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

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**Contents**

List of Tables …………………………………………………………………………………………………….………………… X

List of Figures ……………………………………………………………………………………………………….….…………. X

Declaration of Authorship …………………………………………..………………………………………….………….. X

Definitions and Abbreviations ………………………………………………………………..………….……………….. X

1 Introduction …………………………………………………………………………………………………………………….. X

* 1. Aims …………………………………………………………………………………………………………….……. X
  2. Light-Matter Interactions ……………………………………………………………………………….…. X
     1. Raman scattering ………………………………………………………………………….…. X
  3. Enhancement of Raman Scattering …………………………………………………………………... X
     1. Single nanoparticles
     2. Nanoparticle dimers
     3. Complex nanoparticle aggregates
     4. Mechanisms of SERS enhancement ………………………………………………..… X
  4. Raman Scattering Techniques for Homeland Security Applications ……………….…… X
     1. Introduction ……………………………………………………………………………….……. X
     2. Applications of Raman scattering-based techniques to homeland security applications ………………….……………………………………………….……. X
     3. Conclusions ………………………….…………………………………………………….……. X

1. Methodologies ……………………………………………………………………………………………………….….…. X
   1. SERS ………………………………………………………………………………………………………….………. X
   2. SERS Spectral processing and chemometric analysis ………………………………….……... X
2. Development of a SERS-based detection system for bioaerosols ……………………….…………. X
   1. Introduction …………………………………………………………………………………………………..…. X
   2. Methods ……………………………………………………………………………………………………………. X
   3. Results and Discussion ………………………………………………………………………………………. X
      1. ALL OF THE RELEVANT BITS OF THAT …………………….…………………………. X
      2. ALL OF THE RELEVANT BITS OF THAT ……………………….………………………. X
      3. ALL OF THE RELEVANT BITS OF THAT ……………………….………………………. X
      4. ALL OF THE RELEVANT BITS OF THAT …………………………….…………………. X
   4. Conclusions ………………………………………………………………………………………………………. X
3. Outlook and Future Research …………………………………………………………………….…………………. X
4. Acknowledgements ……………………………………………………………………………………………………... X
5. References …………………………………………………………………………………………..………………………. X

**List of Tables**

**Table 1** List of the CDC’s ’Dirty Dozen’ biological agents, and chemical agents of importance ……………………………………………………………………………………………………………………………………………. X

**Table 2** Summary of basic advantages and disadvantages of three common Raman spectroscopy techniques ……………………………………………………………………………………………...……. X

**List of Figures**

**Figure 1** Major energetic transitions caused by interactions between light and matter. **Left**: Absorption of a photon of energy by a molecule, resulting in the molecule being excited into a new vibrational (νn) or electronic (Sn) state. The type of transition depends on the energy of the incident photon. Infrared photons will promote to a new vibrational state, whilst ultraviolet or visible photons are responsible for electronic transitions. **Centre**: Emission of a photon possessing the energy released by the molecule as it relaxes from an excited electronic state, both spontaneously and by stimulated emission. **Right**: Scattering processes resulting from the excitation of the molecule to a virtual state, and the simultaneous emission of a photon with equal (elastic, Rayleigh scattering), lower (inelastic, Stokes scattering), or higher (inelastic, Anti-Stokes scattering) energy. These processes result in the molecule finishing the interaction with equal, higher, or lower energy than it starts with, respectively. ……………………………………………………………………………………………………………………………………………………. X

**Figure 2** Illustration of the mutual exclusivity of vibrational modes in the centrosymmetric molecule, carbon dioxide. Carbon dioxide possesses four vibrational modes: The symmetric (**left**) and asymmetric (**centre**) stretches, and the in and out of plane bending vibrational modes (**right**). Symmetric stretches produce no change in the dipole moment of the molecule, but do induce a change in the polarizability of the electron cloud. As such, it is Raman active, but inactive in IR. The remaining three vibrational modes each induce a change in the dipole moment of the molecule, and thus are IR active, but Raman inactive. …………………………………………………………….. X

**Figure 3** Diagrammatic representation of the oscillation of the negatively charged electron plasma around a gold nanoparticle. Displacement from the nuclei occurs in response to the oscillating electric field of incident radiation, leading to time-dependent dipole moments on the nanoparticles as the charge across the particle fluctuates. ……………………………………………………………………………………………………………………………………………………….. X

**Figure 4** Diagrammatic representation of the effect of the polarisation of the incident electrical field on the coupling of plasmons between particles in a dimer. **Left**: The applied electrical field is parallel to the longitudinal axis of the dimer, and causes a strong coupling effect that increasingly red-shifts the plasmon as particle separation decreases. **Right**: The applied field is perpendicular to the longitudinal axis of the dimer and leads to a destructive effect that causes only weak coupling between the plasmons. This leads to an increasing blue-shift in the resonance wavelength as separation decreases. ………………………………………………………………………………….... X

**Figure 5** Diagrammatic representation of dimeric plasmon hybridisation. **Top**: Geometry of the problem. **Bottom**: Hybridisation of the plasmons. Plasmons with a given angular momentum, l, interact with plasmons of differing angular moment on the second particle, leading to extra shifts of the plasmon at decreasing values of d. Xi is defined as being the distance between the centre of a particle, i, and a point on the surface of the second particle, j, with a polar angle of θj. Θi is the corresponding polar angle in the coordinate system of i. …….……….. X

**Figure 6** Energy transitions between an analyte and a nanoparticle in the chemical enhancement mechanism of SERS enhancement. In this mechanism, the Fermi energy level of the metal, EF, acts as an intermediate energy level between the HOMO and LUMO of the analyte, allowing HOMO-NP and NP-LUMO transitions to occur at lower energies than would otherwise be required for the HOMO-LUMO transition in the isolated analyte. ……. X

**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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Date:

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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 |
| CCD | Charge Coupled Device (Camera) |
| CDC | Centre for Disease Control |
| CDM | Composite Dipole Modelling |
| 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 |
| LBL | Layer-by-Layer |
| LD(A) | Linear Discriminant (Analysis) |
| LoD/Q | Limit of Detection/Quantification |
| MS | Mass spectrometry |
| NIR | Near Infrared |
| PA | Anthrax Protective Antigen |
| PBS | Phosphate buffered solution |
| PC(A) | 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 |
| SPR | Surface Plasmon Resonance |
| TNT | Trinitrotoluene |
| UV | Ultra-violet |
| VX | Venemous Agent X |

**1 Introduction**

* 1. **Aims**

Threats against military and civilian targets can come in a variety of forms, from active shooters to explosives, such as the improvised devices used in active conflict zones, or to destroy Pan Am flight 103 over Lockerbie, Scotland. Of particular concern in recent years is the risk of a chemical or biological attack against troops or civilian targets. Beginning with the Sarin gas attack on Tokyo’s subway system by the Japanese doomsday cult, Aum Shinrikyō in 1995, the risk that these highly controlled and lethal substances could be deployed by terrorist groups was highlighted to the world at large. Similarly, the 2001 “Amerithrax” anthrax attacks and the 2003 ricin attacks through the US mail system illustrated a continued threat from chemical and biological threat agents to non-combatants. Governments, too, have deployed these lethal agents against civilians in Iraq and Syria, opening the possibility that stockpiles of these agents could also be utilised against armed forces in the theatre of war. As recently as March 2018, a chemical from the Novichok series of nerve agents was used in the attempted assassination of Sergei and Yulia Skripal in Salisbury, England, though it is unclear who was behind the attack. From this history of attacks, it is clear that there is a need for methods by which to detect and identify these toxins when they are deployed.

The extreme toxicity (Sarin: LD50 = 172μg kg-1 in mice[1](#_ENREF_1). Botulinum toxin: LD50 ≈ 0.09μg intravenously up to 70μg orally[2](#_ENREF_2)) and pathogenicity (median infectious dose for *b. anthracis*: 8,000-50,000 spores[3](#_ENREF_3). Ebola, another possible threat agent: 1-10 virions[3](#_ENREF_3" \o "Franz, 1997 #187)) of these threat agents makes the requirement for low limits of detection one of paramount importance in designing sensing modalities. Furthermore, any detection system that is designed must be specific enough that the incidences of false positives and negatives are minimised, or else unnecessary panic from false alarms may be caused, or cases of release of threat agents may go unrecognised. Other desirable properties for threat agent detection systems include: the ability to screen samples quickly, or in real time; the ability to be operated by non-specialists; the ability to operate in a variety of locations, without the need for specific facilities.

Mass spectrometry (MS) is one of a set of commonly used techniques that is deployed for the detection of bioterrorism agents. The developments in this field have been subject to two exceptional reviews since 2008[4](#_ENREF_4), [5](#_ENREF_5). One of the great advantages of this technology is that it can exhibit great specificity, having been able to discriminate between closely related bacterial species via the fingerprints of ribosomal and acid-soluble small proteins[6](#_ENREF_6), and has the capability to achieve reliable discrimination at the strain level[7](#_ENREF_7). However, MS suffers from drawbacks that mean it is not always an ideal fit for the role. For example, whilst miniaturised, handheld MS devices have been described for *in situ* monitoring[8](#_ENREF_8), such miniaturisation comes at the expense of lost sensitivity[4](#_ENREF_4). A recent study using Matrix-Assisted Laser Desorption Ionisation Time of Flight MS achieved the detection of 2.5x106 spores in thirty minutes[9](#_ENREF_9). It should be noted that this limit of detection is significantly higher than the stated infectious dose of *b. anthracis*, and may result in potentially infectious exposure events going undetected. Similarly, whilst a thirty-minute detection window may be suitable for pathogenic organisms that take time to amplify in the body before becoming symptomatic, molecular toxins do not have this lead-in time. As such, it would be advantageous to reduce this detection time as far as possible so that people can be removed from contaminated sites as quickly as possible, reducing their exposure to the toxin.

This thesis aims to address some of the shortcomings in the existing methods of biosurveillance and threat detection by developing a methodology based on Surface-Enhanced Raman Spectroscopy (SERS). As a technique, SERS is widely studied as a tool for a number of biological problems, including diagnostics and the identification of organisms in a label-free, sensitive, and non-destructive manner.

SERS uses the unique optical properties associated with the plasmonics of nanostructured metals, typically gold or silver, to enhance the intensity of Raman signals obtained from materials. These enhancements are extremely large, with reports of enhancements of up to 1010 [10](#_ENREF_10). These enhancements enable highly sensitive detection, comparable to fluorescence[11](#_ENREF_11), [12](#_ENREF_12). Several studies have also reported single molecule detection, including several reviews[13-16](#_ENREF_13). Another advantage of SERS is that it retains the fine spectral resolution and molecular fingerprinting capabilities of spontaneous Raman spectroscopy, facilitating greater specificity than techniques likes fluorescence spectroscopies.

The work presented herein represents the current progress towards the final aim of developing a SERS-based sensing platform for both pathogenic organisms and biological toxins that are of interest due to their potential to be weaponised. The system should ideally use minimal pre-treatment of the sample to reduce the time taken for samples to go from collection to detection, as well as meet the requirements for high throughput, low limits of detection, and high specificity. The intended SERS method introduces the samples to layer-by-layer (LBL) SERS substrates immobilised on filters via an air-sampling pump. This method would bring proteins and small molecules into the enhancing field of the substrate. Larger analytes, such as whole bacteria, are too large to fit within the enhancing field, but their surface coatings and membrane components would still be in range. It has been suggested that there is greater difference between strains and species in their surface layers than their cytoplasmic contents[17](#_ENREF_17), meaning that this ability to only enhance the outermost regions is not particularly problematic. Once the method is established, efforts can be directed towards optimisation of the method for specific agents by investigation of functionalising the substrate with aptamers or other capture strands, and potentially to the development of a microfluidics-based variant to enable more flexibility in the methodology, or allow it to be easily combined with a secondary analysis.

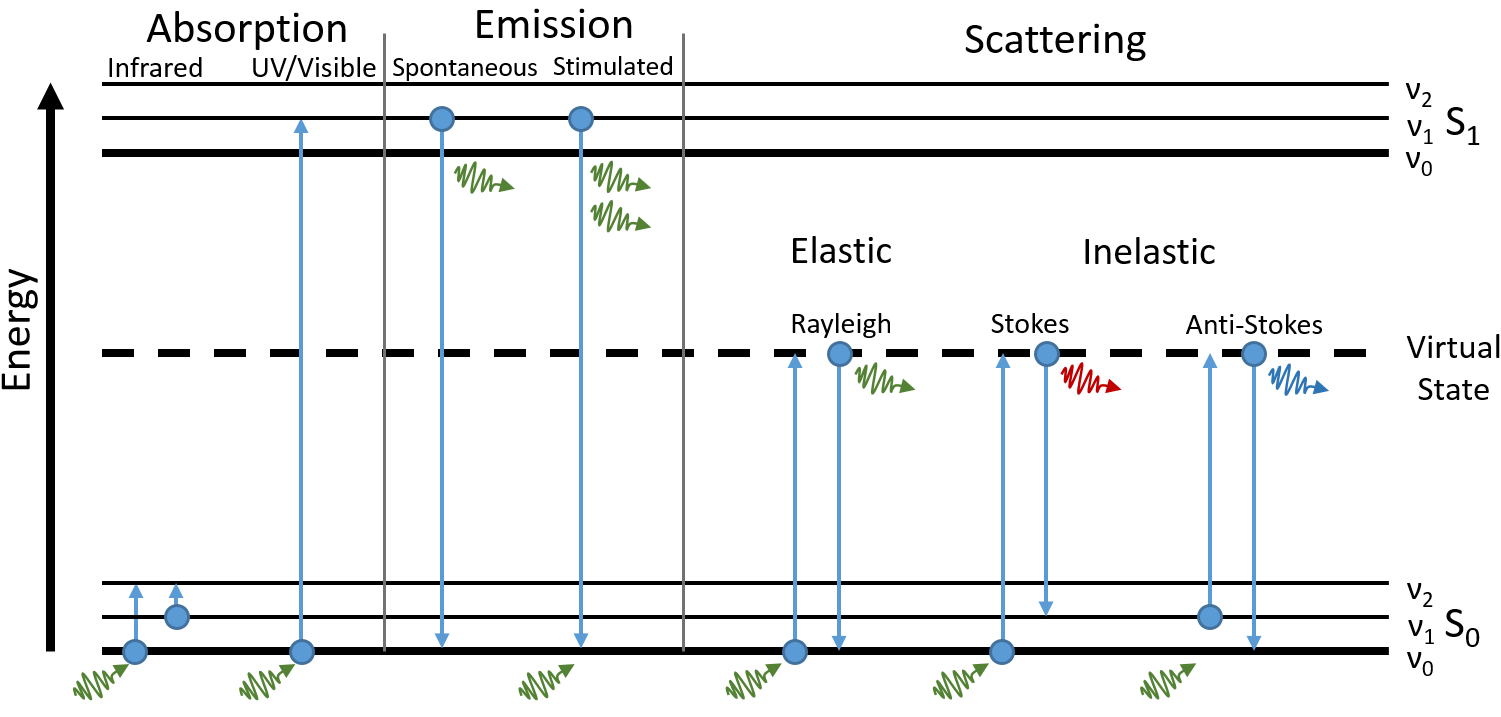
The aims of this studentship are:

* Develop an understanding of theoretical and practical Raman and enhanced-Raman scattering experiments, and their use in detecting both molecules and microorganisms.
* Optimise a substrate for the detection of biological threat agents.
* Develop and optimise an aerosol-sampling platform for use in conjunction with the established sensing substrate.

The first section of this thesis deals with a discussion of the theoretical underpinnings of Raman spectroscopy, as well as the origins of the enhancements arising from metallic nanoparticles in SERS. This discussion is followed by a comprehensive review of the literature surrounding the use of Raman-scattering techniques as applied to homeland security problems. The remainder of the thesis addresses the methods and results gained from the first 18 months of research. The research provides details of the steps taken towards the development of an optimised LBL SERS substrate with an affinity for an important biomarker that shows poor binding with the gold surface of the substrate. It also presents some preliminary work on the detection of aerosols through the combined user of a simple air-sampling unit in conjunction with the sensing substrate. The information gained from this work can then be taken forward and used in developing the system such that it is capable of detecting the aerosol release of spores and simulants of toxic proteins that are of greater interest for any device that would see real-world use.

* 1. **Light-Matter Interactions**

The interactions between light and matter are fundamental to the way in which we understand the world and universe, and are the foundation of the fields of spectroscopy and imaging which play important roles in research. Interactions occur between radiation (which, in accordance with wave-particle duality, is modelled as either particles (photons) or a propagating oscillating dipole) and a material’s defined energy levels. This material can be anything from atoms to complex molecules and mixtures. These interactions cause a variety of different energy transitions that are shown in Figure **1**.

The energy levels of a molecule are comprised of combinations of electronic, vibrational, and rotational states. Of these, the electronic states are separated by the largest difference in energy. The lowest energy, and thereby most stable, is the electronic ground state (S0), with the excited electronic states (S1, S2, …, Sn) above it. The transition between an energetic state and a second, higher, energetic state is achieved by the absorption of a photon (Figure **1**, left). Vibrational states exist as energetic sublevels of an electronic state, and arise from the vibrational state of chemical bonds within the molecule. As with electronic states, vibrational states increase in energy (ν0, ν1, ν2, …, νn,). The variation in energy is caused by the movement of electrons in relation to the nuclei, which are far heavier. The smallest energy differences lie between rotational energy levels (j0, j1, j2, …, jn), and are predominantly relevant to gaseous molecules and rotational spectroscopy, which will receive no discussion.

**Figure 1** Major energetic transitions caused by interactions between light and matter. **Left**: Absorption of a photon of energy by a molecule, resulting in the molecule being excited into a new vibrational (νn) or electronic (Sn) state. The type of transition depends on the energy of the incident photon. Infrared photons will promote to a new vibrational state, whilst ultraviolet or visible photons are responsible for electronic transitions. **Centre**: Emission of a photon possessing the energy released by the molecule as it relaxes from an excited electronic state, both spontaneously and by stimulated emission. **Right**: Scattering processes resulting from the excitation of the molecule to a virtual state, and the simultaneous emission of a photon with equal (elastic, Rayleigh scattering), lower (inelastic, Stokes scattering), or higher (inelastic, Anti-Stokes scattering) energy. These processes result in the molecule finishing the interaction with equal, higher, or lower energy than it starts with, respectively.

Emission processes are the opposite of absorption. In an emission process, molecules in higher electronic states relax to a lower state by emitting a photon with energy corresponding to the difference in energies between the starting state and the end state, as shown in Figure **1** centre. These processes may be either spontaneous or stimulated, where emission occurs as a result of an incident photon matching the energy of the excited state. An example of an emission process is fluorescence. In fluorescence, the molecule emits a photon that is at a longer wavelength than the incident light used to excite it to an excited electronic state. This red-shifting of the emitted light occurs because of non-radiative decay processes occurring between the vibrational levels of the electronic states of the molecule.

Scattering differs from absorption and emission in that there is no stable intermediate electronic state. It is regarded as the instantaneous occurrence of both an absorption and emission event. In scattering, the energy of the incident radiation is insufficient to promote the molecule to an excited electronic state; however, the molecule is promoted to a virtual excited state, which is not a real, stable state of the molecule. As such, it immediately relaxes. The virtual state is an alteration of the polarisation of the electron cloud of the molecule by the exciting radiation. This causes the molecule to adopt a new, higher energy, electronic geometry from which it immediately decays back to the energetically favourable ground state.

Scattering processes can be either elastic or inelastic in nature, meaning that energy of the molecule is either conserved or altered by the interaction with the incident radiation, respectively. Rayleigh scattering is an elastic scattering process that which culminates in the molecule returning to the same energy level from which it started. This means there is no difference in the energies of the incident and scattered photons (i.e. Eout = Ein = E0). This arises because the extremely short-lived virtual state affords the molecule minimal time for nuclear motion in its temporarily altered electron geometry. As such, elastic scattering events are by far the most common scattering mechanism with only one in every million photons being scattered inelastically. This scarcity of inelastically scattered photons means that Raman scattering is inherently very weak by comparison.

Stokes scattering is the process whereby a molecule undergoes an inelastic scattering event and finishes in a higher energy state that it originated in, resulting in it occupying an excited vibrational state. The energy lost by scattered photons is equal to the difference in energy between the initial and final vibrational states. Anti-Stokes scattering is also a process whereby a molecule undergoes an inelastic scattering event; however, in Anti-Stokes scattering the molecule finishes in a lower energy state that it began in. The difference in energy is gained by the scattering photon, such that EAnti-Stokes = E0 + Evib. This gain of energy imposes a requirement that, for Anti-Stokes scattering to occur, the molecule must start in a vibrationally-excited state, meaning that Anti-Stokes scattering is even weaker than its Stokes counterpart. The reason for this can be seen by considering a Boltzmann distribution, which mathematically represents the relative populations of two energy levels.

Here, N represents the extent of population of states n and m by electrons, g is the degeneracy of each state, ΔE is En – Em, T is temperature in Kelvin, and kB is Boltzmann’s constant. In this case, m represents the ground state under normal conditions. When En > Em, this tends towards zero for higher values of En (which corresponds to higher energy states). As such, the ground state is more highly populated than the levels above it, making more electrons available for Stokes scattering than for Anti-Stokes, making the former more common than the latter. The ratio of Stokes to Anti-Stokes scattering decreases with increasing temperature.

* + 1. **Raman Scattering**

Vibrational spectroscopic techniques are commonly used in research and analytical work. They have a selection rule that mandates that the vibrational energy of the transition to change by 1 quanta for it to be observed (Δν = ± 1); however, infrared (IR) and Raman spectroscopy each have additional selection rules that determine the activity of a vibrational mode when irradiated with plane polarised radiation. IR spectroscopy requires that there be a change in the dipole moment (µ) of the molecules electron cloud for a vibration to be observed. By contrast, Raman activity requires that there be a change in the polarizability of the electron cloud. Polarizability, α, is defined as the ease with which the electron cloud of an atom or molecule can be distorted by an electric field. Polarizability contributes to a molecules dipole moment according to the relationship:

Here, E defines the electric field strength that the molecule is experiencing. Dipoles are induced by the displacement of electrons and atomic nuclei in response to their exposure to electric fields, because of their respective charges. The requirement for a change in polarizability to observe Raman scattering can be derived using a classical model of light as an electromagnetic wave with a field strength of E0[18](#_ENREF_18). The scattering of photons occurs in all directions and can be considered spherical.

The electric field strength of incident radiation varies as a function of time (t) and the frequency (ν) of the radiation, maintaining a constant maximum value (E0), which is its amplitude.

This oscillating electric field causes distortions in the electronic field around a molecule, resulting in an induced dipole, μind, such that:

In the event that the molecule under examination already has a dipole moment (µ) prior to the application of the electric field, its total dipole moment (µtotal) is the combination of the existing and induced fields:

It is also necessary to consider the nuclear displacements occurring during the interaction. These displacements cause changes to both the electronic conformation and to the internuclear distances. In the case of diatomic molecules, the displacement, dQ, varies with the vibrational frequency of the mode, νm, and the maximum possible coordinate, Q0.

Both polarizability and the dipole moment can be modelled using a Taylor series expansion, with each new term in the series contributing a decreasing amount to the total.

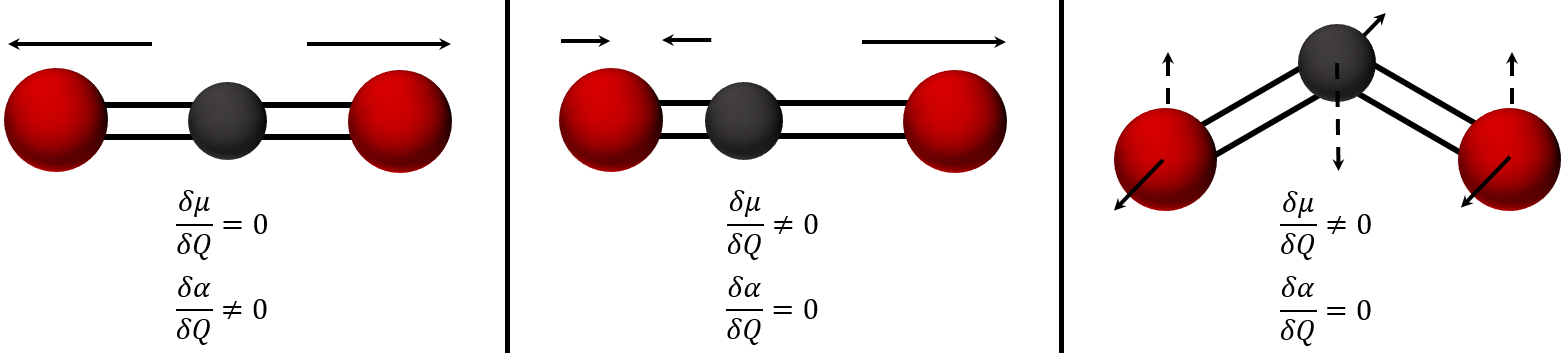
Here, α0 and µ0, represent the values of polarizability and the dipole moment at the equilibrium position, respectively. Assuming a very small nuclear displacement, polarizability and the induced dipole become:

As such, μind becomes:

Combination of the induced and permanent dipole moments then yields a final equation for the total dipole moment.

Expressions for each component of vibrational spectroscopy are observed in the equation above. The left-most component represents the permanent dipole of the diatomic molecule. The second is a non-zero change in the value of the dipole moment with nuclear displacement from the vibration (), giving rise to IR activity. Subsequent terms relate to scattering interactions of the induced dipole with incident radiation. The first contains the incident radiation, and thus is Rayleigh scattering. The final two terms represent Stokes and anti-Stokes interactions with the induced dipole. The equation also shows that in order to probe νm, with the incident frequency, ν, it is required that there be a change in polarizability with nuclear displacement so that .

This can be exemplified using a simple example molecule. In this case, carbon dioxide (CO2) has been chosen. The number of vibrational degrees of freedom that a molecule possesses depends on its symmetry. A linear molecule has 3N – 5 vibrational modes, whilst non-linear molecules have 3N – 6. Here, N is the number of atoms in the molecule. Thus, CO2 has four vibrational modes from its nine degrees of freedom (3N). IR and Raman have a principle of mutual exclusivity in molecules that possess centrosymmetry (such as CO2). This means that any vibrational mode exhibited by the molecule can only induced a change in the dipole or the polarizability, and therefore be active in IR or Raman, but never in both. This principle is illustrated for the case of CO2 in figure **2**. As can be seen in the illustration, the only vibration to induce a change in polarizability is the symmetric stretch, making it the only Raman-active mode for the molecule. In molecules that lack centrosymmetry, intensity in IR and Raman varies. Some modes are more strongly observed with one technique than with the other. As such, IR and Raman can be regarded as complimentary techniques, allowing the acquisition of comprehensive vibrational spectra of a sample. This can be particularly useful in applications where water is likely to be present, such as biological samples), because the IR-active OH stretch is broad and strong, and can mask less intense IR peaks that lie in the same region of the spectrum. By contrast, water does not exhibit Raman activity.

Raman spectroscopy has several advantages as an analytical technique. It is label-free and can be acquired from distance or through containers, and is non-invasive. It also requires little to no preparation of samples, whilst still allowing for the collection of molecular fingerprints for a material. However, as mentioned above, Raman scattering accounts for only one in every million incident photons, leading to low intensities for observed vibrations. These weaker signals can be lost beneath the broad, high intensity fluorescent backgrounds that can be present in some samples. Weak signals can also require the use of long exposure times, or higher laser powers. These may be destructive to delicate samples, or otherwise be unsuitable for the intended application of the method. Thankfully, variations on spontaneous Raman have been devised, helping to enhance the weak signals to enable the mitigation of the aforementioned issues. For example, Surface-Enhanced Raman spectroscopy (SERS) utilises the properties of the plasmons on nanostructured noble metals to provide enhancements of 106-1010 [10](#_ENREF_10).

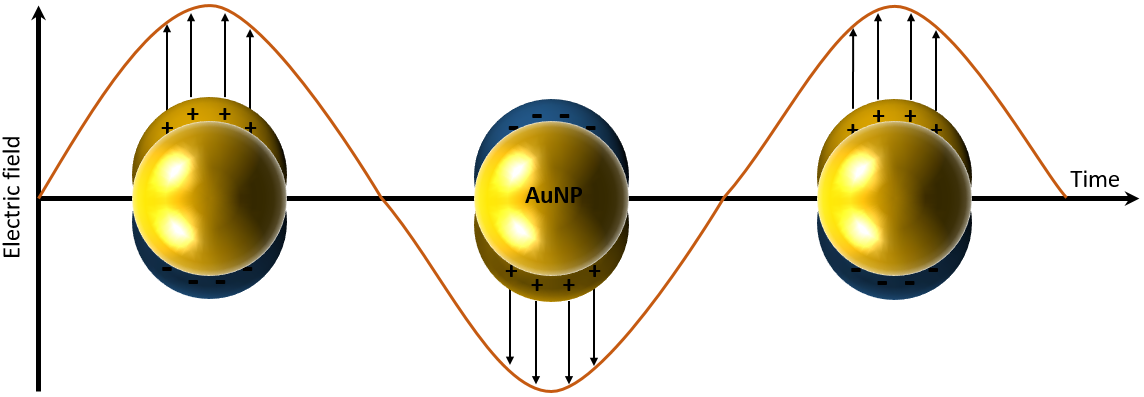
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* 1. **Enhancement of Raman Scattering**

Spherical gold nanoparticles (AuNPs) are perhaps one of the simplest and most common morphologies employed as a SERS substrate. As gold is a conductive metal, its electrons exist within a conductance band, and can be modelled as a free moving, negatively charged plasma on a fixed, positively charged lattice of atomic nuclei. When exposed to plane wave excitation, the electron plasma of the nanoparticle becomes displace from the nuclei, causing a polarisation of the metal particle. The particle oscillates about its nuclear coordinates with the incident radiation, moved by restoring coulombic attractions. This behaviour is shown in figure **3**. The collective oscillation of the surface charge density of the particle is known as the surface plasmon, and is a time-dependent and non-uniform polarisation of the nanoparticle with multiple orders of motion. The frequency of the plasmon resonance varies with the electron density, the effective mass of the unbound electrons, and the size and shape of the distribution of charge[19](#_ENREF_19).

The response of bulk metal to an applied field can be described using the Drude model, through the dielectric functions of the nanoparticle, ϵ, and the surrounding medium, ϵm. In the coming sections, the response of spherical AuNPs will be modelled. First, the case of a single nanoparticle will be addressed, before moving to dimers and finally to more complicated aggregates of particles. Finally, the appearance of the surface plasmon resonance (SPR), will be discussed in regards to the enhancement of Raman scattering, and in terms of the mechanisms by which these enhancements arise.

* + 1. **Single nanoparticles**

Given the dependence of SPR frequency on particle size, it is first necessary to consider the response of a single, spherical particle with a radius substantially smaller than the wavelength of the incident radiation (r << λ). In this case, the dominant contribution to the electronic oscillations come from dipole motion. Higher order polarisation can occur when the plasma is oriented parallel or anti-parallel to the applied field, but these more complex interactions are neglected in this model.

**Figure 3** Diagrammatic representation of the oscillation of the negatively charged electron plasma around a gold nanoparticle. Displacement from the nuclei occurs in response to the oscillating electric field of incident radiation, leading to time-dependent dipole moments on the nanoparticles as the charge across the particle fluctuates.

Adopting a quasi-static view, the electric field, *E*, can be regarded as being constant across the nanoparticle, so interactions are only governed by electrostatics, and not by the electrodynamic contributions that cause dampening effects in larger particles. The response of free conductance electrons in the particle is described using the dielectric function of the metal as described by the Drude-Somerfield model:

This function depends on the frequency of the incident radiation, ω0, and of the bulk plasma, ωp, as well as a damping constant, *γ*. The dielectric function of the metal has a real component, Re, which governs the frequency and position of the resonance, and an imaginary component, Im, which includes broadening effects and the absorptive dissipation of the resonance from damping and dephasing. The frequency of the plasma can be calculated from the number of unbound electrons, N, the effective mass of the electrons, me, and their charge, *e*, and the permittivity of a vacuum, ε0, as follows:

Additionally, it is important to account for the contributions of bound electrons to the dielectric function of a real metal (ϵ∞). These contributions account for interactions such as inter-band electronic transitions from the valence to conduction bands, which are particularly important at the high frequency end of a spectrum[20](#_ENREF_20). These effects require an addition to the standard Drude model formula shown above to correct for them.

Dipole polarizability, α, describes the collective response a spherical nanoparticle’s electrons to the applied field. It is dependent on the volume of the nanoparticle, V, and a shape factor, K. This shape factor is 2 for a spherical nanoparticle, but can be higher for more shapes that are more polarizable, such as triangles and rids, which exhibit higher curvature along one dimension[21](#_ENREF_21).

From the above, it is clear that polarizability, and therefore the local field strength, tends to a maximum when the real component of the functional tends towards the resonance condition seen in the denominator of the equation above:

As the dielectric function of the medium is typically 1, polarizability maximises when the absolute value of tends to zero (ϵ = -2ϵm). From the above, it is clear that the SPR frequency, ωsp, is determined by the frequency of the bulk plasma of the particle, the particles shape, and the medium surrounding it.

When spherical nanoparticles grow larger, such that their size begins to approximate a ≈ λ/2π, effects such as retardation begin to contribute to the dielectric functional through damping. Under the Drude-Somerfield model, plasmons are regarded as being a superposition of many independent electron oscillations. Such oscillations can become dephasing through scattering events with other electrons, lattice ions, the metal surface, or impurities in the material. This scattering causes a population decay through emission of a photon (radiative damping), or through creation of an electron hole pair by electronic excitation (non-radiative damping). As per the Pauli Exclusion Principle, electrons can only transition to empty states in the conduction band, but these transitions can be intraband (within the conduction band), or interband (originating in the d-band) damping. These damping effects can be corrected using Modified Long Wavelength Approximation to introduce electrodynamic effects to the model[19](#_ENREF_19). As before, the dipole moment is still defined as μ = Eα.

and

Here, χ and ξ0 are geometry-dependent parameters, similar to K mentioned above, but extended to a spheroid model. Similarly, a and b are the minor and major axes of the spheroid, respectively. Of course, a=b for a sphere but the equation shows that the damping constant is depending on the aspect ratio of the particle, red shifting the resonance as b2/a2 increases. It is important to note that the denominator contains a dependence on the dielectric function of the material and the surroundings again. In this case, the resonance condition is achieved when.

Accounting for damping in larger particles requires correction to account for electrodynamic contributions to the dipole moment through the radiative correction field, Erad.

where

In the above equation, terms for both radiative and dynamic damping processes are present. The first term accounts for radiative damping, which occurs by spontaneous emission of a photon by the induced dipole, and increases with particle size. This damping reduces the size of the induced dipole and broadens the linewidth of the plasmon. Dynamic damping arises from the ratio of the particle size to the wavelength of the incident radiation. As with radiative damping, it reduces the dipole’s strength as NP size increases. The net effect of these terms is a modified expression for the dipole moment that is multiple by the following expression[19](#_ENREF_19):

In this equation, we see that the contribution of radiative damping is proportional to k3α, whilst the dynamic damping contribution varies with (α/b)k2. This implies that both contributions will be on the same order when the radius of the particle approaches λ/2π. Calculating this for a wavelength of 600nm, we arrive at a value of approximately 100nm (95.49nm) for b.

The above shows that a single nanoparticle possesses a localised SPR that is resonant across a particularly wavelength range, and which is dependent on the dielectric function and the geometry of the particle; however, it is rare to deal only with a single nanoparticle. Indeed, the large signal enhancements observed in SERS predominantly arise in regions of intense electric field, such as those between nanoparticles. To provide a more valid illustration of the nature of SERS enhancements, it is important to examine the nature of the interactions between the plasmons of particles under the influence of the electrical fields of its neighbours.

* + 1. **Nanoparticle dimers**

The next step in the modelling of SERS enhancement is to examine the interaction of plasmons on two neighbouring particles. The nature of conductance electrons is such that they are only loosely bound around a nucleus, allowing them to couple with others. The result of this is that an observed plasmon resonance would actually be that of several nanoparticles. In these regimes, the plasmon of one particle actually needs to be treated as a constituent part of the surrounding medium of its neighbouring particle when examining dielectric functions. The coupling of plasmon properties causes an incredibly strong electric field to arise in the junctions between the plasmon-generating structures, which are known as hotspots. A molecule caught within one of these hotspots will experience considerable enhancements to its Raman signals, though this is not a homogenous effect and is dependent on factors such as the orientation of the vibrational modes relative to the plasmonic oscillation.

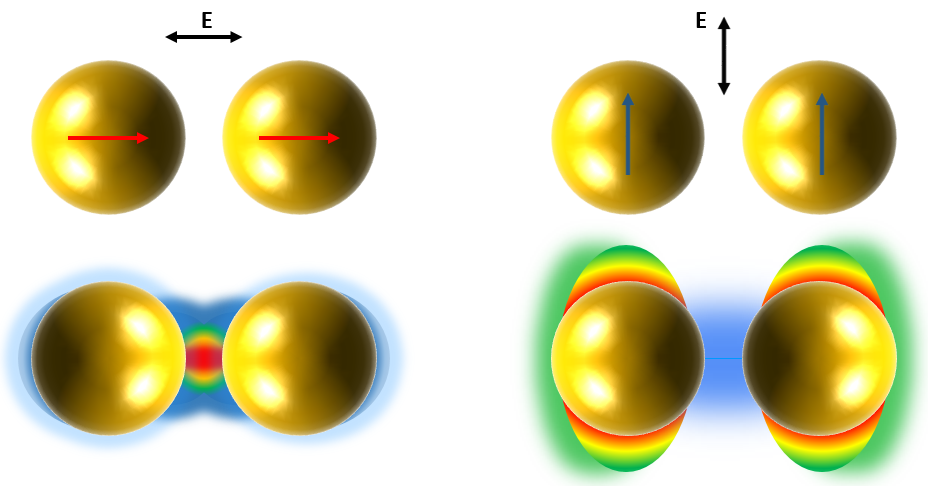
Given that the significant enhancement of SERS come from the junctions between plasmon-generating structures such as nanoparticles, it is clear that it is desirable to have a substrate with a large number of such junctions. A good example of this is aggregated nanoparticles. In this section, the simplest case of an aggregate – the dimer – is examined as a base upon which to develop the theory that is relevant to aggregates that are more complicated. In the case of a dimer that is excited by an electric field polarised along its axis, a strong additive electric field that red-shifts the plasmon is observed[22](#_ENREF_22). Conversely, the effect is destructive if the field is polarised perpendicular to the axis of the dipole, leading to a blue-shifting of the resonance (Figure **4**). This is dependent on the incident field and the field of the neighbouring particle, as mentioned previously.

Where

Here, d is the distance between the particles in the dimer, E0 is the incident radiation field, and ENb is the field of the neighbouring particle. This neighbouring field experiences a dependence on the dipole moment of the particle, μNb, and an orientation effect defined by ξ. θi and θj are angles that represent the dipole moment of the two nanoparticles relative to the axis of the dimer (Figure **5**, top). Edim can experience two modes that contribute to its value via ENb. One of these modes is the parallel-polarised field (longitudinal mode) mentioned in the previous paragraph. Here, ξ = 2. The other mode is the perpendicular-polarised field (transverse mode), where ξ = -1.

For a dimer, the total polarizability is defined as:

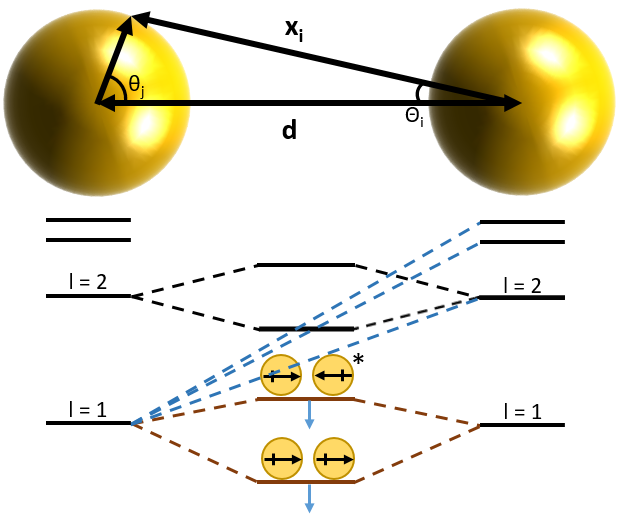
In this instance, the polarizability of a particle tends towards its maximum field enhancement in the resonance condition:

As seen above, the enhancement of the field exhibits a distance dependence in the form of (r/d)3. As d tends to infinity, the equation comes to satisfy the resonance condition of a single particle, causing no coupling to occur.

**Figure 4** Diagrammatic representation of the effect of the polarisation of the incident electrical field on the coupling of plasmons between particles in a dimer. **Left:** The applied electrical field is parallel to the longitudinal axis of the dimer, and causes a strong coupling effect that increasingly red-shifts the plasmon as particle separation decreases. **Right:** The applied field is perpendicular to the longitudinal axis of the dimer and leads to a destructive effect that causes only weak coupling between the plasmons. This leads to an increasing blue-shift in the resonance wavelength as separation decreases.

Plasmon hybridisation between particles bears similarities to the formation of molecular orbitals through the hybridisation of atomic orbitals in chemical bonding[23](#_ENREF_23). As the plasmons approach eachother, they hybridise into new plasmonic modes of higher and lower energy (bonding and antibonding, respectively) than the respective single modes. Using the simple aufbau principle governs that the bonding resonance, with lower energy, is populated by electrons first, achieving a lower-energy resonance. This is depicted pictorially in lower half of figure **5**.

Hybridisation of plasmons can be simply modelled when dielectris effects and the effects of ion cores are neglected. In this model, nanoparticles are regarded as being sufficiently small that the retardation effects can be disregarded (a << λ/2π). The interaction between the surface charges is coulombic. As a function of distance, this parameter, V(d), is defined as:

Where σ(β) is the surface charge of the particle as a function of the solid angle. If the polar axis is defined along the long-axis of the dimer, the interaction is diagonal for the azimuthal quantum number, m, which defines its angular momentum. As a result of this, plasmon modes with differing m values will decouple. A non-interacting dipole is now denoted as n, whilst I defined an interacting mode. Those modes possess energy, ω, and an angular momentum of l on a sphere, N, with a radius of r. Assuming i and j are on the same sphere, is zero. If not, the dependence on distance is modelled as:

**Figure 5** Diagrammatic representation of dimeric plasmon hybridisation. **Top**: Geometry of the problem. **Bottom**: Hybridisation of the plasmons. Plasmons with a given angular momentum, l, interact with plasmons of differing angular moment on the second particle, leading to extra shifts of the plasmon at decreasing values of d. Xi is defined as being the distance between the centre of a particle, i, and a point on the surface of the second particle, j, with a polar angle of θj. Θi is the corresponding polar angle in the coordinate system of i.

integrated across the surface of particle Nj. For spheres plasmon modes with an angular momentum of l and l’, we see that the interaction will disappear as V ∝ d-(l+l’+1).

Interactions Vij and Vji are symmetrical and thus are equal to one another. For m = 0, representing polarisation parallel to the long axis of the dimer, large interparticle distances show weak interaction. In this regime, the hybridisation was analogous to the bonding and anti-bonding combinations mentioned above, and shown in figure **5**. Lowest energy plasmons, where l = 1, show an energy dependence of d-(l+l’+1) (i.e. d-3) that is symmetrical at high values of d. This relationship breaks down at smaller values of d, where the energy symmetry breaks down as the antibonding plasmon gains energy at a slower rate than predicted. This effect arises as the l = 1 plasmons of one particle couple to the plasmons of higher angular momentum on the second particle. The stabilisation of the antibonding orbital is also seen in figure **5**, where it is clear that the stabilisation is arising from attractive interactions between the dipoles of the spheres.

* + 1. **Complex nanoparticle aggregates**

Extending the model beyond simple dimers requires further adaptions of the existing theoretical framework. As discussed previously, close proximity of two nanoparticles produce plasmonic hotspots. Higher assemblies aggregate in an uncontrolled manner, but the response of coupled resonances through 1D chains of particles is significant in the modelling of more complex aggregates through composite dipole modelling (CDM)[24](#_ENREF_24).

A one-dimensional chain of particles is, in essence, a dimer of particles extended over *n* particles with a constant separation, *d*. As before, the orientation of the chain relative to the applied field is an important contributor to the resonance. For a parallel polarisation, longitudinal coupling is achieved. For perpendicular polarisation, the observed plasmon resonance is closer to that of single nanoparticles, as the light creates a weak coupling effect. For the case of a parallel configuration, the red shift of the plasmon resonance is greater than that of a dimer, and shows a dependence on *n*[25](#_ENREF_25). This dependence on *n* is saturable[26](#_ENREF_26" \o "Aguirregabiria, 2017 #211). Evidence of a distance dependence also exists, suggesting greater coupling as *d* decreases[25](#_ENREF_25), [27](#_ENREF_27).

According to CDM theory, the wavelength and width of the plasmon resonance of an aggregate with sub-nanometre inter-particle distance, *d*, is determined by the longitudinal plasmon mode of the longest one-dimensional chain of particles within the aggregate. The properties of the lower energy resonance of the aggregate can be modelled by chains via the spectral extinction coefficient of the aggregate, Aagg.

Here, Vagg is the active volume of the plasmonic surface, ω is angular frequency, c is the speed of light in the medium, Lagg is a depolarisation factor (Lagg for a perfect sphere, is 1/3, though this decreases with particle eccentricity), and ϵNP and ϵm are, as defined earlier, the dielectric constants of the particles and surrounding medium, respectively. Combining the imaginary part of this equation and combining it with the Drude model and compensations for multipolar and retardation factors, it is possible to determine the maximum extinction of the aggregate, .

Where nch is the fraction of the aggregate that exists in the active chain, NP is the effective number of spheres in the chain, and g(NP) is a term that relates the active plasmonic volume to the physical volume of the dominant chain, Vdom. fagg is the product of the square of the damping constant, γ, and the inverse of the total active chain length, a. (i.e. fagg = γ2a-1).

By reference to the extinction peak and simulations of 1D particle chains, it is possible to determine the proportion of nanoparticles contributing to the low energy resonance, η[24](#_ENREF_24" \o "Taylor, 2012 #212).

Where the subscripts ‘agg’ and ‘sp’ represent the properties of the aggregate and of single particles, respectively.

For the purpose of experiments using SERS, localised hotspots are of paramount importance for increasing the quantum yield of the weak Raman scattering processes, as the fluctuations of the surface charge density of the substrate enhance the local electrical field strength experienced by analytes within close proximity to the nanostructured surface. The mechanisms underpinning this process are discussed in the coming section.

* + 1. **Mechanisms of SERS enhancement**

The details of the mechanisms of SERS enhancement are not certain, but two predominant theories are thought to contribute to the phenomenon. The predominant theory is that of electromagnetic enhancement; the second is chemical enhancement. This section will discuss both mechanisms in turn, but will begin with a discussion of the electromagnetic theory.

As demonstrated above, an increase in the electric field experienced by a molecule is key to increasing the degree of inelastic scattering that it produces. If the interaction between the local surface plasmon resonances of neighbouring particles is ignored, it is possible to model the electric field enhancement by a simplification of electric field strength at distance *r* (E*r*) caused by a single particle’s local surface plasmon resonance’s with an incident field of strength E0.

where

From this, it can be seen that the local field strength is dependent on the energy of the incident radiation, ν0, the relative angle of the radiation and polarisation of the nanoparticle (θ represents the transverse or longitudinal modes), the radius of the nanoparticle, *a*, distance from the particle, 1/r3, and a match between the dielectric constants for the metal and medium to the resonance condition discussed earlier (ϵNP = -2ϵm). If these parameters are properly optimised for the experiment being undertaken, the enhancements can be sizeable. This is particularly true when the analyte is localised in a plasmonic hotspot. Here, the enhancement of electric fields around a nanoparticle in response to incident and inelastically scattered radiation, as described by Moskovits, is explained[28](#_ENREF_28).

When incident laser light is resonant with a dipolar plasmon on a metal particle, the particle radiates light that is characteristic of dipolar radiation. This radiation is a coherent process with the incident field, and which can be described as a spatial distribution of electric field magnitudes that reach a steady state a few femtoseconds after the incident radiation is switched on.

Calling the average field enhancement across the particle *g*, and the magnitude of the incident field E0, it is possible to determine the average magnitude of the local near field radiated by the particle, Es, by the formula: . The average molecule adsorbed at the surface of the particle will therefore experience an electric field of magnitude Es, and the Raman scattered light will have a field strength (ER) such that:

Where αR is a Raman tensor of the scattering material. From this, it can be seen that the field strength of the Raman scattered light varies with the field strength of the incident radiation and average field enhancement. The Raman scattered fields, like the incident field, can also be enhanced by the metal particle, meaning the metal particle can scatter light at a Raman shifted wavelength with an enhancement factor of *g’*. The amplitude of this SERS-scattered field, ESERS, is: and the average SERS intensity is proportional to the absolute value of the square of the SERS scattered field.

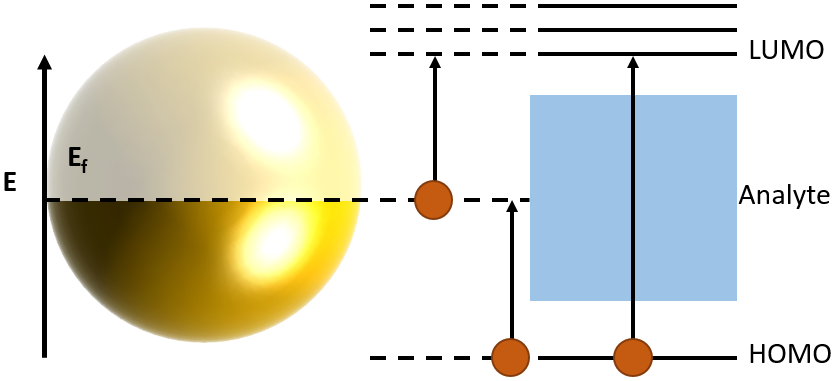
Where I0 and ISERS are the intensity of the incident and SERS-scattered fields, respectively.

In the case of low wave numbers, g approximates g’ the, SERS intensity is enhanced by a factor with proportionality to the fourth power of the enhancement of the local near field of the particle, EL, (i.e. ). This does not hold for higher wavenumber Raman modes, where the SERS intensity is a more complex function with a dependence on plasmon resonance properties of the enhancing particle that vary with the wavelengths of the incident field and Raman scattered light.

The SERS enhancement, G, is defined as the ratio of the intensity of Raman-scattered intensity in the presence of the particle to the intensity of its value when there is no enhancing particle present. As such:

Where αR and αR0 are the Raman polarizabilities of the enhanced and isolated molecules, respectively. This shows that the majority of SERS scattering originates from the plasmons, and not from the molecules themselves.

There are three other important things to note. The first is that, though SERS intensity for low wavenumber modes varies with the fourth power of the local near field, the effect is actually a linear optical effect with a dependence on the first power of I0. Second, the term αR is referred to as the Raman polarizability of the molecule, but it actually refers to the polarizability of the Raman scatterer. In the case of a molecule adsorbed to a metal, this polarizability *includes* the molecule, but also contributions from the metal. This can have significant effects on the magnitude, symmetry and resonance properties of the Raman polarizability when compared to the isolated molecule. This is particularly relevant in systems where charge transfers can occur between the molecule and the metal, and contributes to the chemical enhancements that will be discussed shortly. The third consideration is that SERS excitation is a near-field phenomenon. This near field has spatial components that decay more rapidly with distance than the special components of the far field. This can cause relaxations in the ordinary selection rules, and means that some vibrational modes that are normally forbidden can be observed in the SERS spectrum.

Whilst electromagnetic enhancement by surface plasmons provides a large and non-discriminatory enhancement to local fields, it is also observed that some vibrational modes are always selectively enhanced[29](#_ENREF_29). Chemical enhancement occurs because of an interaction between the analyte and the surface of the metal particle, which is said to be weak chemisorption (weak covalent bonding). The charge transfer enhancement is regarded as being a new resonance Raman process between the electronic ground state and excited energy levels of the complex. These processes are attainable in cases where the highest occupied and lowest unoccupied molecular orbitals are symmetrical around the Fermi energy of the electrons in the metal, forming intermediate levels that facilitate electronic transitions at lower energies that would be possible in the isolated molecule (Figure **6**). These transfers of charge can occur between the metal and the molecule, or vice versa.

**Figure 6** Energy transitions between an analyte and a nanoparticle in the chemical enhancement mechanism of SERS enhancement. In this mechanism, the Fermi energy level of the metal, EF, acts as an intermediate energy level between the HOMO and LUMO of the analyte, allowing HOMO-NP and NP-LUMO transitions to occur at lower energies than would otherwise be required for the HOMO-LUMO transition in the isolated analyte.

It is thought that both of the mechanisms of enhancement discussed above operate independently of one another, it is difficult to observe one without the other. Often, a charge to the experiments has repercussions for both mechanisms, making their isolated study a complex matter[29](#_ENREF_29).

* 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 that 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[[30](#_ENREF_30)]. | | | |
| **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* |  |

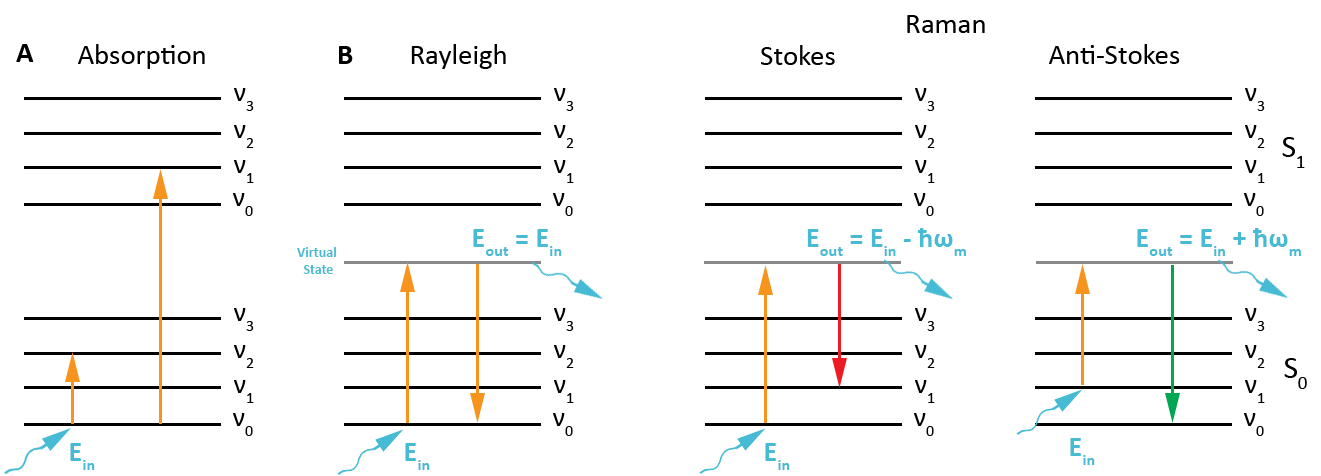
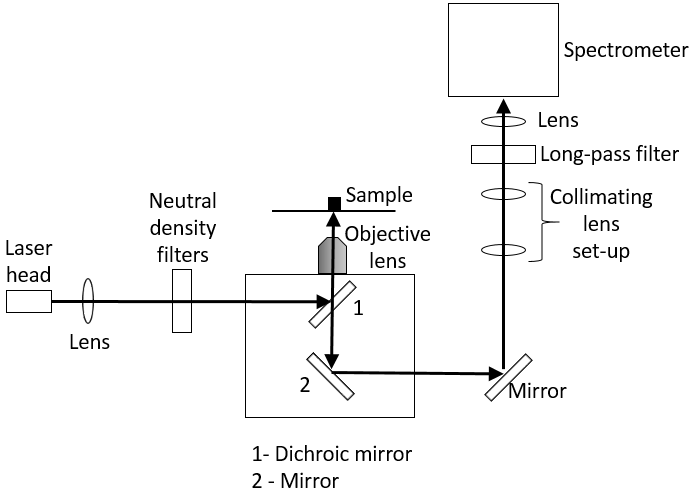
# 1.4.2 Theory of Raman spectroscopy

# 1.4.1.2 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[[31](#_ENREF_31)], but it was not until the work of C. V. Raman in 1928 that the first experimental observations of the phenomenon were made[[32](#_ENREF_32)]. 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 2** 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 3** 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[[33](#_ENREF_33)]. 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[[34](#_ENREF_34), [35](#_ENREF_35)]. 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[[36](#_ENREF_36)].

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[[37](#_ENREF_37)]. 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[[33](#_ENREF_33)].

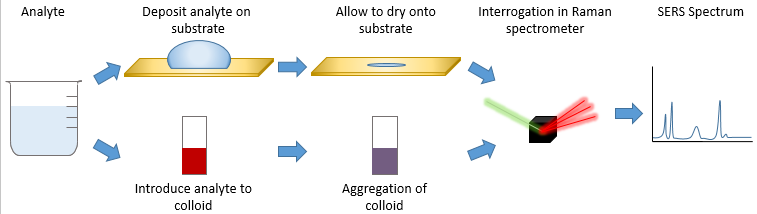
Another key problem with RS is fluorescence that may arise from unintentional electronic excitation of the molecule caused by the laser. It should be noted that this fluorescence may not arise from the target analyte, and may arise from sources like impurities or the environment[[38](#_ENREF_38)]. Fluorescence 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[[39](#_ENREF_39)].

**1.4.1.3 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[[40](#_ENREF_40)]. 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[[41](#_ENREF_41),[10](#_ENREF_10)], which has been demonstrated to be sufficient for single molecule detection[[41](#_ENREF_41),[42](#_ENREF_42)]. 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[[43](#_ENREF_43)]. 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[[44](#_ENREF_44), [45](#_ENREF_45)], 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[[46](#_ENREF_46)].

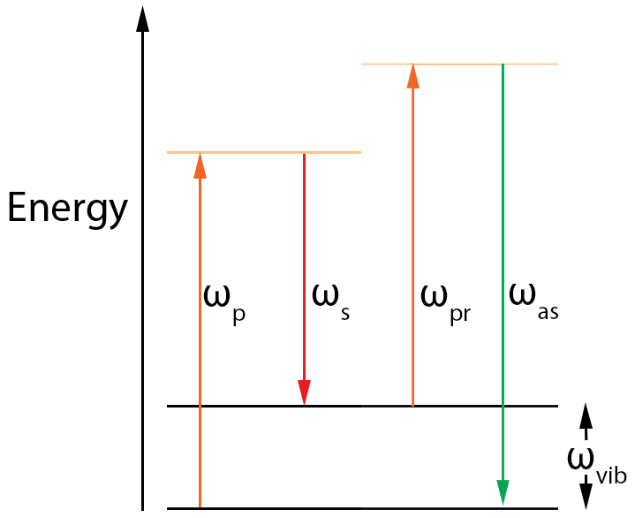
**Figure 4** Diagrammatic representation of two primary means of undertaking a SERS experiment. Top – deposition of analyte onto a substrate such as Klarite. Bottom – Mixing with colloidal nanoparticles and aggregation with a material such as salt.

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[[47](#_ENREF_47)], silver or gold coated microspheres[[48](#_ENREF_48)], metal-polymer nanocomposites[[49](#_ENREF_49)], 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.

**1.4.1.4 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[[50](#_ENREF_50)].

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*[[50](#_ENREF_50), [51](#_ENREF_51)].

**Figure 5** 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[[52-56](#_ENREF_52" \o "Bowman Pilkington, 2016 #24)]. Additionally, the frequency of the Stokes beam can be swept across a range to obtain a spectrum of intensity of *ωas* against *ωpr* – *ωs*[[51](#_ENREF_51)]. 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[[57](#_ENREF_57)]. Additionally, CARS in solution-based analyses can display a significant background that can be difficult to suppress[[50](#_ENREF_50)].

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

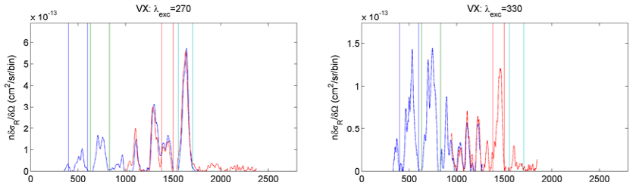
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Table 2:** Summary of basic advantages and disadvantages of three common Raman spectroscopy techniques. | Disadvantages | Fluorescence can dominate spectra.  Scattering events are rare, causing weak signals. | Signal enhancement relies on close proximity 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[[55](#_ENREF_55), [56](#_ENREF_56), [58-69](#_ENREF_58)]. 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[[58-61](#_ENREF_58), [70-75](#_ENREF_70)].

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[[70](#_ENREF_70)]. 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[[59](#_ENREF_59), [61](#_ENREF_61), [67](#_ENREF_67), [70](#_ENREF_70)].

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 **5**). No such changes were found for sulphur mustard (HD) and Tabun (GA)[[61](#_ENREF_61)].

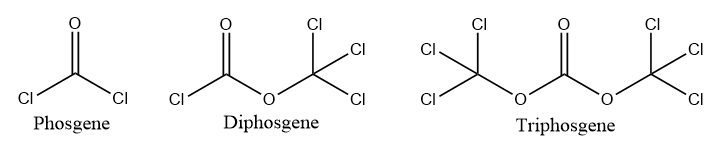
To help overcome the issue of poor Raman scattering cross-sections of many of these analytes, SERS and CARS have been investigated as detection methodologies, owing to their large degree of signal enhancement when compared to spontaneous Raman spectroscopy. This has included several studies on live agents[[58](#_ENREF_58), [60](#_ENREF_60), [73](#_ENREF_73), [75](#_ENREF_75), [76](#_ENREF_76)] and simulants and/or hydrolysis products[[58](#_ENREF_58), [60](#_ENREF_60), [62-65](#_ENREF_62), [68](#_ENREF_68), [69](#_ENREF_69), [73](#_ENREF_73), [76](#_ENREF_76)]. 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[[60](#_ENREF_60)].

**Figure 6** 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.

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[[75](#_ENREF_75)]. The structures of these compounds are shown in Figure **6**. 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[[75](#_ENREF_75)].

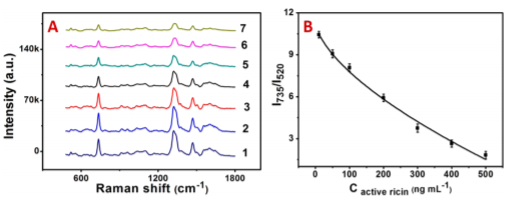
**Figure 7** Structures of all three phosgene agents.

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[[55](#_ENREF_55), [56](#_ENREF_56)]. 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[[56](#_ENREF_56)].

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

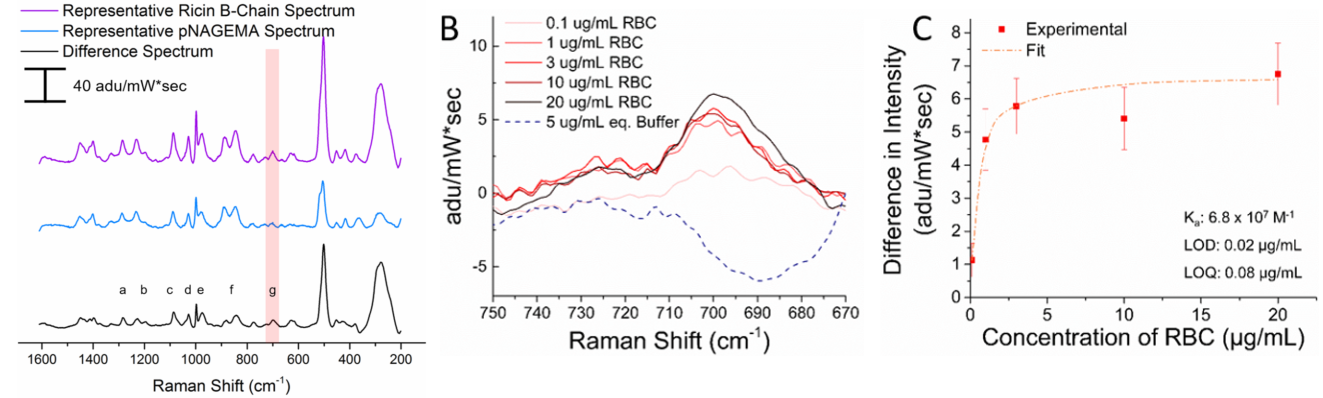
Table 1 shows that biological warfare agents (BWAs) can be either whole pathogens, or toxins such as the toxic proteins, ricin and botulinum toxin (BTX). Raman spectroscopy has been able to detect proteins for some time[[69](#_ENREF_69), [77](#_ENREF_77), [78](#_ENREF_78)], and its application to the detection of ricin has been demonstrated in numerous studies that have used the harmless A chain (RAC)[[79](#_ENREF_79)] or B chain (RBC)[[30](#_ENREF_30), [79-82](#_ENREF_79)], as well as whole ricin[[79](#_ENREF_79), [83](#_ENREF_83), [84](#_ENREF_84)]. Detection in complex media such as might be found in diagnostic, or in food that had been maliciously contaminated[[80](#_ENREF_80), [83](#_ENREF_83), [85](#_ENREF_85), [86](#_ENREF_86)], 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[[87](#_ENREF_87)]. Aptamers have been studied for detection of bioweapons in numerous studies. As examples, aptamers have been tested on RBC[[82](#_ENREF_82), [85](#_ENREF_85)], Anthrax Protective Antigen (PA)[[88](#_ENREF_88)], Anthrax Lethal Factor[[89](#_ENREF_89)], and *bacillus* bacteria[[90](#_ENREF_90), [91](#_ENREF_91)].

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[[83](#_ENREF_83)].

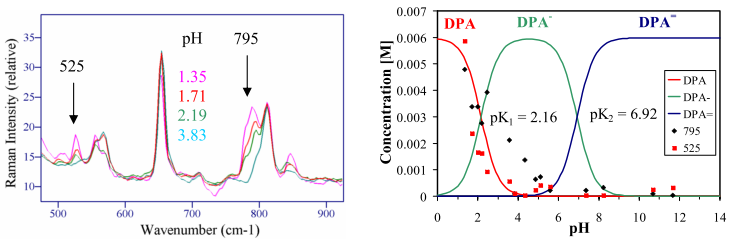
**Figure 8** (A) Signal attenuation of adenine at differing concentrations of intact ricin. (B) Calibration curve for the determination of ricin concentration. Taken from Tang et al. 2016

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[[80](#_ENREF_80)].



**Figure 9** (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 achieved[81](#_ENREF_81). 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[[92](#_ENREF_92), [93](#_ENREF_93)]. An important consideration for the detection of DPA is that is spectral features depend on pH, due to its diprotic nature (Figure **9**)[60](#_ENREF_60). Various studies have reported low levels of detection for pure DPA, including detection in the low micromolar range[[94](#_ENREF_94), [95](#_ENREF_95)].

**Figure 10** 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[[94-97](#_ENREF_94" \o "Bell, 2005 #79)]. 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](#_ENREF_96)].

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)[[97](#_ENREF_97)]. 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.

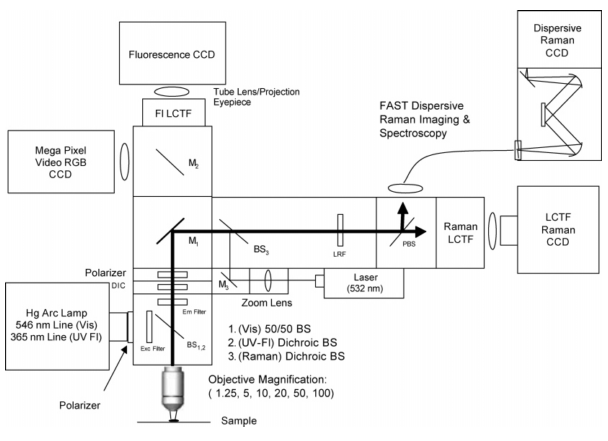
* + - 1. **Pathogen detection**

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[[17](#_ENREF_17), [30](#_ENREF_30), [35](#_ENREF_35), [62](#_ENREF_62), [69](#_ENREF_69), [84](#_ENREF_84), [90](#_ENREF_90), [91](#_ENREF_91), [93](#_ENREF_93), [96](#_ENREF_96), [98-103](#_ENREF_98)], Yersinia pestis and other *yersinia* bacteria[[30](#_ENREF_30), [64](#_ENREF_64), [84](#_ENREF_84), [101](#_ENREF_101)], *burkholderia mallei*[[30](#_ENREF_30), [84](#_ENREF_84)], *francisella tularensis*[[30](#_ENREF_30), [84](#_ENREF_84), [104](#_ENREF_104)], and *brucella abortus and related bacteria*[[30](#_ENREF_30), [84](#_ENREF_84), [101](#_ENREF_101)]. 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[[64](#_ENREF_64), [101](#_ENREF_101)], between spores and vegetative cells[[101](#_ENREF_101), [105](#_ENREF_105)], and potentially between living and dead bacteria[[64](#_ENREF_64)]. Various studies have also demonstrated the ability of Raman techniques to discriminate between bacteria at the sub-species and strain levels[[104-107](#_ENREF_104" \o "Fey, 2007 #62)].

Though conventional RS is a very weak phenomenon, it is possible to obtain whole-organism spectra from single spores or cells[[84](#_ENREF_84), [99](#_ENREF_99), [108](#_ENREF_108)]. 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[[99](#_ENREF_99)].

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[[17](#_ENREF_17)]. 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[[84](#_ENREF_84), [109](#_ENREF_109)]. 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[[109-112](#_ENREF_109" \o "Rösch, 2006 #109)]. 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[[113-123](#_ENREF_113)], but detection of phages by spontaneous Raman coupled with electrokinetic capture of virions has been demonstrated[[124](#_ENREF_124)]. 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[[118](#_ENREF_118)]. 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[[116](#_ENREF_116), [117](#_ENREF_117), [120](#_ENREF_120)].

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.

PCA is perhaps the most common chemometric technique used in threat agent detection, with numerous studies having investigated its utility in threat agent identification, including chemical[[56](#_ENREF_56)], protein[[80](#_ENREF_80), [81](#_ENREF_81), [85](#_ENREF_85)], bacterial[[84](#_ENREF_84), [98](#_ENREF_98), [105-107](#_ENREF_105), [125](#_ENREF_125)], and viral threats[[119](#_ENREF_119)]. 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[[56](#_ENREF_56)]. Additionally, PCA has proven to be a valuable tool in detecting threat agents in complex media, like food and paper[[80](#_ENREF_80), [81](#_ENREF_81), [85](#_ENREF_85)]. HCA has frequently been paired with PCA as a means of classifying spectra[[84](#_ENREF_84), [106](#_ENREF_106), [107](#_ENREF_107), [117](#_ENREF_117), [122](#_ENREF_122)]. 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[[102](#_ENREF_102), [103](#_ENREF_103), [106](#_ENREF_106)]. 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[[103](#_ENREF_103)].

In addition to these two techniques, a range of other techniques have been explored, including the use of support vector machines[[102](#_ENREF_102)], partial least squares (and partial least squares discriminant analysis)[[69](#_ENREF_69), [116](#_ENREF_116), [119](#_ENREF_119), [120](#_ENREF_120)], multivariate adaptive embedding[[30](#_ENREF_30)], and soft independent modelling of class analogues[[69](#_ENREF_69)] 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[[66](#_ENREF_66), [78](#_ENREF_78)]. 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[[126](#_ENREF_126), [127](#_ENREF_127)]. 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[[70](#_ENREF_70)]. Using UV lasers with a wavelength shorter than 250nm also has the added benefit of avoiding the fluorescence associated with biological samples[[70](#_ENREF_70)].

Despite the difficulties of collecting the Raman signal at distance, systems have been designed that can detect Raman signals at distances of over 100m[[128](#_ENREF_128)], 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[[129](#_ENREF_129)], 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[[126](#_ENREF_126)]. The same system has also been used to detect explosive materials 25m[[127](#_ENREF_127)].

CARS has also been explored for stand-off detection, with early demonstrations of this technique working effectively at 12m arriving in 2008[[130](#_ENREF_130), [131](#_ENREF_131)]. Subsequent work as demonstrated CARS imaging and low LoDs of explosive materials[[132](#_ENREF_132), [133](#_ENREF_133)]. 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.

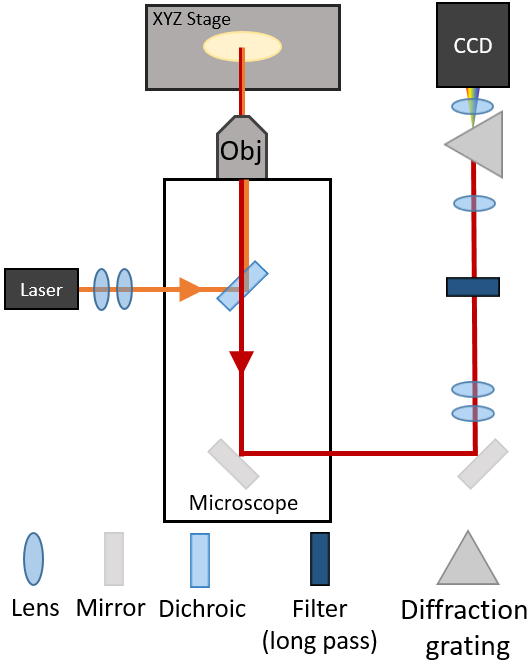
In future work, it would be beneficial to see further development of techniques for biological toxins and whole pathogens that do not rely on lengthy extraction steps. This would further expand the limits of Raman scattering techniques as a candidate for a detect-to-warn platform that can be used against 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**

All methods relating to preparation of samples and specific experimental procedures are addressed within the chapters of this thesis to which they are relevant. This section addresses the details of the Raman spectrometer used for the collection of spontaneous Raman and SERS spectra contained within the manuscript, as well as discussion of the processing and chemometric analysis used in the experimental chapters.

* 1. **Raman Spectroscopy and SERS**

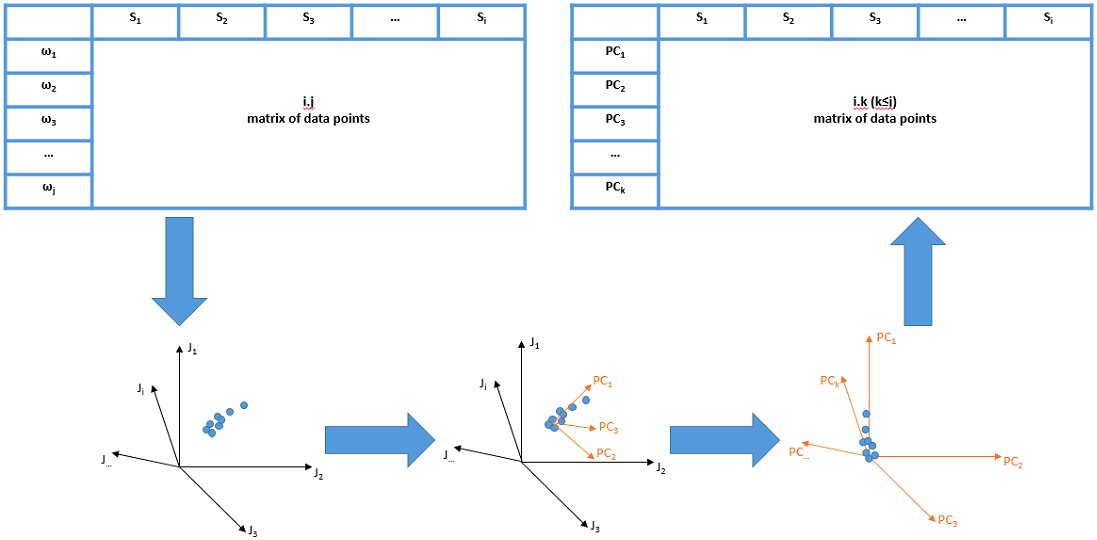
Raman and SERS measurements for this thesis were performed on a system built in-house. The system utilises a continuous wave diode laser at 785nm. The laser light is expanded to fill the entrance to a Nikon Eclipse Ti-E inverted microscope, and then collimated by a pair of lenses. Within the microscope, the light is directed up to the objective (selected for use) by a 785nm RazorEdge dichroic beam splitter. Red-shifted Raman scattering is backwards collected, passing through the 785nm beam splitter in the microscope and out to a pair of collimating lenses. The collimated scatter passes through a 785nm RazorEdge long pass filter to eliminate Rayleigh and Anti-Stokes scattered light. Finally, the light is focused into the spectrophotometer, utilising an Czerny-Turner monochromator with a blazed grating (780nm, 300 lines/mm) to diffract the light into its constituent wavelengths for detection with a charge-coupled device (CCD). This setup is depicted in figure <FIGURE NUMBER 1>. Andor SOLIS software (version X) was used to control acquisition parameters, whilst incident laser power was controlled through an automated filter wheel controlled through the dedicated ThorLabs software. A polystyrene standard (620.9cm-1, 795.8 cm-1, 1001.4 cm-1, 1031.8 cm-1, 1450.5 cm-1, 1602.3 cm-1) was used as a calibrant for all experiments conducted on the system. Spectra were saved as SOLIS data files (.sif), and converted to text files in later steps.



**FIGURE NUMBER 1** Schematic representation of the Raman spectrometer on which experiments were performed. Monochromatic laser light at 785nm is collimated and passed into an inverted microscope and focused onto the sample by an objective lens. The backscattered Stokes shifted light is filtered by at 785nm long pass filter to exclude Rayleigh and Anti-Stokes scattering. The remaining light is passed through a Czerny-Turner monochromator to split it into its constituent wavelengths by a CCD.

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

Raman and SERS experiments yield raw spectra that contain a large amount of data, and can often require sensitive and careful processing in order to fully utilise and evaluate the information presented. This is particularly true in applications where differentiation of biological samples is required, as the spectra of different proteins and microorganisms are often very similar and may not be readily discriminated by simple visual examination. Spectra obtained from these experiments were pre-processed in order to improve the quality of the spectra, including truncation to wavenumbers below 2000 cm-1, wavelet denoising and background subtraction. These pre-processing steps were performed using the iRootLab toolbox[134](#_ENREF_134) for Matlab (Mathworks). The background subtraction step uses a partial least squares method to iteratively fit a polynomial of a specified order to the baseline of the spectrum. This method removes broad background features such as sample fluorescence from the spectrum, leaving behind the sharper Raman peaks. Wavelet denoising is a process of smoothing that helps to remove noise from the baseline of the spectrum.

Given the large data matrices mentioned above, evaluating data from SERS and Raman experiments often requires the use of techniques to reduce the dimensionality of the data whilst preserving as much of the information and variability to allowed for the extraction of useful characteristics and chemical information as possible from the spectrum[135](#_ENREF_135). PCA is a widely used unsupervised multivariate statistical technique for dimensionality reduction that relies on the creation of new variables, called principal components (PCs) from existing ones. These PCs are orthogonal linear functions of the original data that are generated to maximise variation in the data. At its simplest, this can be considered as a transformation between two coordinate systems, such that the axes of the new coordinate system are the PCs. This is represented diagrammatically in <FIGURE NUMBER 2>.

**FIGURE NUMBER 2** Diagrammatic representation of the PCA process. Top left: Initial dataset of i spectra at j wavelengths. Bottom left: Projection of i spectra into j-dimensional space. Bottom middle: Generation of k PCs. Bottom right: Projection of i data points onto newly generated k dimensional PC space. Top right: Table of scores for i data points at each of k PCs.

Consider a matrix (X), comprised of i columns that represent spectra and j rows representing intensity at a specific wavenumber. The rows of X are vectors in j dimensional space and can be combined as:

, which can be simply expressed as

Here, T is a new vector, generated from a linear combination of X variables and is plotted in the same space, and w is a vector of wj, where j = 1, …, j. The amount of variation in the new vector, T, can be expressed by its variance, which can be used to summarise the X variables as a single variable and, in this way, reduce the dimensionality of the dataset. To ensure that the newly created vector describes the maximum possible variance in the data, it is necessary to pre-process the data by mean centring X, such that the data is normalised so ||w||2 = 1. Without the pre-processing step, T may correspond more or less to the mean of the data.

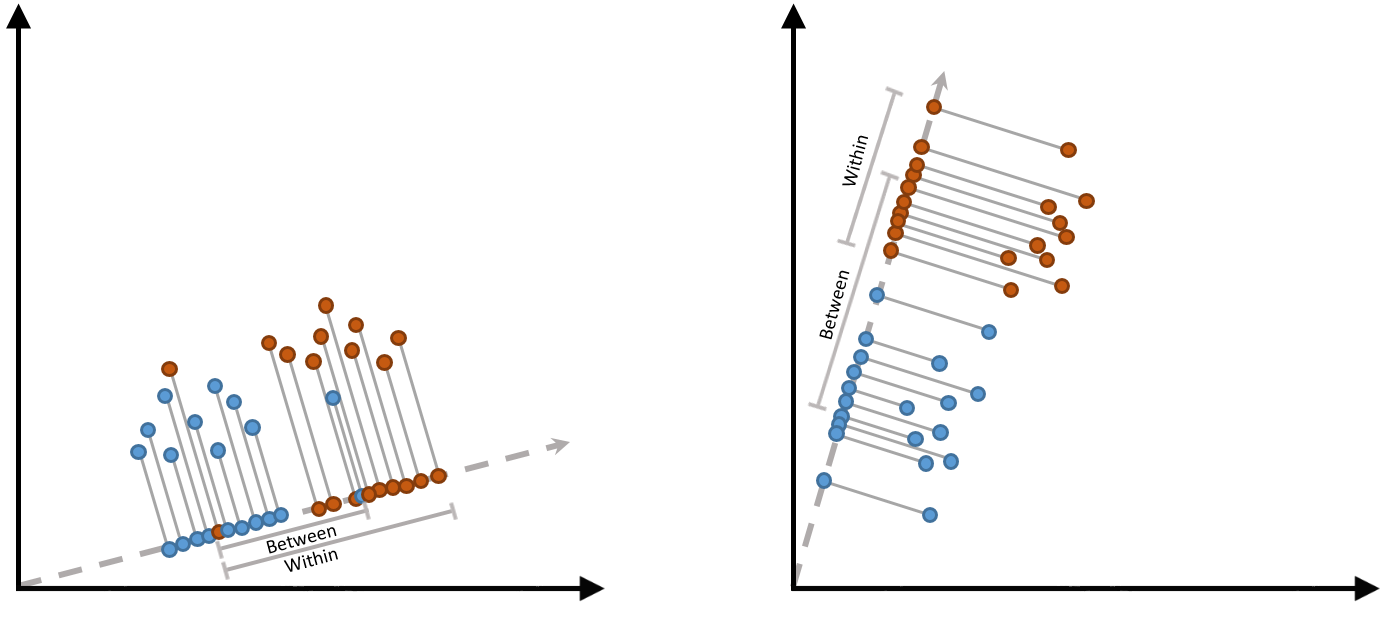
The capacity of T to summarise X is measured by projecting the rows of X onto the vector and calculating the residuals using a regression equation:

Here, P is a vector of regression coefficients (in this case, equal to w), and E is the matrix of residuals. In this way, X is modelled by TPT, where T is fixed and P is a coefficient to be determined. Here, P is normalised to 1, and known as the loadings vector, whilst T is the PC scores vector. Noise in spectral measurements is accounted for in E. Whilst it is not included in the PC model, it can be used in judging the summarisation of X by T, given the percentage of explained variation (%ExVarT) via:

The variance explained by each successive PC becomes progressively smaller with each iteration. PC scores are visualised as a plot of each spectrum onto the axes defined by selected PC scores as a single data point. In this way, spectra with similar spectral features will be assigned similar scores across the PCs and will cluster together when plotted in PC space. By extension, dissimilar spectra will have dissimilar PC scores and appear separated. Loadings are coefficients that describe the weight of each individual variable (row in X) in defining a particular PC, and can be visualised as a plot of variable against loading for that variable. This allows for the identification of the original variables that most significantly contribute for representation of X. In the case of Raman and SERS spectra, this would correspond to intensities and specific wavenumbers, and therefore to the chemical bonds which most strongly characterise the data.

Additionally, PCA can be combined with LDA as PC-LDA[136](#_ENREF_136). LDA is another multivariate technique, though it differs from PCA in that it is a supervised method. In LDA, new vectors (LDs) are sought that maximise the variance between specified classes within the dataset whilst minimising variance within each class, rather than simply finding the axes along which the entire dataset exhibit’s maximum variance. In PC-LDA, these LDs are generated from the PCs exhibited by a dataset, rather than from the original data. PCA has been widely used in conjunction with Raman scattering techniques for homeland security applications, including the detection of CWAs[56](#_ENREF_56), as well as biological threats such as toxic proteins[80](#_ENREF_80), [81](#_ENREF_81) and bacterial threat agents[107](#_ENREF_107).

As mentioned above, LDA is another multivariate statistical technique. It bears similarities to PCA in that both are methods that attempt to explain a dataset by examining combinations of variables within it, and can be used in conjunction with dimensionality-reduction methods[136](#_ENREF_136). Unlike PCA, LDA is supervised, meaning that it requires some *a priori* knowledge about the sample so that a categorical dependence variable (a class label) can be assigned to each sample in the dataset. In LDA, as with PCA, the data is transformed from its original space into a new coordinate system (here, LD space) by the linear combination of features within the *n* data sets, creating *n-*1 or *p* (where *p* is the number of predictors) projections, whichever is smaller[136](#_ENREF_136).

In short, LDA attempts to locate orthogonal linear functions, called Linear Discriminants (LDs), in the data, such that variances between classes are maximised and variance within a class is minimised, as shown in FIGURE NUMBER 3. This class-dependence for classification is missing from PCA.

**FIGURE NUMBER 3** Diagrammatic representation of the underlying principle of PCA, showing how the model attempts to transform the data into a new subspace (LD space), such that the maximum variance between classes is produced and variance within a class is minimised. The projection of the data points onto the grey dashed line visualises the improvement in the right projection by comparison to the left.

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

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* 1. **Methods**
  2. **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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