such as bacteria. Spectra were differentiated by PCA and canonical discriminant analysis (CDA), and showed good clustering when plotted. This was particularly evident in CDA, where the species were widely separated, and clustering was especially tight (within 1% of score range).

However, SERS has been combined with other analytical procedures, for example lateral flow assay devices. The use of lateral flow immunoassays (LFIAs) are common immunochromatographic tests for target substances. They operate under the same principle as ELISA assays, are simple to use, economical to produce, and typically offer results in under 30 minutes. Typically, a positive result is indicated by the presence of one (a test line), or two (a test and control line) coloured lines, such as in a home pregnancy test. Wang *et al* have developed LFIA kits based of SERS-detection for three important pathogens: Y*. pestis*, *F. tularensis*, and *B. anthracis*110. The test uses gold nanoparticles labelled with Malachite green isothiocynate (MGITC) as a Raman reporter. The intensity of peaks in the SERS spectrum of MGITC in the test line can then be used for quantitative analysis for the amount of pathogen present in each of the test strips. Each assay took only 15 minutes, and required just 40µL of pathogen solution. Reported limits of detection for *Y. pestis*, *F. tularensis*, and *B. anthracis* were estimated to be 43.4 CFU/mL, 45.8 CFU/mL, and 357CFU/mL, respectively. This are approximately 3-4 orders of magnitude better than commercial lateral flow assay kits. The nature of the pathogen-antibody interaction means these tests have excellent specificity for their chosen analytes, but also means they lack generalizability, and that testing *in situ* would require an inference or *a priori* knowledge about the nature of the pathogen that may be present, which can limit their applicability.

Although it may not be relevant for field-based detection, methods such as laser trapping have been used to bring the SERS substrate to the pathogen to enable its detection. Using laser trapping SERS substrates were generated by confining four silver nanoparticles - but they were unstable due to Brownian motion and interactions between the nanoparticles; however, addition of *B. subtilis* enabled the trapping of a greater number of nanoparticles109. SERS of the resulting bacteria-nanoparticle mixture was then achieved by excitation of the trapped volume with a 532nm laser with an integration time of 30s. This method produced high quality, feature-rich spectra of the spores, with good reproducibility between spectra.

Raman spectroscopy has been combined with other techniques to help circumvent issues such as the long acquisition times due to weakness of signals. This can be a detriment to high-throughput analysis, or to real time detection. To mitigate this, 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.80, 117 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.

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 lack of reproducibility can arise from an inability to optically visualise regions in which some SERS substrates, such as colloids, are closely co-localised with the target analyte to enable SERS enhancement to occur. To help combat this problem, Jarvis *et al* combined SERS with electron microscopy118. The use of secondary electron detection on a scanning electron microscope allowed the authors to detect regions of co-localisation between bacteria and the silver colloid (their SERS substrate), 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. subtilis* and *E. coli* from one another using PCA. Previously, the requirement for an SEM would have limited the application of this method significantly, however, the increasing miniaturisation of SEM into desktop systems offers potential to further develop this combined SEM-SERS technique as a part space-efficient of a mobile laboratory.

An important aspect of chemical and biological hazard detection is the capability to detect the release of these agents into the atmosphere. Incidents such as the Tokyo Sarin attacks, the use of chemical weapons on Syrian civilians, as well as the reported testing of aerosolised ricin toxin on animals by Ansar al-Islam in 2002, illustrate the potential for release of threats into the air. Agents such as ricin and *B. anthracis* are particularly dangerous if aerosolised, and so it is vital that appropriate detection methods be developed. Studies have reported on the detection of bioaerosols by Raman scattering techniques, showing promise for these techniques to fulfil this need117, 119-121. Additionally, the portable Resource Effective Bioidentification System (REBS) from Battelle is a robust system designed for this role122. Batelle report that this device costs 4 cents per analysis and can identify over 100 biological materials, with a demonstrated sensitivity of 25 agent containing particles per litre of air, and a time to alarm of under thirty minutes.

## Pathogen detection - viruses

Viruses are also potential candidates for use as BWAs. In 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.

For the most part, work on viruses seems to centre on the use of SERS123-133, but detection of phages by spontaneous Raman spectroscopy (RS) coupled with electrokinetic capture of virions has been demonstrated134. This body of work on viruses shows that Raman scattering techniques have the 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 via a SERS-based immunoassay has been demonstrated, offering promise that SERS could be deployed in complex real world samples of these important threat agents128. Discrimination between three types of avian influenza has also been demonstrated by Song *et al.* using a handheld Raman system77. Similarly, other groups have also demonstrated species and strain level discrimination of viruses126, 127, 130. As has been discussed previously, the relative scarcity of study in this field would require a considerable degree of library-building and protocol standardisation if SERS and RS methods are to become mainstay technique in the analysis of these materials.

More recently, Zhang *et al* used SERS to detect two viruses using a variation of the SERS technique that they called Volume-Enhanced Raman Spectroscopy (VERS). To achieve VERS, the researchers used a SERS substrate that was comprised of microbowls with hollow nanocones in the bottom135. This configuration enabled both surface and bulk hotspots. As the virus particles entered the nanocones, the majority of the volume of the particle would be within the enhancement range of a hotspot, which therefore produces SERS signals. Relative to samples of viruses on gold nanoparticle films, the VERS spectra showed significantly clearer features across the entire Raman fingerprint region. Further, when examined by PCA, VERS spectra clustered substantially more tightly than their nanofilm counterparts, suggesting less variation between the spectra.

Another recent work, published by Xiao *et al* describes the detection of avian influenza (AIV) viral material using a SERS-based LFIA that was capable of returning results in twenty minutes136. The LFIA strip was comprised of a test and a control line, and used an AIV specific antibody to capture the virus, and a novel core-shell material, AuAg4-ATP@AgNPs, as a Raman probe. Viral capture was visually indicated by the appearance of a coloured test line, and quantitative analysis could be performed based on the peak intensities of the SERS spectra of this line (R2 0.998). The authors report a limit of detection of 0.0018HAU, which is three orders of magnitude better than corresponding haemagglutination assays136. Given the small size of these test strips, and the prevalence of portable Raman spectrometers, systems such as this may offer potential for deployment with military personnel or civilian first-responders as a quick test in the event of a suspected biological incident; however, further work would need to be conducted to broaden the range of test strips available, so that a broader range of materials of interest could be tested.

Similarly, Chen *et al* have reported a SERS imaging-based aptasensor for detecting influenza virus A (H1N1)137. The method involves 3D gold ‘nanopopcorn’, functionalised with an aptameric DNA strand. 3μL drops of virus suspension were deposited onto the substrate, and interact with the DNA strands, bringing them close to the gold and inducing a conformational change in the aptamer that decreased the signal intensity associated with the Cy3 reporter molecules hybridised onto the capture strands. A limit of detection of 97PFU/mL was reported, which is a thousand times better than corresponding ELISA assays. With an assay time of 20 minutes, this method overcomes the time- and labour-intensiveness of PCR methods, and is significantly more sensitive than lateral flow assays.

One of the significant problems of Raman spectroscopy for biological hazard analysis is the large degree of qualitative spectral similarity between many biological agents, particularly between closely 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 and computational techniques are powerful tools that can help resolve this issue. This issue will be discussed in the next section.

# Chemometrics 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 infer the class or identity of the sample by comparison to training models of known reference spectra. Perhaps the most common statistical techniques for this task with Raman spectra are 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. 138 As a technique, LDA attempts to model the difference between classes of data, while PCA looks at similarities. 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. The output of HCA is often diagrammatically represented as a dendrogram139.

The use of chemometrics has been applied to examination of both chemical and biological hazards, allowing for the detection and identification of specific agents, as well as biological toxins and organisms. These applications are discussed below.

PCA is perhaps the most common chemometric technique used in chemical and biological hazard detection, with numerous studies having investigated its utility in threat agent identification, including chemical48, 54, protein76, 77, 81, bacterial80, 100, 108, 112-114, 116, 118, and viral threats129, 135. 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 set48. Additionally, PCA has proven to be a valuable tool in detecting threat agents in complex media, like food and paper76, 77, 81. HCA has frequently been paired with PCA as a means of classifying spectra80, 113, 114, 127, 132. 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 on basis of K-nearest neighbour calculations, or other metrics.

Choi *et al* have examined a large number of chemical warfare agents, including nerve agents, nitrogen mustard, CS gas, as well as several simulant chemicals and other materials, using 248nm excitation to obtain Raman spectra54. They reported on the unique peaks associated with each of the materials that would assist in the discrimination of each from the others. In particular, G-series agents with similar structures all still presented unique structural features, though they were not easily resolved from each other when subjected to PCA; however, they did form a cluster that was distinct from other types of materials, enabling the presence of the correct family of CWA to be classified.

Akanny *et al* recently used PCA and Partial Least Squares Discriminant Analysis (PLS-DA) to discriminate between the SERS spectra of *B. subtilis*, *E. coli*, and *L. rhamnosus116.* The study used uncoated gold nanoparticles as an enhancing substrate, providing stronger enhancement than silver nanoparticles. This improvement was up to 15 times better in the case of *E. coli*. Spectra were of high quality, and resolved well by PCA and PLS-DA. The authors noted that numerous bands in the spectra overlap, making accurate assignment of vibrations difficult; however, the use of PLS-DA Variable Importance Projections allowed them to locate the most discriminating spectral region, which pertained primarily to cell wall peptidoglycans, adenine-based compounds, and small purines.

LDA has also been used to study bacterial threat detection106, 107, 113. Stöckel *et al* published a study in 2012, showing their work on detecting *Bacillus* spores in powder samples by LDA. 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 over fit 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 particles107. The same group have also published a study that was able to classify strains from four different species of *Bacillus* endospores. 140 Their method used a two-stage classifier that used unsupervised HCA to divide the *Bacillus* species into two groups. This was then fed into three classifiers: artificial neural networks with a feed-forward topology, SVM, and LDA. Their method achieved an accuracy of 99% accuracy for viable endospores, but this decreased to 81-89% for spores that had been inactivated in 20% formaldehyde. Given that the array of possible matrix materials is so vast, the ability to detect the material of interest in a previously unknown matrix is of particular interest. It allows for greater generalisation of the technique, and highlights the versatility that can be achieved by the combination of chemometric techniques and RS data.

In addition to these three techniques, a range of other techniques have been explored, including the use of support vector machines106, partial least squares (and partial least squares discriminant analysis)53, 126, 129, 130, multivariate adaptive embedding18, and soft independent modelling of class analogues53 have also been applied within the scope of threat agent detection and classification. With such a range of techniques, chemometric techniques can be a powerful tool in the detection of these chemicals and organisms. However, for hazard detection in the field the Raman detection instruments and the methods need to be coupled with engineering platforms for providing effective solutions. This has manifested in a range of remote monitoring and detection techniques.

# Stand-off and robotic detection

To avoid the risk of accidental contamination or harm to first responders or military personnel, detection and identification of highly toxic, dangerous, or suspected chemical/biological hazards is best performed from a distance and ideally remotely or through automated/robotic assistance. This detection-at-distance is often referred to as stand-off detection.

Raman spectroscopy is a good candidate for applications for detection at a distance, thanks to its ability to be able to acquire signals over potentially very long distances using intrinsically collimated lasers and ability to be coupled with fiber optics. The examples for biohazard detection remotely using optical fibres are limited though141, 142. This could be due to the high background from silica based optical fibres but recent hollow core fibre technologies offer hope143, 144. Nevertheless, technologies such as portable Raman spectrometers permit the design of compact robotic devices that can be mounted on unmanned ground vehicles (UGVs) 50, 74. 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 returned light is reduced as the square of the distance to the detector, with further signal loss from absorption and scattering of light by the environment in the beam path. 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 emitting145, 146. 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 260nm62. Using UV lasers with a wavelength shorter than 250nm also has the added benefit of avoiding the fluorescence associated with biological samples62.

Despite the difficulties of collecting the Raman signal at distance, systems have been designed that can detect Raman signals at distances of over 100m147, with one study claiming detection at a 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 kilometre148, indicating that true stand-off detection by Raman may be 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 DeltaNu145. The same system has also been used to detect explosive materials at a distance of 25m146.

Time-gated Raman spectroscopy has been a focus of recent attention for stand-off detection applications. The ability to gate the collection of signal allows this approach to mitigate some of the fluorescent signal, which takes place over a longer time scale than the very rapid Raman scattering process, as well as exclude interference from daylight, and atmospheric gases, like N2 and O2, which would otherwise contribute to the detected signal to a measurable extent. Several studies have reported on this technique in the last two years, detecting explosive materials at distances from 50cm to over one mile19-21, 37, 149, as well as on a range of materials, such as soil, wood, and grass19, 21. The ability to detect samples of interest on these kinds of materials is a vital component of Raman’s development into a viable tool for security applications, as real-world samples are often contaminated, or on less-than-ideal surfaces.

Recently Misra *et al* utilised time-gated for collection of Raman spectra of chemicals at a range of 1.75km during afternoon daylight149. The authors utilised a Q-switched Nd:YAG laser to provide 532nm excitation in 10ns pulses of 100mJ/pulse. The beam was expanded and collimated to illuminate distant targets, and signal was collected via a telescope that coupled into the spectrometer via a 50mm f/1.8 camera lens. Signals for naphthalene with 1s of exposure, using a 100ns gate width and gate delay of 11.75µs, even at a distance of 1.75km show strong features and good reproducibility between acquisitions. Similarly high quality results were reported for urea, ammonium nitrate, nitrobenzene, and potassium chlorate. These results further demonstrate potential of stand-off Raman spectroscopy as a tool for the detection of chemical hazards.

Following the development of a spatial heterodyne Raman spectrometer by Gomer *et al* in 2011150, Hu *et al* have utilised the technology to build a stand-off system capable of detecting CWA simulants both *in situ* and at a distance of 11m, using off-the-shelf products59. To achieve stand-off detection, the group simply replaced the imaging optics for *in situ* detection with a telescope. With only 26mW of power, the system was capable of a SNR of greater than five, and yielded reasonable spectra with only 10s of integration time.

CARS has also been explored for stand-off detection, with early demonstrations of this technique working effectively at 12m arriving in 2008151, 152. Subsequent work has demonstrated CARS imaging and low LoDs of explosive materials153, 154. 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.

# **Conclusions and outlook**

Rapid and accurate detection and identification of chemical and biological threat agents is possible through Raman spectroscopy techniques. Raman scattering based techniques have evolved over the years and have resulted in a suite of advanced methods that can be applied advantageously to detection of a range of hazardous materials. While Raman spectroscopy itself has been augmented to permit higher signals through pre-concentration or to obtain information from deep within containers through techniques such as SORS, there are now many other techniques that are strong contenders for in-field detection. SERS and its variations such as VERS, as well as waveguide-enhanced Raman and coherent Raman techniques show promise to enable trace-level detection with further development. All these techniques can actually be combined with each other, as well as with engineered and robotic systems to enable remote detection. 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. With further work to develop and train chemometric methods, it may be possible to use Raman spectroscopy to discriminate between closely related samples, and suspected samples in a range of complex matrices. For Raman techniques to develop into common tools for this role, spectral libraries of materials of interests and close analogues must be developed, and constantly maintained and updated as new hazards emerge. This library-building, in turn, requires that standardised methods be established to allow for the comparison of results between machines and labs across the globe. While work in this direction for developing photometric standards155 and calibration procedures for spectrometers156 has been going on it is yet to become a standard practice adapted across research labs. Furthermore, there can be shifts in vibrational frequencies between RS and SERS and some peaks can be absent in the latter, making inter-technique comparison challenging. Hence, there exists are plethora of spectra of different hazardous materials and agents but small shifts in vibrational frequencies and relative peak intensities make interpretability difficult. For widespread adoption of Raman based techniques for critical analytical applications such as for homeland and security there is an urgent need to develop machine-independent spectral libraries and universal detection protocols.

In future research work, it would be beneficial to see further development of rapid techniques for biological toxins and whole pathogens that do not rely on lengthy extraction steps or the development of aptameric substances to aid in isolation of the materials. 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. Development of sophisticated machine learning tools and artificial intelligences could play a part in this expansion, enhancing the detection of materials of interest from within the enormous variety of matrices, breakdown products, and confounding environmental contaminants that are often associated with these materials. These classification systems would likely be aided by combination of Raman spectra with fluorescence and morphological characteristics, such as size and aspect ratio, that can acquired simultaneously.

Further, additional work to develop autonomous, stand-alone - for static deployment or mounting on unmanned vehicles - 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 in the field.

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