Surface-Enhanced Raman Spectroscopy and Homeland Security: A Perfect Match?

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ABSTRACT This Nano Focus article reviews recent developments in surface-enhanced Raman spectroscopy (SERS) and its application to homeland security. It is based on invited talks given at the "Nanorods and Microparticles for Homeland Security" symposium, which was organized by one of the authors and presented at the 238th ACS National Meeting and Exhibition in Washington, DC. The three-day symposium included approximately 25 experts from academia, industry, and national laboratories and included both SERS and non-SERS approaches to detection of chemical and biological substances relevant to homeland security, as well as fundamental advances. Here, we focus on SERS and how it is uniquely positioned to have an impact in a field whose importance is increasing rapidly. We describe some technical challenges that remain and offer a glimpse of what form solutions might take.

When it comes to nanoscience and nanotechnology, surface-enhanced Raman scattering (SERS)[1] is the spectroscopist's poster child: in no other measurement technique does the role of nanostructure play such a critical role. Discovered in the late 1970s, the field of SERS appears to be undergoing a renaissance, with an explosion in the synthesis of novel shapes of SERS-active nanoparticles, significantly enhanced capabilities in lithography and related techniques, and increasingly sophisticated and accurate theory and modeling. With this torrent of activity, the field's optimists might say that SERS, after a very long infancy, has finally reached adolescence, and that the recent development of nanoscale tools was the catalyst for growth. Pessimists might counter that SERS has been sufficiently mature for two decades yet still remains an academic curiosity with no useful commercial applications; thus, there must be an intrinsic feature of SERS that fundamentally limits its utility in chemical analysis and/or bioanalysis.

Both sides have an element of truth. However, the measurement challenges associated with homeland security may render the argument moot. Prior to September 11, 2001, the definition of homeland security would have encompassed solely biological warfare agents (*e.g.*, anthrax), their chemical counterparts (*e.g.*, sarin), and explosives detection. It is recognized now that homeland security encompasses a much broader scope of measurement scenarios that includes forensic science, monitoring of controlled substances (*i.e.*, narcotics), food safety, water safety, and even detection of counterfeiting (which generates income for terrorists). The range of analytes to be detected or quantified runs the gamut from viruses and bacteria to proteins, DNA, lipids, and/or low molecular weight (LMW) species and are found in a wide range of sample matrices, from solid food to air. To add to the challenge, measurements typically need to be carried out in the field, in real time.

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Because of its unique attributes, SERS may be the best-positioned among all measurement techniques to have a significant impact on homeland security. To that end, the ACS Division of Colloid and Surface Chemistry organized a symposium at the 238th ACS National Meeting (August 16–19, 2009, Washington, DC) entitled "Nanorods and Microparticles for Homeland Security". Drawing mostly but not exclusively from the symposium, this article

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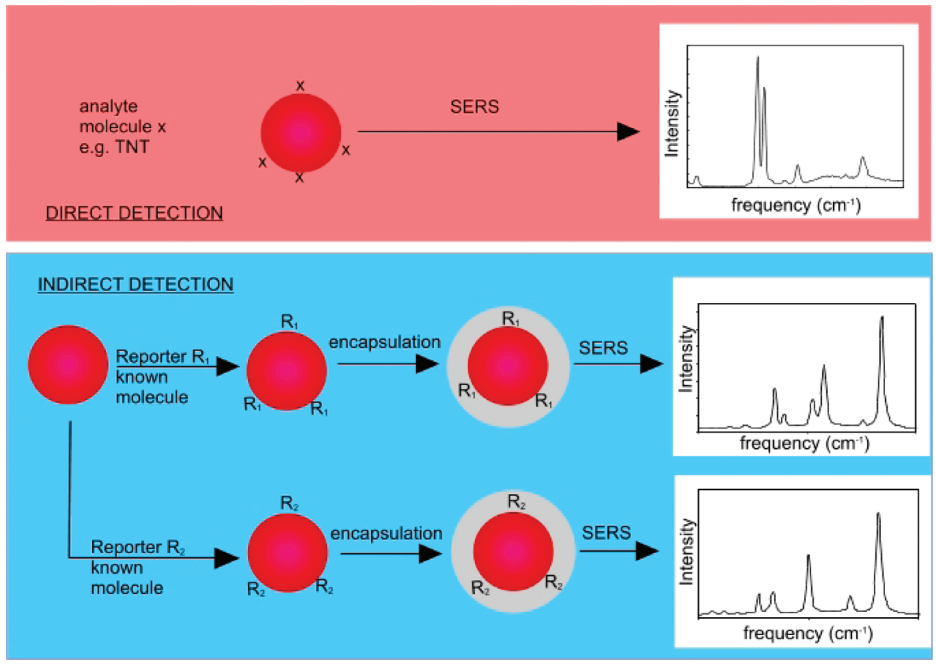

Scheme 1. Illustration demonstrating methods used for direct detection *via* SERS (top panel) and preparation of labels for SERS-based indirect detection (bottom panel).

summarizes some advances in SERS-active materials and in applications of SERS to problems in homeland security, ending with a discussion of some opportunities for the field as a whole.

Detection and Quantitation in Homeland Security. Chemical and biochemical analysis are mature disciplines, and entire industries are based on sensitive, accurate monitoring of species in the gas phase (H_2, CO) and in aqueous solution (*e.g.*, glucose, cholesterol, prostate-specific antigen). What factors make analyte detection so difficult in homeland security applications? (1) The molecular weight range of substances to be detected is very broad, from LMW species to cells. (2) In some cases, the species may not be known or may not appear in databases. This is particularly true for biothreats but may also be the case for chemical warfare agents or even impurities in food or water. (3) Exceptionally low error rates (both positive and negative) are required because the cost for a mistake can be enormous. (4) Many of the species are at very low abundance, either by design or by dilution, pushing the envelope of detection capabilities. (5) The potential for continuous monitoring must exist. (6) Consumables are not ideal because they have to be carried in, and any instrumentation needs to be small, light, and battery-operated. (7) Tests developed must be able to work in extremes of temperature, humidity, and wind conditions. (8) In most cases, answers are needed in real time, that is, in 5 min or less. (9) Finally, tests must be able to handle a wide variety of matrices, from swabs of surfaces (*e.g.*, envelopes) to powders to aqueous solutions to air samples composed predominantly of sand and dust.

Fundamentals of SERS. In SERS, molecules in extremely close proximity to Au and Ag nanostructures sized 5−200 nm give rise to million-fold or higher increases (known as enhancement factors, EFs) in Raman scattering efficiency. The phenomenon depends intimately on the interplay between the excitation wavelength and the nanostructure geometry at the 1, 10, and 100 nm scales.[2,3]

Three key features of Raman in general and SERS in particular appear to be a perfect match for the technical specifications required for homeland security detection. First and most importantly, SERS is a vibrational spectroscopy that furnishes molecular-level, chemical-fingerprint-like information. This enables detection of many different species and also permits multiplexing, whereby multiple species are detected in a single measurement. Second, the instrumentation meets the needs for point-of-use measurements. Hand-held spectrometers with sufficient spectral resolution and sensitivity are commercially available from multiple vendors. Excitation wavelengths can be chosen in the visible or the near-infrared spectral regions according to the needs of the application. Since the phenomenon is based on scattering, it is possible to "point-and-shoot" and collect high-quality spectral data in seconds.

The third important attribute of SERS for homeland security is the capability to be used for both direct and indirect detection. As shown in the top of Scheme 1, direct detection refers to the case when the species of interest adsorbs directly to a SERS-active surface. Examples of direct detection described below include bacteria, drug molecules, and the chemical contaminant melamine. SERS is the only optical detection technique capable of analyte detection and identification at nanomolar to picomolar concentration levels. Indirect detection, by contrast, makes uses of nanoparticles as SERS-active quantitation labels, similar to the way fluorophores are used. These SERS nanotags[4] (Scheme 1, bottom) are made by coating a SERS-active particle with an adsorbate ("the reporter") that has a unique spectral fingerprint and then encapsulating in silica. The resulting spectrum of the tag depends solely on which reporter is used (R_1 *vs* R_2). Conjugation of antibodies or DNA (or some other molecular-recognition motif) to the outer silica surface allows the particles to be used as quantitation labels in ways that are exceedingly familiar to developers of immuno-diagnostic or molecular diagnostic assays. The ability to generate multiple spectrally unique tags enables quantification of multiple analytes in a single measurement.

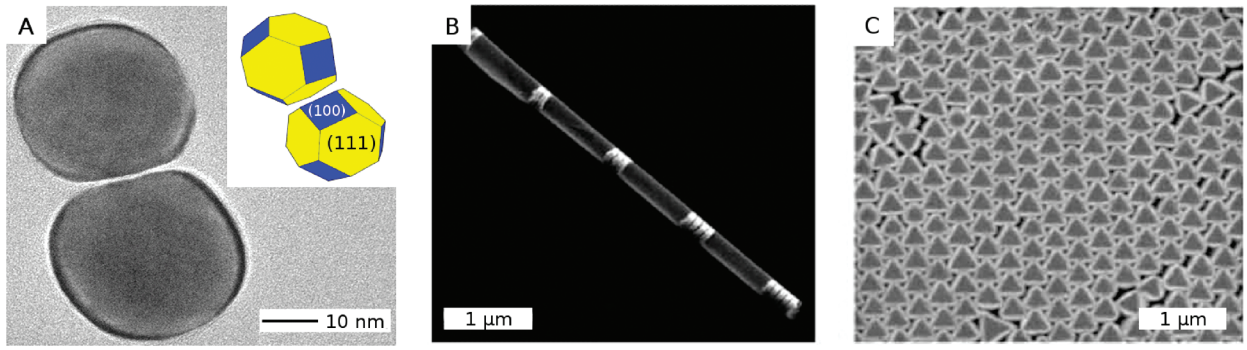

Figure 1. Particle-based approaches for SERS: (A) TEM image of a silver nanosphere dimer, with a cartoon inset depicting their architecture. (B) Field emission SEM image of a Au nanodisk array prepared using on-wire lithography (OWL). The disks (bright segments) are 120 nm in diameter with a 30 nm separation. (C) SEM image of silver octahedra assembled into a close-packed film. Panel A reproduced from ref 18. Copyright 2009 American Chemical Society. Panel B reproduced with permission from ref 19. Copyright 2006 National Academy of Sciences, USA. Panel C reproduced with permission from ref 20. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA.

Other Methods. To be sure, there are other viable approaches to detection of analytes for homeland security applications. At this meeting, Shawn Mulvaney of Naval Research Laboratories described an innovative fluidic force assay based on magnetic beads and has demonstrated sensitive assays for staphylococcal enterotoxin B.[5] George Farquar from Lawrence Livermore National Laboratories described a single-particle aerosol mass spectrometry system for detection of intact bacteria.[6] Keating,[7] Halas,[8] Wei,[9] and Odom[10] have all developed novel metal nanoparticles or nanostructures that can be used for non-SERS-based detection schemes. Thomas Huser of University of California, Davis described how super-resolution microscopy might prove to be a useful detection technique, especially for single bacteria.[11] Even colorimetric approaches are viable, as was described by Allen Apblett of Oklahoma State University.[12] In addition, there are numerous companies with active commercial programs connected to homeland security such as Tetracore,[13] ICx Technologies,[14] and Smiths Detection.[15] However, as of today, SERS is the *only* detection technique that is both (i) field portable without a significant loss in performance and (ii) capable, *via* direct or indirect modes, of identifying LMW species (known and unknown), lipids, proteins, DNA, and cells. It is this unique set of attributes that makes it so well-suited to address the particular challenges associated with homeland security.

Novel SERS-Active Particles and Particle Assemblies. There are two fundamentally different approaches to development of SERS-active materials with "hotspots", the particle junctions responsible for the highest enhancements: bottom-up assembly, starting with Au or Ag nanoparticles, and top-down synthesis. whereby macroscopic SERS-active substrates are made using lithography, selective etching, or related approaches. Below, we describe the advantages and disadvantages of each and highlight selected approaches from the recent literature and from the ACS symposium.

In the past decade, advances in the understanding and control of nanoparticle synthesis have been nothing short of spectacular.[16] A wide variety of regular shapes and sizes of particles ranging from a few nanometers to a few hundred nanometers in size have been described. Metal nanoparticles offer multiple advantages for SERS experimentation. (1) They comprise the fundamental SERS "building block" and are easy to isolate. This enables detailed physical and optical characterization, which in turn provides high-quality data for comparison to theoretical modeling.[17] (2) They are the most straightforward to use from an experimental standpoint. In many cases, simple addition of an analyte to a solution containing metal nanoparticles causes aggregation, leading to "hotspots" containing two or more particles with entrapped analytes. These structures are strongly enhancing for SERS. (3) Solution-based synthesis allows SERS-active materials to be made in all three dimensions of space at once. In contrast, top-down synthesis creates materials only in two dimensions. Thus, with the appropriately sized reaction vessel, it is possible to create enough material for many millions of SERS experiments in a few minutes without additional processing. This cannot be done using the top-down approach.

There are three corresponding disadvantages to metal nanoparticles for SERS. (1) Only a fraction of the many novel particle shapes are SERS-active as prepared. Many are covered with a surfactant used to direct shape during synthesis, which inhibits analyte adsorption. Removal of surfactant leads both to instability and, in the case of anisotropic particles, to changes in the particle shape by decreasing the aspect ratio. (2)

There are two fundamentally different approaches to development of SERS-active materials with "hotspots", the particle junctions responsible for the highest enhancements: bottom-up assembly and top-down synthesis.

Aggregates of nanoparticles in solution are inherently unstable and are very difficult to isolate. (3) Most importantly, few methods exist for assembly of particles into stable, well-defined hotspots, ideally on a macroscopic scale. During the ACS symposium, however, several novel approaches to hotspot creation were described.

The Xia group has pioneered controlled synsthesis of anisotropic metal nanoparticles and has now turned their attention to *in situ* synthesis of particle dimers with built-in hotspots.[18] Their approach combines the well-known polyol process in which ethylene glycol serves both as a solvent and a precursor to the reducing agent for metal salts. Controlled addition of NaCl leads both to oxidative etching and particle dimerization (under the proper conditions of dilution). The Ag nanospheres obtained using this protocol were actually truncated octahedrons with a rounded profile (see the inset of Figure 1A), which are enclosed by a mix of (111) and (100) facets. SERS spectra of 4-methylbenzenethiol led to enhancement factors at the hot spot of 1.9×10^7.

Chad Mirkin described his group's continuing efforts to make and characterize SERS hotspots using on-wire lithography (OWL).[19] In OWL, a striped nanorod is made by electrochemical reduction of metal ion salts in cylindrical templates, with alternating stripes of Au and Ni. The width of the metal segments can be controlled down to the few-nanometer level by coulometry. After template release and evaporation of a silica "backing", etching out of the Ni leads to voids between two or more Au disks (Figure 1B). This approach results in SERS-active structures with variable numbers of disks and, using templates with different diameters, control of disk dimensions. Using pulsed deposition, it was shown that the rough surfaces between the gap can be smoothed by a factor of 5-fold to a roughness of 5 nm, which leads to sharper plasmon resonances but lower SERS intensities.[21] Also described was recent work placing the OWL wires on adjacent electrical pads. This enables electric-field-based trapping of molecules and control of electrochemical potential in SERS experiments, leading to a 10-fold increase in enhancement.

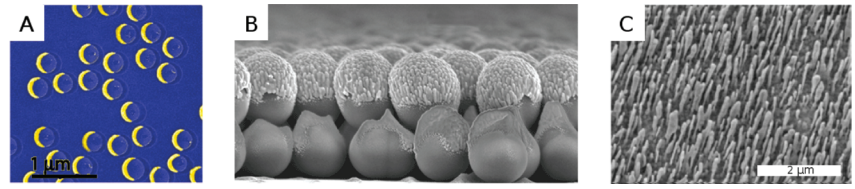

Figure 2. SERS substrate approaches: (A) Schematic of nanocrescents prepared using nanosphere lithography. (B) SEM image of a silver nanorod film grown over 600 nm diameter silica spheres. (C) Silver nanorod array prepared by oblique angle vapor deposition onto a glass substrate. Panel A reproduced from ref 26. Copyright 2009 American Chemical Society. Panel B courtesy of Richard Van Duyne of Northwestern University. Panel C reproduced from ref 28. Copyright 2008 American Chemical Society.

The Yang group at University of California, Berkeley, has developed a Langmuir–Blodgett method to assemble a variety of metal nanoparticle shapes into close-packed, macroscopic structures. Initially developed for nanowires,[22] more recent activity has focused on regular Ag polygons such as cubes and truncated octahedra (Figure 1C).[20] One key feature of these surfaces is that the dense array of nanoparticles leads a broadband scattering profile that allows for excitation at a variety of wavelengths. The particles are made by the polyol process but are capped *in situ* with poly(vinylpyrrolidone) (PVP), which serves both to stabilize the Ag to oxidation/passivation and to enable analytes such as arsenate ($[AsO_4]^{3-}$) to be detected at trace levels.

Novel Macroscopic SERS-Active Substrates. Macroscopic substrates offer several benefits for SERS. (1) Macroscopic surfaces are typically stable and can be stored for long periods between fabrication and use. (2) Because they are typically planar (and often highly reflective or scattering), it is easier to make measurements where the excitation beam is properly focused. (3) SERS has its origins in the surface science community, and it is easier to probe nanostructures on surfaces (*e.g.*, by AFM) than in solution. (4) Macroscopic surfaces are easier to interface externally, for example, to electrochemical instrumentation, to allow accurate measurement of surface area or to control surface potential.

Macroscopic surfaces also have shortcomings for SERS. (1) It is more difficult to control architecture of larger surfaces completely from the nanometer to micrometer level; accordingly, it has proven more difficult to achieve spot-to-spot and substrate-to-substrate reproducibility for macroscopic surfaces than for aggregated colloids, even despite the latter's instability. (2) As mentioned above, they can only be fabricated in two dimensions, making them far more expensive to use on a per-measurement basis than colloidal materials. (3) Macroscopic SERS-active surfaces are often very mechanically fragile.

Despite this, there continues to be a steady stream of new and interesting SERS-active substrates described in the literature, several of which were described at the meeting.[23–28] For example, the Lee group has published work on a variety of crescent-shaped particles and voids, such as the random array nanohole antennas shown in Figure 2A.[26] A variety of wafer-scale lithographic techniques have been used to create novel architectures, and at the meeting, Lee described "beak-shaped" structures made by shadow angle interference lithography that allowed detection of 1 fM BPE [*trans*-1,2-bis(4-pyridyl)ethene], a favored adsorbate molecule among SERS practitioners.

The Van Duyne group has made countless contributions to the field of SERS, including the seminal paper in the field.[29] Most recently, they described a new wrinkle on the seemingly well-studied Ag film over nanosphere (AgFON) surface[27] that comprises Ag films evaporated onto close-packed arrays of 600 nm diameter silica nanospheres (Figure 2B); namely, that the best EFs come from surfaces with spherical arrays of Ag rods on the silica particles. This finding underscores one of the difficulties of macroscopic surfaces for

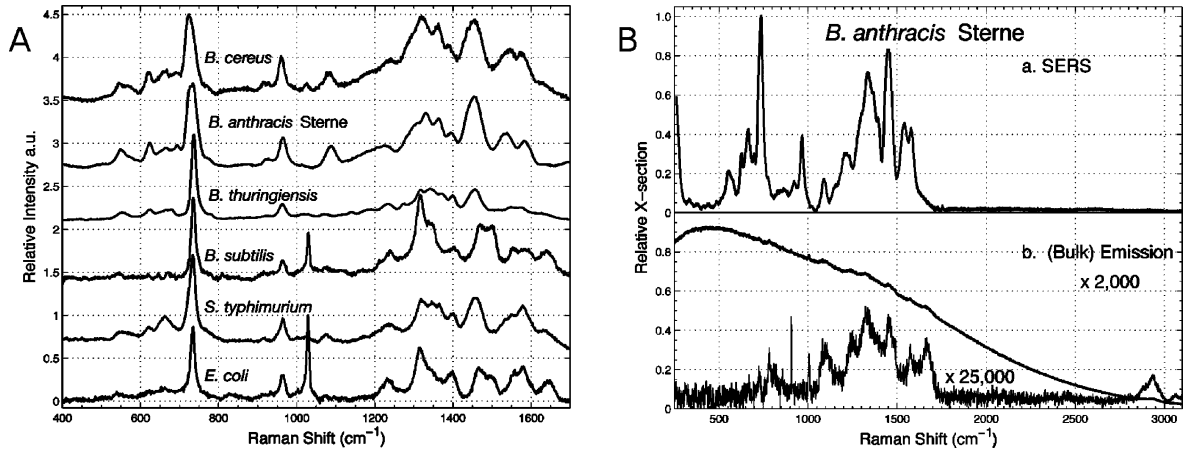

Figure 3. (A) SERS spectra of six bacterial species obtained under identical conditions. Spectra are offset for clarity and are arranged vertically according to their phylogenetic relationship. (B) Raman scattering obtained from *B. anthracis* using SERS (top) and non-SERS (bottom) methodologies. Spectra were acquired using 785 nm excitation. The bottom trace shows the resulting Raman spectrum after subtraction of the broad background associated with the bulk Raman measurement. Panels reproduced from ref 30. Copyright 2005 American Chemical Society.

SERS: it is necessary to understand the structure on the many-micrometer scale (sphere packing), the 100 nm scale (the gross structure of the Ag film), and the 10−50 nm scale (the relevant rod dimensions). It would not be surprising to learn in the future that the rod surface roughness (which is on the 0.1−5 nm scale) also exerts an influence on the SERS behavior.

The Dluhy group has perfected oblique angle deposition as a means to fabricate stable, strongly enhancing Ag rod arrays.[28] In this approach, evaporation onto glass (or metal) substrates at angles close to the grazing angle leads to shadowing of nucleation sites and subsequent oriented rod growth. Figure 2C shows a typical array, with rod dimensions on the order of 80−90 nm in diameter, and depending on the conditions, lengths of roughly 800 nm, and with a mean inter-rod spacing of 140 nm. Each of these parameters can be adjusted by controlling the oblique angle, the temperature, and the deposition rate/length. Enhancement factors of 10^8 with spot-to-spot variations of less than 10% were routinely achieved for BPE. As described below, these and similar substrates have proven useful for direct SERS measurements on bacteria.

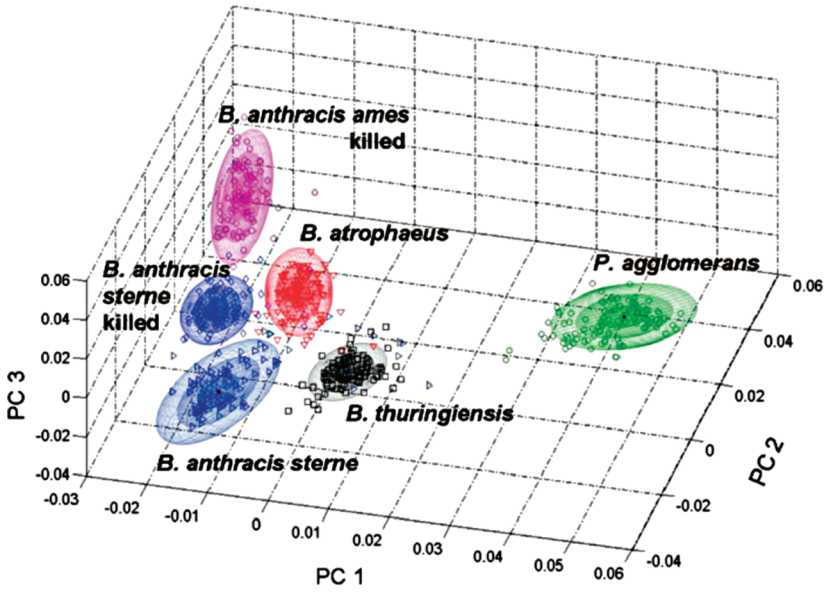

Figure 4. Discrimination of five *Bacillus* spore samples and *P. agglomerans* is shown in this principal component (PC) analysis plot based on their SERS emission. The lighter-color spheroids denote the 2σ and 3σ standard deviations, respectively. Reproduced from ref 34. Copyright 2008 Society for Applied Spectroscopy.

Direct Detection of Biological Warfare Agents. Perhaps the most popular image of biological terrorism is that of anthrax spores in the form of a "mysterious white powder". Ultimately, though, the field must be thought of in much broader terms, encompassing everything from biological toxins (*e.g.*, botulism toxin) to the so-called "weaponized" strains of bacteria and viruses, to the potential threat of such agents being introduced into food or water sources. Additional concern must be given because even a small-scale attack (both in pathogen quantity and number of targets) could result in a large outbreak if an infectious pathogen is able to effectively propagate and spread from one host to another. Thus, it is critical not only to detect such obvious examples as the aforementioned white powders but to monitor high-risk targets consistently, to detect very low levels of pathogens, and also to differentiate and to identify very similar species and strains.

It follows that detection of such pathogens would ideally be ultrasensitive, rapid, require no extrinsic labeling steps, and differentiate species and strains of pathogens. SERS is perhaps the only technique that has shown the promise to satisfy all such criteria. The tremendous signal enhancements obtained from SERS, coupled with the large scattering cross sections of bacterial cells, allow rather routine acquisition of spectra from individual bacterial cells.

Ziegler and colleagues have shown that SERS spectra can be obtained after deposition of bacteria onto a colloidal Au-based substrate, and that these spectra are approximately 10^4-fold more intense than the corresponding bulk Raman signals.[30] Distance-dependent enhancement mechanisms enhance preferentially those vibrational modes within close proximity to the substrate, resulting in simpler spectra compared to native Raman scattering. Importantly, these simple spectra were shown not only to be distinct for six different bacteria species (Figure 3A) but also to provide better Raman differentiation among the species than their native Raman spectra. Presumably, this is because the cell surface compositions of bacteria are more specialized for their function and environment than are the components of the cytoplasm, so the extra "weighting" of SERS measurements is advantageous.

Fluorescence has long been an issue when acquiring Raman spectra, as the resultant background often masks the much weaker Raman scattering bands. Since these problems are most often encountered in the visible-light regime, Raman instruments incorporating near-infrared (NIR) excitation sources have recently become commonplace, most often utilizing 785, 830, or even 1064 nm laser sources. Even with NIR excitation, though, one can still encounter fluorescence from bacteria.[30] In addition to enhancing the Raman scattering intensity, metal surfaces also have the ability to quench such endogenous fluorescence and produce much higher quality data. After depositing the same bacteria onto a SERS substrate, the Raman spectrum becomes the dominant feature, allowing relatively straightforward analysis (Figure 3B).

An underlying theme that must accompany all discussion of direct SERS measurements is that of data analysis. Much effort has recently been devoted to the accurate differentiation of species/strains of bacteria and virions[31,32] using principal component analysis (PCA) and hierarchical clustering analysis (HCA).[33−36] Guicheteau demonstrated PCA discrimination between five *Bacillus* species/strains and *Pantoea agglomerans* that had been dried in the presence of colloidal silver.[34] Of special note was the differentiation between the killed and live versions of *B. anthracis sterne*, which is clearly shown in their PCA experiments (Figure 4). A similar sample set was tested by Ziegler, but PCA was also performed on first- and second-derivative spectra, resulting in improved discrimination.[33] Furthermore, using an advanced barcoding method (based on the sign of the second-derivative spectra) as an input for PCA consistently provided additional benefits.

Even with the recent advancements in detection of pathogens by SERS, a number of issues must be resolved before effective field implementation, some of which are presented in the recent overview by Zeiri.[37] One of the biggest concerns is general sample preparation, which includes the exact substrate or colloid used to generate enhancement and the actual bacterial sample. While most reports suggest high levels of reproducibility within a study, there have been mentions[33,37] of identical strains of bacteria producing quite different SERS responses across different laboratories. This could be caused by a number of factors, but is most likely due to using different SERS substrates and acquisition conditions: Zeiri's work clearly demonstrates a difference in spectra obtained from bacteria incubated with Au or Ag. Çulha also discusses the manner in which samples are prepared and shows how substantially different spectra are generated based simply on the sample pH, both because it affects the adsorption of colloid to the bacteria and because it impacts the ionization state of the bacteria's functional groups.[38] Another suggested factor is the possibility of graphitization of samples, which is even more likely in the presence of metal substrates.[37] Graphitization manifests itself as an intense background that tends to overwhelm the desired spectrum but can be mostly avoided by use of low laser powers or slight defocusing of the laser beam. Low levels of degradation could slightly distort spectra, causing reduced species discrimination while being rather difficult to monitor.

Finally, sample collection is a major concern. Detection of individual organisms still requires they at least pass through the focal spot of a laser beam that might be smaller than the organism itself. Similarly, most samples tend to be somewhat inhomogeneous (due to low pathogen concentration or nonuniform SERS substrates) and thus require time-consuming sample manipulation, spatial averaging, removal of "spurious" spectra, or a combination of all of the above to obtain consistent responses. Even with such manipulation, most PCA experiments require additional filtering and spectral processing to obtain optimal differentiation. Thus, implementing automated field-operable systems will require substantial efforts, meaning that, for now, determination of pathogens using SERS remains best suited to laboratory testing.

SERS for Direct Chemical Detection. Although there have been reports in the SERS literature over the past decade on detection of toxic gases,[39−43] a quantitative approach toward SERS gas detection has been deficient from the literature. Van Duyne and colleagues from Northwestern University presented data representing a considerable advance toward the real-time gas detection of chemical warfare agents (CWAs). In this work, they measured the kinetics of benzenethiol adsorption from the gas phase onto the SERS-active AgFON surfaces described above and developed a comprehensive adsorption model intended to improve accuracy and quantitation of measurements. The key finding was that adsorption was unexpectedly slow, likely due to a requirement for S−H bond scission. In a recent publication, methods for improving the adsorption kinetics have been have identified.[44] This study provides insight into routes to use SERS as a gas-phase detection modality.

For the detection of airborne molecules with parts per trillion sensitivity and high specificity, Martin Moskovits and co-workers have developed a field-portable chemical detector that combines free-surface microfluidics with SERS.[45] They demonstrated that this

analytical platform is ideal for the detection of polar explosive molecules that readily absorb into the microchannel flow. In their approach, airborne molecules that are absorbed and accumulated in a microfluidic channel are flowed through a field containing Ag colloid. The colloid aggregates and is detected downstream (as aggregation continues) at the location where the highest signal is obtained. A nice feature of this approach is that only small volumes of colloid are used per measurement, with the remainder not exposed to ambient, meaning the device can be used repeatedly with no degradation of signal.

John Lombardi presented data from a collaborative research effort that utilized SERS for trace identification of the controlled substances morphine, codeine, and hydrocodone (unpublished results). In this research, Ag nanoparticles were used as SERS substrates,[46] providing signal enhancement in the visible-to-NIR region. Because the analytes of interest have an intense fluorescence band, NIR SERS allowed for amplification of the analyte signal with concomitant suppression of the fluorescence interference. Given the similar structure of the molecules in question, density functional theory calculations were used to supplement SERS measurements to elucidate spectral differences.

SERS has likewise proven useful for analysis of chemical contaminants. For example, Mengshi Lin discussed the feasibility of using Au substrates for direct SERS detection of melamine, a milk and pet food contaminant that has garnered recent worldwide attention.[47] In this work, trace amounts of melamine were quickly and accurately detected and characterized using commercially available Klarite[48] SERS substrates. The detection limit of melamine was found to be 33 ppb, which was much lower than that of high-performance liquid chromatography (HPLC) analysis (100 ppb) in samples of wheat gluten and chicken feed. For the detection of melamine in milk, the detection limit was 2 ppm due to the fact that melamine binds to proteins in milk causing it to be more difficult to detect *via* direct SERS measurements.

Challenges for Direct SERS Detection. Reproducible, low-cost, strongly enhancing, and stable SERS substrates were prepared by Au and Ag colloid self-assembly over a decade ago.[49] Likewise, SERS-active macroscopic substrates were made by lithographic techniques a decade before that.[50] Given the availability of these and several dozen other SERS substrates, what has prevented SERS from being used to solve problems in analytical and/or bioanalytical chemistry?

The main limitation to direct detection by SERS is its intrinsic lack of selectivity. Real-world samples are complex and contain numerous SERS-active species (*e.g.*, chloride) that bind strongly to SERS-active surfaces. For example, a molecule that can be detected at 1 ppb in pure water using SERS can typically be detected only at parts per million levels when dissolved in a 5 mM aqueous solution of NaCl. Likewise, proteins tend to bind to and denature on bare metal surfaces, complicating analysis of biological samples. This phenomenon contributed to the aforementioned poorer limit of detection (by 2 orders of magnitude) for melamine in milk compared with water.[47]

There are four approaches to solve this problem: (1) Work only on samples that are intrinsically clean. Obviously, this is very limiting. (2) Clean up the samples to remove adsorbing impurities. The question then becomes, "is SERS a significantly better detection method on 'clean' samples relative to mass spectrometry, surface plasmon resonance (SPR), immunoassays, electrochemical detection, and so on?" Our view is that it is not. (3) Use chemometrics and sophisticated spectral analysis tools to tease out information about adsorbed analytes. As shown above, this approach has merit for analysis of bacterial surface proteins and lipids associated with biological warfare agents, but it is unclear how well the methods will work on challenging, dirty samples in complex matrices. (4) Develop permselective coatings for SERS-active surfaces that have weak Raman spectra, prevent adsorption of unwanted species, and ideally, promote adsorption of the analyte, imparting a level of selectivity. There are few examples of this in the literature. Vo-Dinh used a coating of PVP to improve the performance of SERS-active surfaces for detection of gas-phase molecules.[39] Carron used an organothiol self-assembled monolayer (SAM) coating on a Ag substrate to improve detection of aromatic amines, which do not absorb strongly to SERS-active surfaces.[51] Van Duyne has used organothiol SAM coatings to detect glucose directly by SERS.[52] Recently, Haes has developed[53] and described at the ACS meeting a porous silica coating for SERS-active nanoparticles that allow diffusion of low molecular weight (LMW) species through the pores to the particle surface. Though it is not clear whether the coating provides selectivity based on molecular dimensions, charge, or a combination of factors, it is definitely an excellent first step to creating SERS-active particles (or surfaces) that do not adsorb all species in solution.

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Indirect SERS Detection for Homeland Security Applications. An alternative to addressing the selectivity issue associated with direct SERS is to use indirect SERS detection and to obtain selectivity through molecular recognition. Oxonica Materials Inc. (Mountain View, CA) has developed a series of assays that use the SERS tags described in Scheme 1. These particles offer numerous advantages for detection and identification of analytes and bioanalytes. (1) Because the silica coating locks in the reporter molecules, the SERS signal is fixed, eliminating issues with surface fouling. Indeed, the signal is impervious to matrix effects, ionic strength, and other effects. (2) It is simple to attach oligonucleotides or antibodies (Abs) covalently to the silica outer surface. (3) Importantly,

Highly enhancing SERS-active media are not currently a limiting factor in the use of SERS.

only the SERS spectrum of the reporter is detected (as opposed to the Abs, molecules bound to Abs, or any species in solution. (4) The tags can be designed for NIR excitation (typically 785 or 1064 nm), allowing their use in dirty samples without regard to removal of fluorescent impurities.

Oxonica recently reported a novel "no-wash" sandwich immunoassay using a magnetic particle functionalized with a capture Ab and a SERS tag functionalized with a detection Ab.[54] After an Ab-mediated two-particle "sandwich" is formed around the analyte, the magnetic particles are concentrated into a pellet from which a SERS spectrum is acquired. The signal intensity is proportional to the number of bound SERS tags, which in turn is proportional to the analyte concentration. No wash steps or processing steps are required; the entire reaction and detection takes place in an Eppendorf tube. Sensitive detection of circulating tumor cells in whole blood was demonstrated. During the symposium, Michael Natan presented data from two separate sets of experiments that demonstrated the use of this platform for the detection of aflatoxin B1 in peanut butter and 2,4,6-trinitrotoluene (TNT) from air. In both cases, a competitive immunoassay format[55] was employed because these analytes are too small to be bound by multiple Abs. For the alfatoxin study, samples were extracted into methanol prior to introduction in the SERS-magnetic bead-based assay, and a limit of detection of ~0.7 ng aflatoxin B1/mL was achieved. TNT was extracted from air samples *via* bubbling TNT-tainted air through deionized water. Analysis of the resulting aqueous samples yielded a TNT limit of detection 0.56 ng/mL.

Marc Porter and colleagues have developed an effective variation of the indirect SERS detection method that utilizes an immunoassay chip platform in conjunction with SERS tags as optical labels.[56] Analytes are first captured onto an antibody-functionalized organothiol SAM-coated Au slide. The SERS detection agent is then introduced, which comprises a Au nanoparticle coated with a succinimidyl ester-terminated SAM with a built-in reporter, with a detection Ab attached through the succinimidyl ester. The group has focused on optimizing the platform by determining factors such as the optimal particle sizes for the detection tag and ideal binding distances from the surface. For instance, they have determined that the SERS enhancement factor increased by an order of magnitude when a 60 nm particle was immobilized 1.2 nm above the gold substrate as compared to that for a 30 nm particle.[57] The group has also worked to improve the assay kinetics, allowing the platform to be more effective in real-world applications. Because diffusion of molecules to a two-dimensional substrate is slow, the team has developed a method of rotating the capture substrate, which improved binding kinetics from 24 h to 25 min, and resulted in a 10-fold improvement in the limit of detection.[58] Using this indirect approach, detection of low levels of cancer markers, biowarfare simulants, viral pathogens, and bacteria has been accomplished.[56,59]

Using a format and materials identical to that used by Oxonica Materials Inc., General Electric developed a magnetic bead-based sandwich immunoassay for rapid identification of five different bacterial strains of *Escherichia coli*.[60] Also presented were results of a study on assay robustness for the detection of anthrax in the presence of common household items and environmental matrices.

Another area where synthesis can have an impact is in the development of general approaches to make SERS hotspots.

While not yet as plentiful as work on SERS substrates, there is an increasing body of work on alternative materials for indirect SERS detection.[61−66] In addition, Amar Flood at Indiana University described an interesting approach to indirect detection whereby chemical receptors are used to detect analyte binding events in solution-phase measurements. If the receptor is designed such that the receptor−analyte complex can be resonantly excited (*i.e.*, surface-enhanced resonance Raman scattering), a significant improvement in signal-to-noise can be achieved. His group is applying the method to the detection of TNT.

OUTLOOK

In the immediate future, the making of new nanoparticles, new nanostructures, and new substrates must give way to better characterization of existing materials. Highly enhancing SERS-active media are not currently a limiting factor in the use of SERS. Moreover, since the Raman spectra of adsorbed impurities are enhanced to the same extent as that of adsorbed analytes, SERS-active surfaces with increased EFs do not lead to any improvement in signal-to-noise (though it could lead to less expensive detectors). Thus, the impetus should be to understand the fundamental aspects of performance (what is responsible for the enhancement, wavelength response, analyte generality, stability, durability, repeatability) rather than optimization of the EF, a number that is never even reported in analytical or bioanalytical measurements.

There must also be a move to the NIR region of the spectrum. As discussed above, one way SERS is differentiated from other analytical methods is that it can operate on "dirty" samples, an attribute of obvious importance for the point-of-use mandate for homeland security. However, real-world samples invariably contain fluorescent impurities, and a high fluorescent background renders SERS signals undetectable. As the excitation wavelength moves fur-

ther to the red, fluorescence diminishes. SERS experiments that are impossible to carry out using 514 nm excitation because of background fluorescence become possible at 633 nm, easy at 785 nm, and trivial at 1064 nm.

In the near term, the indirect detection, no-wash format of SERS is most likely to have an impact for homeland security because commercial products are actively being developed. The indirect method circumvents the "elephant in the room" of selectivity in direct SERS detection. Furthermore, it leverages the ability to use NIR excitation on unprocessed samples and the here-and-now availability of hand-held Raman readers. The capability to multiplex can be used to detect simultaneously multiple biothreat targets or to detect several epitopes on a single target to minimize false positives or false negatives. Finally, indirect detection using reporter-loaded SERS-active nanoparticles can easily take advantage of improvements in nanoparticle size/shape/aggregation state to make tags with optimal brightness and wavelength response.

In the intermediate term, SERS and homeland security will both benefit by an infusion of organic synthesis. Sophisticated permselective materials will have a significant impact on all SERS applicatons. These materials, likely polymers, will serve to concentrate analytes near or at the SERS-active surface, while blocking all other SERS-active (or inactive) species. The concentration of analytes near the surface will be especially important for SERS detection of agents (such as nerve gases) that might not adsorb strongly to uncoated Ag or Au.Another area where synthesis can have an impact is in the development of general approaches to make SERS hotspots.

Another area where synthesis can have an impact is in the development of *general* approaches to make SERS hotspots. In her talk, Jennifer Shumaker-Perry described a synthetic route to make dimers of any particles.[67] The approach involved binding particles to a surface, coating the particles with a SAM, and then lifting off the particles to expose the uncoated region protected by the surface. Functional groups can be added to this naked region of the surface that leads to selective dimerization. While such dimers would not be useful for SERS (since the entire particle surface and especially the region between particles is already coated with tightly bound molecules), it nonetheless constitutes new thinking on how particles can be brought together in a controlled fashion.

In the longer term, when these advances come to fruition, SERS stands to have a major impact not just for homeland security but for a wide variety of analytical and bioanalytical measurements. Pam Boss showed coating of magnetic particles with Au colloid coated with pentachorothiophenol (PCTP) could be used to improve the performance of a direct SERS measurement for napthalene.[68] By forming a magnetic pellet, she effectively concentrated a dispersed SERS substrate, and the PCTP coating extracted and concentrated the naphthalene from aqueous solution. From an operational perspective, this experiment is identical to the no-wash, indirect detection assay. Thus, one could imagine combining such assays in a single tube, enabling, for example, ultrasensitive direct detection of a LMW analyte and ultrasensitive indirect detection of a protein *via* a sandwich immunoassay in a single measurement. Such a measurement is not possible with any other detection modality.

The first measurements on fluorophores attached to proteins were made over 60 years ago; since then, the integrated impact of fluorescence on life science has been incalculably large. We believe a similar age is dawning for SERS and look forward to seeing both the analytical/bioanalytical insights and practical, real-world advancements this nanoscale phenomenon will enable.

Conflict of Interest: M.J.N. declares that he is Chief Executive Officer of Oxonica Materials Inc.

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