Chapter 10

SERS Biomedical Applications: Diagnostics, Forensics, and Metabolomics

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1 INTRODUCTION

1.1 Biomedical Applications and Vibrational Spectroscopy

Vibrational spectroscopies, such as infrared (IR) absorption and Raman scattering, are attractive methods for a wide range of biomedically related applications because no additional labeling is required for identification. The vibrational motions of the molecules in the often complex mixtures relevant to bioanalytic investigations are the inherent “labels” of interrogated samples. Secondly, unlike UV/Vis absorption spectra, vibrational spectral features are narrow thus providing multiple and highly accurate identification markers for a given specific molecular signature, and hence IR- and Raman-based techniques are inherently multiplexing approaches. Vibrational signatures of several components in a complex mixture can be identified in a single spectral signal acquisition especially when combined with sophisticated multivariate data analysis techniques and reference spectral libraries. In addition, optical approaches are generally easy to use and rapid, which are important additional attributes for clinical and point-of-care use. Finally, these spectroscopic techniques are nondestructive and thus samples may be further tested for additional characterization by complementary methodologies following vibrational spectroscopic examination.

Consequently, the label-free molecular specificity of IR absorption and Raman spectroscopy has been exploited for many biomedical applications, including diagnostics, for many years [1–11]. The ability to avoid masking strong water absorption signals inherent to biological samples and the greater spatial resolution afforded by shorter excitation wavelengths make Raman a more attractive vibrational spectroscopic probe than IR for many medical applications. Furthermore, recent advances in solid-state laser diodes have resulted in the rapid development of portable Raman instrumentation allowing implementation of clinical point-of-care use. Thus, Raman spectroscopy, in particular, emerges as a potentially very powerful vibrational approach for the study of biological materials.

There are some limitations, however, associated with the use of Raman for biomedical applications. Conventional Raman spectroscopy is a relatively weak effect. Thus, for good-quality (large signal-to-noise ratio) Raman spectra, high concentrations of molecular species are required in order to make accurate analytic identifications, and thus detection and identification of trace amounts of key biological components may not be possible. Secondly, depending on the excitation used, biological samples may be fluorescent, and thus such broad emission features may mask the weaker discrete Raman bands. Finally, given the complex mixtures that often characterize biomedical samples, the clear observation of molecular specific features of disease or treatment relevance may be difficult to distinguish in the ensemble Raman (or IR absorption) signal. As illustrated in this review, the greatly enhanced effective Raman cross sections for some molecular specific components resulting from the molecular
proximity to nanostructured plasmonic surfaces make surface-enhanced Raman spectroscopy (SERS) an ideal tool for many applications in medical diagnostics, in metabolomics, and in forensic science. For the applications summarized here, SERS is found to combine the desirable attributes of Raman spectroscopy: speed, ease of use, on-site data acquisition, spectral specificity, multiplexing capability, nondestructive to sample, with the added qualities of high sensitivity, fluorescence-free backgrounds, and improved specificity to address several unmet urgent biomedical and forensic science needs. Furthermore, this review highlights the importance of understanding these biomedically related SERS spectra in terms of their chemical and biochemical origins. Providing these molecular interpretations is essential in particular for the adoption of this relatively new optical approach into the broadly defined biomedical community, for the optimization of the best sample preparation protocols, and for the development of new SERS applications. In this review we will summarize our recent efforts to develop SERS for the rapid diagnostics of urinary tract infections, sexually transmitted diseases of chlamydia and gonorrhea, and bacteremia (blood infections), all resulting from pathogenic bacteria, and for the detection and identification of trace amounts of human body fluids related to forensic science investigations.

1.2 Surface Enhanced Raman Spectroscopy (SERS)—Brief Introduction

SERS was discovered about 35 years ago when it was first observed that the efficiencies of Raman scattering increased by factors of $10^4$–$10^8$ for molecules near ($\leq$ 5 nm) nanostructured metal surfaces [12,13]. This phenomenon arises predominantly from a resonance effect between the incident and Raman-scattered electric fields and the surface plasmon excitations of the nanostructured metal surfaces [14–17]. Ag and Au are the most commonly employed metals in part because their surface plasmon resonances (SPR) for 10–100 nm structures are in the visible to NIR where inexpensive, robust, and efficient solid-state lasers are readily available. Au substrates are generally more chemically stable than Ag substrates. A large number of bioanalytic applications have been explored via different kinds of SERS substrates ranging from simple colloidal solutions to highly controlled and precise nanoengineered arrays [16,18–21]. A so-called chemical effect also plays an important role in determining which vibrational degrees of freedom appear in SERS spectra [22–24]. Thus the relative intensities of a SERS spectrum may differ from those of the normal or non-SERS spectrum (NRS) of the same molecule, in addition to the large ($>10^6$) increase in effective scattering cross section per molecule, at least for those moieties physisorbed or very close to the SERS active regions of the nanostructured substrates.
1.3 Advantages of SERS for Biomedical Analyses

SERS has been increasingly exploited in a number of biomedical applications for the identification of molecular markers of biological activity [25–27]. The most obvious advantage that SERS offers for biomedical analyses is that the large increase in scattering cross section permits the identification of dilute solutions and analytes of low concentration in biologically relevant samples. Molecular components in the μM-to-mM regime can be readily monitored with just a few milliwatts (or less) of incident laser power as a result of enhancement factors in the $10^6$–$10^8$ range for molecules near SERS active surfaces. Secondly, SERS offers sensitivity to different molecular species in a complex mixture than found for normal Raman resulting from the molecular selectivity to nanoparticle adherence [28]. The competitive metal surface binding of different molecular species in a biological fluid preferentially selects for some components (sulfur-containing multiple nitrogen ions) over others in the mixture. This selectivity attribute of SERS is probably underappreciated for biological mixture sample applications. It allows for discriminations via SERS that are not possible via normal Raman spectroscopy and offers supplementary information about inherently multicomponent biological fluids compared with other analytic techniques, such as mass spectrometry, as shown here. A further important advantage of SERS, as compared with normal Raman, is that fluorescence is effectively quenched in SERS due to efficient energy transfer to the nearby metal [29]. Thus broad variable fluorescent backgrounds, often observed in Raman spectra of biological materials and cited as a challenge and complication for normal Raman identification methodologies of forensically relevant human body fluids [30], vanish in SERS. Speed, fluorescence elimination, and enhanced sensitivity and specificity characterize this optical approach relative to normal Raman spectroscopy providing the basis for SERS-based solutions in clinical settings and for forensic investigations.

2 BRIEF SUMMARY OF SERS EXPERIMENTAL CONSIDERATIONS

A SERS-based platform for biomedical applications requires four key components: sample preparation, SERS substrate, Raman instrumentation, and software/multivariate data analysis techniques for identification [31]. Although often overlooked, sample preparation protocols are an important component for the effective use of SERS spectra for biomedical uses. Procedures for collecting or enriching cells from biological samples and the consequent biochemical response or the chemical response to pH or temperature effects can offer an additional degree of control over the effectiveness of SERS for medical applications of biologically relevant samples. For example, the ability to bring a cell to the focus of the Raman microscope may be the limit determining the clinical sensitivity for infectious disease diagnostics in human blood or urine or for pathogenic cancer cell determinations.
Secondly, nearly all of our SERS work results from the use of a Au or Ag nanoparticle covered SiO2 chip produced by a metal-ion-doped sol-gel procedure [29]. These substrates are found to yield strongly enhanced, reproducible SERS signal when excited by 785 nm excitation. The typical SERS active Au nanoparticle chips used for the studies summarized in this review are substrates that have clusters of 2–15 80 nm Au nanoparticles physically adsorbed to the outer surface of the SiO2 matrix. An ensemble averaged SERS enhancement factor of $\sim 5 \times 10^7$ has been estimated for this substrate as measured by the Raman scattering of glycine [29]. The production cost for this Au nanoparticle covered SERS substrates is low and by insulating the chips in an inert Ar environment, storage time of up to 5 months has been achieved. These attributes are central to point-of-care use and the portability of this methodology. Ag can be substituted in the recipe for the production of these in situ grown substrates as well and has achieved similar and for some analytes even larger enhancements [32]. The sol-gel Au nanoparticle “chips” have proved to provide the best signal/noise for bacterial identification purposes of those we have thus far developed [33–35].

All of the SERS spectra described here have been acquired by the use of a Renishaw RM2000 SERS microscope excite by a few mW or less of 785 nm excitation at the focus of a 50× objective. Similar or better performance has been achieved with portable Raman microscope (cooled CCD array, $\sim 5 \text{ cm}^{-1}$ spectral resolution, piezo-driven sample positioning stages, and submicron imaging resolution) that has been developed with BioTools, Inc. (Jupiter FL) for these biomedical application purpose.

The final key component for a SERS-spectra-based identification and detection platform is a software procedure for robust classification of unknown spectra within a previously developed spectral library. We have developed a novel multivariate data analysis technique that enhances accurate distinction between the SERS spectra of closely related spectra when combined with a previously developed reference library [36]. SERS spectra are converted to a series of “zeros” or “ones” based on the sign of the second derivative of the normalized spectrum at each wave number. This barcode procedure reduces the effects of broad variable background and minimizes the effects of all sources of inhomogeneities (nanoparticle substrate or sample based) and has been shown to result in greater analytic sensitivity and specificity when combined with well-known multivariate data analysis approaches such as principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA).

3 INFECTIOUS DISEASE DIAGNOSTICS: GENERAL INTRODUCTION

The ability to rapidly detect and identify bacterial cells in human body fluids at relatively low cost and in point-of-care settings is a continuing health-care need
particularly as infections resulting from drug-resistant bacteria are increasingly recognized as a growing major public health problem [37]. Currently, the gold standard method for diagnosing bacterial infections is culture growth of the suspected infectious body fluid, blood, urine, sputum, etc. The major limitation of the cell culture growth method is that it is slow. It takes \( \sim 24 \) h (or longer for fastidious organisms) to establish the presence of a bacterial pathogen and another \( 24–48 \) h (or longer) for antimicrobial susceptibility to be determined. Consequently, physicians will typically prescribe broad-spectrum antimicrobial regimen to kill a large range of different microorganisms until culture results are known. The practice of treating with broad-spectrum antibiotics first undoubtedly contributes to the increasing prevalence of drug-resistant bacteria. Ideally, a physician would have near-instant identification of the causative bacterium and its antibiotic susceptibility profile, as delays in appropriate antibiotic therapy can increase morbidity and mortality [38]. More generally, as a result of this long waiting period due to cell culture growth, broad-spectrum antibiotics are often prescribed based on presentation symptoms contributing to ineffective treatment, chronic infections, increased care costs, and the enhanced proliferation of drug-resistant bacterial strains. Nucleic acid amplification (NAA) techniques, such as PCR, or immunoassay techniques are more rapid molecular based approaches but suffer from limitations due to background contamination from exogenous sources of DNA for the former [39] and sensitivity, needed antigen amounts, and the time required for seroconversion, for the latter [40]. A rapid diagnostic that provided microbial identification and drug susceptibility within the time frame of a patient presenting at a clinic (<1 h) would allow optimal, narrow-spectrum drug treatments, thus improving outcomes more quickly and limiting the proliferation of bacterial resistance more generally. As described below for UTI, STD, and bacteremic diagnostics, SERS has the potential to meet this unmet biomedical need for rapid, specific identification of viable bacterial cells enriched from human body fluids.

4 URINARY TRACT INFECTION DIAGNOSTICS

4.1 General Introduction

Urinary tract infections (UTI), evidenced by the appearance of bacteria in urine, are among the most common types of infections in humans and consequently are among the leading reasons for treatment in primary care medicine [41]. Approximately 50% of all women will have at least one UTI in their lifetime, 20% will get a second infection, and a large percentage of this group will suffer from chronic UTIs [42]. In the United States alone, UTIs are responsible for more than 7 million doctors’ office visits and over one million hospital admissions at a cost of \( \sim \$1 \) billion per year [43,44]. Furthermore, UTIs have become the most common hospital-acquired infection, accounting for as many as 35% of nosocomial infections [45].
Strains of *Escherichia coli* are the predominant bacterial species responsible for UTIs and account for \( \sim 70\% \) of UTI clinical presentations [45]. Other UTI etiologic pathogens include strains of *Staphylococcus saprophyticus* (5%–10%), *Klebsiella pneumoniae* (4%–7%), *Proteus mirabilis* (4%–5%), *Pseudomonas aeruginosa*, and *Enterococcus faecalis* [41,46]. The current “gold standard” for UTI diagnosis is a quantitative urine culture that requires \( \sim 24 \) h to produce results, and antibiotic susceptibility testing minimally requires another 24 h or more via conventional clinical growth methods [47]. A midstream bacterial count of \( 10^5 \) cfu/mL in free collection urine is generally defined as a positive indication of UTI in symptomatic patients and the recommended level for antibiotic treatment [48]. The successful development of a rapid, growth-free, optical SERS diagnostic with drug susceptibility specificity will guide the use of narrow-spectrum therapeutics resulting in the more judicious use of antibiotics, earlier initiation of appropriate therapy, and better real-time measures of therapeutic efficacy for patients presenting with UTI symptoms.

### 4.2 SERS Spectra of UTI Clinical Isolates

We recently reported on the ability of SERS to provide strain-specific identification of infectious urine samples resulting from spiking processed human urine at the minimum level for a UTI diagnosis (\( 10^5 \) cfu/mL) [49]. The 12 bacterial strains used in this study were all isolated from clinical urine specimens, and their antimicrobial susceptibility profiles, which varied among this set of clinical isolates, were determined for a wide range of antibiotics by a BD Phoenix automated microbiology system [50] prior to these SERS measurements. The 785 nm excited SERS spectra of these 12 bacterial strains of UTI clinical isolates grown to log phase in urine and enriched from \( 10^5 \) cfu/mL urine solutions are shown in Fig. 1. Six strains of *E. coli* and two strains each of *S. saprophyticus*, *K. pneumoniae*, and *E. faecalis* are displayed in this figure. The strains of these four species are color-coded in the figure. Each of the displayed spectra is an average of \( \sim 10 \) individual SERS spectra acquired on our Au nanoparticle substrates and exhibits SERS spectra with excellent S/N as seen in this figure. The gray-shaded region on each displayed spectrum corresponds to \( \pm 1 \) standard deviation of the average normalized intensity at each scattered frequency and thus is a measure of the excellent reproducibility of these SERS spectra.

### 4.3 SERS UTI Diagnostic Capabilities: Identification and Antibiotic Susceptibilities

The high quality of these spectra (Fig. 1) demonstrates that SERS has the sensitivity to report on the presence of bacteria enriched from urine at the minimal required level (\( 10^5 \) cfu/mL) and given the observed S/N, even higher sensitivities should be possible. Sample preparation, consisted of centrifugation and washing the spiked urine samples and accounted for most of the \(<1 \) h total time.
required for the SERS diagnostic result of these infectious urine samples following receipt of the urine samples and enrichment via the employed benchtop prototype protocol. For the most part, the differences between the SERS spectra of the four species studied here, *E. coli*, *K. pneumonia*, *E. faecalis*, and *S. saprophyticus*, are greater than the difference between the strains of a given species type. A number of vibrational bands, for example, strong bands in the 725–735 cm\(^{-1}\) region and at \(~660\) cm\(^{-1}\), 960 cm\(^{-1}\), 1240 cm\(^{-1}\), 1315 cm\(^{-1}\), and 1450 cm\(^{-1}\), are common to several of these bacterial spectra.

In order to demonstrate how a UTI diagnostic procedure could be developed based on these SERS spectra of urine-enriched bacterial cells, a PLS-DA
classification analysis was performed on this set of clinical isolate SERS spectra [49]. The barcode reduced spectra served as the input vectors for this procedure for the 150 spectra in this data set. Optimized PLS-DA classification performance resulted for a model that included 24 latent variables (LV) and cross validation was accomplished by the random subset method employing 27 different test sets and 21 iterations. The excellent results of this cross validated PLS-DA classification procedure demonstrate the potential for SERS spectra of bacterial strains grown in urine to serve as a rapid diagnostic at clinical concentrations. The analytic sensitivity (true positive rate) and specificity (true negative rate), averaged over all 12 classes, was 95.8% and 99.3%, respectively, for this classification model. The average corresponding classification error for the 150 SERS spectra defining this data set was just 2.3% [49]. It is important to note that this procedure allowed classification of not only different species but also successfully separated strains within a given species. Since drug susceptibility profiles were predetermined for this group of clinical isolates [49], the successful SERS-based classification also resulted in a determination of the effective antibiotic treatments for each infected urine sample that exhibited some strain and species dependence. This antibiotic susceptibility information was thus known for each spiked urine sample in less than 1 h by our SERS-based procedure.

In order to test the robustness of this SERS diagnostic classification model based on the SERS spectra of these 12 strains grown in processed urine and the ability of this SERS-based library to identify a UTI clinical isolate grown and enriched in nonprocessed human urine, we applied this methodology to a sample of one of the clinical isolates, *E. coli* BD 6594 spiked into normal or nonprocessed urine. Overnight culture of this clinical isolate was spiked into a fresh urine sample that was not subjected to prior solid material removal via centrifugation. Following this inoculation and growth, the infectious mixture was diluted in urine to 10^5 cfu/mL, and bacterial cells were enriched from this sample via a four-stage filtration procedure and then centrifuged and washed prior to being placed on the SERS substrates for spectral acquisition. The spectra of spiked *E. coli* BD 6594 are virtually identically to those obtained from growth in processed urine and demonstrate that the different sample handling protocols have no significant effect on the nature of the bacterial SERS spectrum. However, more importantly, when the SERS spectra of this separate sample, grown in unprocessed urine and enriched via the filtration/centrifugation procedure, was treated as an unknown and classified via the PLS-DA procedure developed for the 12 strains of bacteria grown in processed urine, all 21 spectra of this “unknown” data set were correctly identified as the *E. coli* BD 6594 strain with a predetermined drug susceptibility profile, as shown in Fig. 2. Thus these studies illustrate the potential for a SERS-based diagnostic to provide identification of a bacterial strain from an infectious urine sample at clinically relevant concentrations and, with predetermined drug susceptibility profile, offer antibiotic-specific recommendations for targeted narrowband treatment.
5 STD DIAGNOSTICS

5.1 General Introduction: Chlamydia and Gonorrhea

Sexually transmitted diseases (STDs) continue to be a significant cause of morbidity in the United States with ~$15.9 billion spent annually on health-care costs related to their diagnosis and treatment [51]. Chlamydia, the most common sexually transmitted disease (STD) in the United States, is caused by infection from the Gram-negative bacterium *Chlamydia trachomatis* [52]. More than 1.5 million cases of chlamydia were reported to the US Center for Disease Control and Prevention (CDC) in 2015, an increase of nearly 6% over 2014, and recent European Centre for Disease Prevention and Control data also demonstrate an increase of reported chlamydia infections in Europe [52,53]. Long-term, untreated chlamydia infection can lead to severe consequences such as pelvic inflammatory disease, a major cause of infertility, ectopic pregnancies, and chronic pelvic pain in women [54]. Furthermore, chlamydia increases the risk of HIV transmission and infection [55], offers complications for newborns via perinatal transmission [56], and is the cause of trachoma, a major cause of blindness in the developing world [57].

The *C. trachomatis* organism is an obligate intracellular bacterium that lacks the enzymes for many biosynthetic and metabolic pathways and hence requires a eukaryotic host cell to complete its life cycle [58]. This bacterium has a complex biphasic developmental cycle that involves a metabolically active and non-infectious form called reticulated body (RB) and a metabolically inactive but infectious form called an elementary body (EB). Following invasion of eukaryotic cells, the EBs are converted to RBs, the intracellular replicating form of this
organism and after multiple divisions, the RBs differentiate into EBs, and these infectious cells are released to initiate new rounds of infection. EBs are relatively small particles (~0.3 μm diameter) with a rigid, disulfide cross-linked outer membrane protein complex that helps maintain the structural integrity of the EB [59] and, as the infectious agents, are the cells of diagnostic interest including for our SERS studies [60].

Gonorrhea is the second most commonly reported STD in the United States, with ~400,000 cases reported in 2015 and results from infection by the gram-negative bacterium *Neisseria gonorrhoeae*. After decades of decreasing reported rates had reached an all-time low in 2011, the prevalence of gonorrhea in the United States and Europe has shown a steady increase in the last 5 years [52,53]. Patients with gonorrhea are often asymptomatic until complications arise such as pelvic inflammatory disease, ectopic pregnancy, and infertility. Untreated gonorrhea can also lead to disseminated gonococcal infection when *N. gonorrhoeae* spreads to the blood or other parts of the body [61], and gonococcal infections have also been shown to facilitate the transmission of HIV infection [62], as found for chlamydia. Of particular relevance to the SERS diagnostic capabilities, ~30% of patients infected with *N. gonorrhoeae* are coinfected with *C. trachomatis*, and thus patients treated for gonococcal infection are often treated routinely with an antibiotic regimen that is effective against *C. trachomatis* infection [63]. Thus, the capability to distinguish one from the other and mixtures would be an important advance in reducing the over prescription of antibiotics and their consequent impact on the evolution of drug-resistant strains.

Although sensitive and specific, traditional cell culture methods for chlamydia diagnosis require specialized culture media, culture conditions and skilled staff for the technically demanding procedure and are very slow (≥72 h), making it virtually impossible for routine and point-of-care diagnostics [64]. Similarly, *N. gonorrhoeae* is also a fastidious organism requiring enriched media in a CO₂ atmosphere for lab-cultured growth for ≥48 h. More nonculture methods for STD diagnostics, such as enzyme immunoassay (EIA) and direct fluorescent antibody stain (DFA)-based techniques, have been recently developed [65]. However, nucleic acid amplification tests (NAAT) are the current best technology recommended by the CDC for the detection of chlamydia and gonorrhea offering sensitivity and specificity comparable with the culture method with a faster turnaround time [65,66]. However, the susceptibility to cross contamination from exogenous genetic material, cost, inability to distinguish bacterial viability (i.e., live vs dead cells), the presence of inhibitory factors, and the need for experienced technicians in laboratory settings, which necessitates a second clinical visit for some patients, are limitations for the NAAT approach [67,68]. Furthermore, although NAAT is considered the “gold standard,” same-day results for most NAAT are not usually available because they are classified as being of moderate-to-high complexity, usually requiring 1–2 days for results to become available [69]. Thus, the development of alternative, low-cost, easy-to-use,
rapid, point-of-care approaches for the detection and simultaneously differentiate \textit{C. trachomatis} and \textit{N. gonorrhoeae} in clinical setting and in a useful time frame (≤1 h) for narrow-spectrum antibiotic drug prescription remains a critical strategy for improving reproductive and sexual health worldwide.

5.2 SERS Spectra of STD Causative Bacteria

SERS spectra of \textit{C. trachomatis} serovar D (\textit{Ct}) and \textit{N. gonorrhoeae} FA1090 (\textit{Ng}) on our Au and Ag SERS substrates excited at 785 nm are shown in Fig. 3. The data illustrated in this figure are illustrating two important points. Firstly, the SERS spectra of \textit{Ct} and \textit{Ng} exhibit dramatically different vibrational features from each other on both the Au and Ag SERS substrates. Whether SERS spectra are obtained on Au or on Ag substrates, the SERS spectra of these two STD causative bacteria are readily distinguishable. Thus, in addition to identifying biological samples with on or the other of these infectious agents, resolving infectious mixtures of these two organisms should also be possible. Furthermore the SERS spectra of \textit{C. trachomatis} EBs are completely different than the SERS spectra of all other bacteria we have observed (Fig. 2) [29,32,36,49,70–73]. Secondly, the spectra in Fig. 3 demonstrate that for some biomedical samples, SERS spectra can be very different on Au or Ag

![SERS spectra of N. gonorrhoeae and C. trachomatis](image)

**FIG. 3** SERS spectra of \textit{N. gonorrhoeae} and \textit{C. trachomatis}, the causative agents of gonorrhea and chlamydia, on Au and Ag nanoparticle substrates.
nanostructured surfaces. As seen for *N. gonorrhoeae* (Fig. 3), SERS spectra show relatively little difference on Au or Ag substrates [32]. Small frequency shifts and some changes in relative intensities usually characterize the difference found for a given vegetative bacterial sample on Au or Ag SERS active substrates. However, for *C. trachomatis*, whose SERS spectrum does not resemble any other bacterium, the differences between the Ag and Au spectra are very dramatic. The very broad (~150 cm\(^{-1}\)) bands centered at ~900, 1200, and 1550 cm\(^{-1}\) of the Au substrate spectrum are replaced by more typical narrow vibrational bands at 1003, 1375, 1450, and 1600 cm\(^{-1}\) in the *Ct* spectrum on Ag. The origins of these SERS bands and the metal dependence will be discussed further below.

Furthermore, in addition to the different molecular origins of the vibrational bands correspond to the *Ct* and *Ng* spectra, a distinct and reproducible time dependence is observed for the characteristic SERS spectra of these two bacterial species as shown in Fig. 4 for *Ng* on Au. Over a period of about 1 h, the spectrum of *Ng* evolves with time. In particular, a strong band at 1050 cm\(^{-1}\) prominent in the initial spectrum disappears, and a feature at 733 cm\(^{-1}\) blue shifts to 735 cm\(^{-1}\). In contrast, although the overall intensity decreases by a factor of 5 over this same time period, the SERS characteristic signature of *Ct* remains unchanged in terms of relative intensities and anomalously broad shapes on Au. Thus this temporal affect also distinguishes these two STD Gram-negative pathogens. Although we have not yet optimized a sample preparation protocol for the enrichment of these gram-negative bacteria from body fluids, we have demonstrated detection sensitivity as low as 10\(^2\) and 10\(^4\) cfu/ml for *C. trachomatis* and *N. gonorrhoeae*, respectively. These values are consistent with those required for chlamydia and gonorrhea diagnosis [74,75]. Thus,

![FIG. 4](image)

**FIG. 4** The post-water washing time dependence of the SERS spectra of *N. gonorrhoeae* on Au. The observed spectra (black) and the best fits (red) due to time-dependent contributions of NADH/NAD\(^+\), adenine, and guanine are shown. Bar graph representation of the time dependence of these spectral components is shown on the left.
these preliminary results indicate that SERS may be developed as a rapid, inexpensive, growth-free diagnostic for these most prevalent bacterial STDs.

6 BACTEREMIA DIAGNOSTICS

6.1 General Introduction

Bacteremia is defined as the presence of bacteria in the blood that is a nominally sterile fluid. It can develop from a severe infection at another site, a surgical wound infection, or contaminated catheters or other implanted prosthetic devices. Bacteremia can lead to a number of serious health conditions, including sepsis. Sepsis ranks 11th overall among the causes of death in the United States with annual estimates of ~750,000 cases of severe sepsis and mortality rates ranging from 20% to 52% [76,77]. Sepsis was the single most expensive condition treated in US hospitals in 2009 [78] and the number of sepsis cases is expected to continue to rise due to the aging American population, spread of antimicrobial resistance, growth of immunosuppressed populations, and increased use of invasive catheters and prosthetic materials [79]. Every hour without the appropriate therapy translates to increased mortality [80]. Thus a diagnostic that can shorten the time to diagnosis can have a significant effect on all measures of health outcomes.

Currently, the gold standard method used to diagnose bacteremia relies on blood culture with results taking 24–48 h or longer. However, enriching bacteria from blood is much more challenging than enrichment from urine, as required for UTI SERS diagnostics described above. Detecting pathogens directly from blood is very challenging due to the low concentration of a variety of infectious cells (often <10 cfu/mL) and a complex blood matrix containing ~10^9 blood cells (RBCs, WBCs, platelets, etc) per mL and high protein content. However, to exploit the rapid diagnostic capability of SERS for blood infections, we developed a platform that consists of a universal sample preparation system for viable bacteria coupled with the rapid SERS identification procedure [73]. By integrating SERS detection with this universal sample preparation process, the demand for a rapid, sensitive, and specific bacteremia diagnostic method from primary blood samples can be fulfilled.

6.2 SERS Detection and Identification of Bacteremia Developments

The developed bacteremia diagnosis system provides physician identification of the pathogens directly from whole, uncultured blood within 7 h. This method relies on a preferential blood cell lysis process and centrifugal forces to concentrate the microorganisms. To demonstrate the effectiveness of the combined sample preparation and SERS detection methodology, we designed a blinded study wherein 10 mL human blood samples were inoculated with log-phase bacteria (*E. coli* ATCC 25922 or *S. aureus* ATCC 25904) or remained sterile.
Since this was a preliminary demonstration of the complete system, we chose a common Gram-negative rod and a Gram-positive coccus to demonstrate our system’s utility. Of the 91 samples tested, 59 samples were inoculated with log-phase *E. coli* or *S. aureus*, and the rest of the samples were negatives. These samples were immediately processed using our universal sample preparation process, incubated at 37°C, analyzed by SERS, and identified using our algorithm, which was optimized for identifying bacteria from blood. At starting concentrations ranging from $10^1$ to $10^4$ cfu/mL in blood, we achieved 97% specificity and 88% sensitivity after processing the spiked whole blood and analyzing the results of the SERS spectra using the barcode-based PLS-DA algorithm [73]. Of the spiked samples, both bacteria were also correctly identified 97% of the time. The positive predictive value was 98%, and the negative predictive value was 82%. Given these figures of merit and the relatively short processing time (7 h), which is considerably reduced from the 24–48 normal culture growth timescale, it is demonstrated that this process has the potential to help physicians more rapidly identify pathogens from a bacteremic patient. The development of other novel schemes and approaches for bacterial enrichment will only serve to reduce this SERS-based diagnostic time further.

7 ORIGINS OF BACTERIAL SERS SIGNALS

7.1 Chemical/Molecular Origins of the SERS Spectra of Bacteria

Despite the large number of research groups that have reported SERS spectra of bacteria over the past 20 years [29,32,36,81–113], there has been little agreement on the assignment of the observed vibrational signatures in terms of the chemical species and biological basis for these signals. In order to maximally exploit the capabilities of SERS for bacterial identification, it is essential to understand the molecular and corresponding biochemical origins of these SERS vibrational signatures. Such information informs the design of optimal bacterial preparation and enrichment procedures from infectious human body fluids in order to provide maximally robust and reproducible SERS signals. It also provides a basis for understanding the capabilities and limitations for diagnostics by this optical approach and is a requirement for the adoption of this novel rapid diagnostic in the medical community. Finally, a biochemical understanding of these cellular molecular markers has the potential to broaden the uses of SERS for other biomedical applications and provide an even larger chemical biology impact beyond rapid, growth-free bacterial diagnostics.

A variety of assignments have been previously offered for the observed vibrational features in the SERS spectra of bacteria. A set of representative observed SERS spectra of 10 species are shown (in black) in Fig. 5. Due to the distance dependence of the SERS enhancement mechanisms [114] it has been generally assumed that the observed vibrational bands appearing in the SERS spectra of whole vegetative bacterial cells are dominated by the contributions of their outer cell wall components. Furthermore, the SERS vibrational
features of whole bacterial cells are different than the peak frequencies seen in the normal, non-SERS Raman spectra of bacteria, consistent with cell wall molecular components accounting for SERS bacterial signals [29,100,115,116]. Correspondingly, the current consensus had become that SERS spectra of bacteria are due to a mix of vibrational modes of cell wall components, such as peptidoglycan, lipids, lipopolysaccharides or membrane proteins, and nucleic acids [29,32,36,81,83–87,90,91,95–98,101,102,105,106,109,111–113] although the reported bacterial SERS spectra did not correlate with cell wall architecture, that is, Gram-positive versus Gram-negative. A few studies also suggested the possibility that small molecules, such as adenine, may contribute to the observed bacterial SERS spectra as well [84,98,111,112]. The generally strongest feature in the 785 nm excited bacterial SERS spectra at \( \sim 725–735 \text{ cm}^{-1} \) (see Fig. 5) has

FIG. 5 785 nm excited SERS spectra of 10 bacterial species on Au nanoparticle substrates. Displayed experimental spectra are averages of 4–6 individual spectra and normalized to the intensity of the strongest feature in each spectrum. Empirically determined best fits of the experimental bacterial spectra due to linear combinations of purine (adenine, hypoxanthine, xanthine, guanine, uric acid, and AMP) SERS spectra are overlayed on the observed spectra. Excellent fits are obtained for all bacterial SERS spectra.
been assigned variously to the glycosidic ring or C—N stretching mode of
N-acetyl-D-glucosamine (NAG) or N-acetylmuramic acid (NAM), the major
components of peptidoglycan, a key bacterial cell wall biopolymer
[86,90,101,102,105,106,111,113], and a symmetrical O-P-O vibrational mode
of the phosphate group [99], adenosine [91], adenine [84], or adenine-containing
molecules such as flavin adenine dinucleotide (FAD) or nucleic acids
[96–98,102,104,106,110–113]. Thus, there had been considerable uncertainty
about the biochemical origins of these bacterial SERS signatures.

7.2 Purines Dominate the Bacterial SERS Spectra

Recently we unequivocally established that the 785 nm excited SERS spectra of
bacteria are primarily due to metabolites of the purine degradation pathway that
result from the rapid onset of the bacterial starvation response [72]. Some of the
evidence establishing the chemical identity of these molecular signatures is
summarized in Fig. 6. We initially recognized that the SERS spectra of some
bacterial samples very closely resemble the SERS spectra of some purines.
For example, the 785 nm excited SERS spectrum of *E. faecalis* ATCC

![SERS spectra of *S. aureus* (ATCC 25904) grown on both 14N and 15N growth media compared with 14N- and 15N-labeled purines. Experimental 15N isotopic vibrational red shifts of *S. aureus* spectra match those observed for 15N-labeled adenine. Comparison of SERS spectra of (B) *E. faecalis* (ATCC 29212) and (C) *A. baumannii* (ATCC 17978) with hypoxanthine and xanthine SERS spectra, respectively. The SERS spectra of the model compounds are shown below the SERS spectra of the bacteria which have been raised by 0.25 units for viewing convenience. Contributions from these compounds appear dominant in each of these bacterial SERS spectra. Comparison of the SERS spectra of (D) *E. faecalis* (ATCC 29212) and *B. cereus* ATCC 14579 (E) (blue) and their corresponding enriched supernatant (red). The supernatant has been enriched by approximately an order of magnitude before analysis. Each displayed spectrum is normalized by its band maximum, and enriched supernatant spectra are offset for better viewing.](#)
29212 (Fig. 6B) and *A. baumannii* ATCC 17978 (Fig. 6C) closely resembles the SERS spectrum of hypoxanthine and xanthine, respectively. Although other vibrational bands are also evident in the SERS spectra of these bacterial samples, the contribution of these compounds to their respective bacterial spectra is evident. Secondly, the traditional vibrational method of isotopic labeling further confirmed the chemical identify of these bands. As seen in Fig. 6A, the observed isotopic shifts of the prominent bands of the SERS spectrum of *S. aureus* (ATCC 25904) grown in $^{15}$N culture media relative to those cultured in normal $^{14}$N media exactly match isotopic shifts in the SERS spectra between the $^{14}$N- and $^{15}$N-labeled purine base, adenine. Furthermore, the 785 nm excited SERS spectra of bacterial cells and their corresponding enriched supernatants are nearly identical as shown in Fig. 6D and E for *E. faecalis* and *B. cereus*, respectively. The supernatants following water wash of the vegetative cells have been enriched by about an order of magnitude based on volume reduction as a result of lyophilization before being placed on the SERS substrate. Consequently, these comparisons reveal that these bacterial SERS signatures are *not* due to structural bacterial cell wall features and must arise from small molecules, purines in particular, sufficiently water soluble at biological concentrations, which have been secreted from the bacterial cells and collect in the exogenous regions of these organisms. The SERS signals are larger for samples containing the cells where these molecules are more highly concentrated near the cell wall regions.

Given the above evidence suggesting that 785 nm excited bacterial SERS spectra are due to small purine-like molecules, each of the observed bacterial SERS spectra shown in Fig. 5 was fit to a linear combination of the purine SERS spectra of adenine, hypoxanthine xanthine, guanine, uric acid, and AMP. Excellent fits to all the observed bacterial spectra are achieved by this procedure as seen in this figure. Nearly all vibrational features and their relative intensities seen in each of these bacterial SERS spectra are captured by this fitting procedure. Since all bacterial vibrational features result from these small purine molecules and can also be seen in the SERS spectra of the corresponding cell supernatant (Fig. 6D and E), no components of cell walls, that is, peptidoglycan or any of its constituents, proteins, carbohydrates, or nucleic acids, are required to explain the features evident in these bacterial SERS spectra, and the dominant features in 785 nm SERS spectra can be assigned to a handful of purine molecules.

Consequently, the best-fit determined relative amounts of the purine components contributing to each of these bacterial spectra explain the chemical differences for the different SERS vibrational signatures for these 10 bacterial species are determined by this best-fitting procedure. The resulting relative amounts of the identified purine molecular component are indicated in this color-coded bar graph for each of these 10 bacterial species in Fig. 7. Normalized SERS spectra of neat water solutions of these compounds were used for the fitting procedure. When these values are corrected for the different relative
SERS susceptibility, the relative number of these various components can be determined [72]. As seen in Fig. 7, adenine makes the dominant contribution to the *S. pneumoniae* TIGR4 and *S. aureus* NCTC8325 SERS spectra, hypoxanthine makes the largest molecular contribution in the SERS spectra of *B. anthracis* Sterne, *E. faecium* DO, and *E. faecalis* ATCC 29212, and high uric acid contributions are only observed in the *P. putida* S16 and *A. baumannii* ATCC 17978 SERS spectra. *S. pneumoniae* TIGR4 and *S. aureus* NCTC8325 SERS spectral differences result from the different relative amounts of the purines making smaller contributions to these spectra (hypoxanthine, guanine, xanthine, and AMP). These fitting results unequivocally prove that the different 785 nm excited SERS signatures of vegetative bacterial cells are due to the unique characteristic concentration of purines in the extracellular metabolome surrounding the bacterial cells in contradistinction to the consensus of researchers in this area who attribute these signals to bacterial cell wall components.

Analogously, we fit the SERS spectra of the 12 UTI causative bacterial clinical isolates shown in Fig. 1. Just as found for the 10 SERS spectra in Fig. 5, excellent fits to the UTI clinical isolates were found for a linear combination of normalized SERS spectra of the six purine compounds discussed above plus one more additional purine-based molecular component, guanosine [49]. Again, nearly every vibrational feature can be assigned to a band or overlapping

![FIG. 7 Bar graph representation of the molecule components contributing to the SERS best fits (red) shown in Fig. 5 for 10 representative bacterial species. Color-coded purine components are given in terms of the normalized SERS spectrum of each component.](image)
bands of these purines. The differing amounts of these purine components in the extracellular region near the bacterial cells account for these distinguishable characteristic SERS spectra. Analogously, a color-coded bar graph representation of the relative contribution of the purine spectral components to the SERS spectrum for each UTI causative strain, grouped by species, is given in Fig. 8. Qualitatively, it is evident from Fig. 8 that the differences between species are generally larger than those between strains for a given species. However, the SERS spectra of each species appear to have a similar, although not identical, mixture of purines contributing to these spectra. For example, guanosine and hypoxanthine are the main contributors to the \( K. \) pneumoniae strain spectra, and adenine is the largest contributor to the \( E. \) faecalis spectra with guanosine second in relative importance. Hypoxanthine makes the largest contribution to the \( E. \) coli spectra with significant amounts of hypoxanthine, xanthine and guanine also contributing. Finally, adenine is also the largest contributor to the \( S. \) saprophyticus SERS spectra with various amounts of all of the other purine components appearing in the SERS spectra of this species. The spectral differences between the strains within a given species and hence the spectral

FIG. 8 Bar graph representation of the relative contribution of each of the seven purines found to contribute to the SERS spectra of 12 UTI causative clinical isolates. The success of the PLS-DA classification model is due to the reproducible differences in these relative contributions of purines to the strain-specific SERS signatures. Although strain-specific differences are evident, it can be seen how the pattern of contributing purines is more different between the four species than between strains of a given species.
signatures that provide the successful classification results obtained here arise from the differing amounts of these purine components within these general species patterns, at least for the clinical isolates we have thus far studied (Fig. 8). We again note that Fig. 8 only indicates the relative concentrations of the different normalized purine spectra and hence these values need to be corrected by the relative SERS susceptibilities to yield the actual relative abundances characteristic of the extracellular metabolome for each pathogen [72].

7.3 Biochemical Origins of the SERS Spectra of Bacteria

The purine metabolic pathways are a highly conserved network of enzymatic reactions in all organisms and are responsible for the degradation of nucleotides and nucleic acids [117]. The end products of these degradation processes in bacteria are the set of purines that are found to dominate the SERS spectra shown here: adenine, hypoxanthine, xanthine, guanine, uric acid, AMP, and guanosine. The observed SERS spectra of bacteria result from these metabolites that have been secreted during sample preparation and manipulation. The relative concentration of these purines for each organism is dependent on several factors, but the most crucial appears to be the specific set of enzymes that are present for a given species (strain) in the nucleotide metabolic pathways. The genetically determined absence or presence of specific enzymes in these pathways accounts for the large range of relative concentrations of these purines in the metabolome surrounding these cells and thus determines the 785 nm excited SERS spectra. The enzymes that are present or missing in the purine metabolic pathway for six representative bacteria whose SERS spectra are given in Fig. 5 given by the Kyoto Encyclopedia of Genes and Genomes (KEGG) [117] are shown in Fig. 9. Each box corresponds to a specific nucleotide, nucleoside, or purine base in the degradation of RNA, DNA, or di- or triphosphate nucleotides. The arrows correspond to the enzymes present that convert the indicated product to reactant. In addition, some enzymes act reversibly depending on the relative concentrations, coenzymes, and other allosteric regulation mechanisms and are indicated by a double-sided arrow. This reversibility also indicates how these enzymatic processes function as a purine salvage pathway in addition to a purine degradation pathway. As seen in this figure, the end products are the free purine bases that dominate the bacterial SERS spectra: adenine, hypoxanthine, xanthine, guanine, and uric acid. Furthermore, this is just a subset of enzymatic reactions that impact the purine metabolic pathways but appear to be most directly linked to the appearance of the main constituents of the SERS spectra reported here. The enzymatic reactions feeding into the nucleoside monophosphate reactants at the top of this diagram [117] are not explicitly included here. Guanosine and AMP are also evident as intermediates in these reaction pathways.

The distinct set of active purine metabolism enzymes for a specific bacterial species or strain can be used to explain or at least rationalize the observed
FIG. 9 The purine metabolic pathways given by the KEGG database [117] for 6 of the 10 bacterial species shown in Fig. 7 that result in the formation of the free nucleobases: adenine, hypoxanthine, xanthine, and guanine and uric acid and AMP. Single- and double-sided arrows correspond to enzymes present for each of the indicated reactions.
different SERS signatures. For example, adenine dominates the \textit{S. aureus} SERS spectrum (Fig. 5). Correspondingly, the degradation of adenine-containing nucleotides is only metabolized to adenine by adenine phosphoribosyltransferase via a reversible enzymatic reaction for \textit{S. aureus} NCTC 8325 (Fig. 9). In contrast to the other metabolic pathways shown in Fig. 9, no enzymes are genetically coded for this organism that permit this nucleotide to be directly converted to inosine or hypoxanthine. \textit{A. baumannii} on the other hand, has no enzymatic pathways for adenine-containing nucleotides to form adenine (Fig. 9), and hence no adenine is apparent in the SERS spectrum of this organism (Fig. 5). Instead, reversible enzymatic pathways are available to form guanine, xanthine, hypoxanthine, and uric acid as indicated in Fig. 9, with accumulations of xanthine and uric acid dominating the observed \textit{A. baumannii} SERS spectrum (Fig. 7). Enzymes are present in \textit{A. baumannii} that can convert hypoxanthine and guanine into xanthine (Fig. 9). In contrast, \textit{P. putida} S16 has the enzymes converting inosine and xanthosine to hypoxanthine and xanthine and some ability to produce adenine and guanine. Correspondingly, the \textit{P. putida} S16 SERS spectrum exhibits xanthine, guanine, uric acid, and a very small amount of adenine, and AMP. \textit{B. anthracis} lacks the enzymes converting adenosine to inosine (adenosine deaminase), hypoxanthine to xanthine, and guanine to xanthine (See Fig. 9). Consequently the SERS spectrum of \textit{B. anthracis} is due to large concentrations of hypoxanthine and guanine and a smaller contribution from adenine (Fig. 7). The \textit{E. coli} ATCC 8739 and \textit{E. faecalis} ATCC 29212 strains have the enzymes that can interconvert adenine, hypoxanthine, xanthine, and guanine, but this \textit{E. faecalis} strain is missing enzymes that convert XMP and IMP to xanthosine and inosine, respectively (Fig. 9). Hypoxanthine makes the largest contribution to both of the SERS spectra of these organisms with lesser amounts due to xanthine, guanine, and adenine in different proportions presumably resulting, in part, from the indicated differences in enzymes present for these two species.

The specific purine metabolite concentrations giving rise to the SERS spectra that provide both species and strain level diagnostics, as shown above for UTI causative organisms, result from not only the absence or presence of specific enzymes in the purine degradation pathway as discussed above but also other factors, such as strain-specific enzyme turnover rates, secretion kinetics, and catalytic feedback mechanisms. Other metabolic profiling techniques have reported metabolic diversity at the strain level. For example, two-dimensional high-performance thin-layer chromatography (2-DHPTLC) metabolite fingerprinting has also shown significant biochemical diversity for strains of a given species (\textit{E. coli}) [118].

Other SERS-based experiments further confirmed that the molecular and chemical origins of the bacterial SERS signals result from the metabolic degradation of purine nucleotides and illustrate how SERS may be employed as a probe of cellular metabolic reactivity more generally. For example, SERS spectra of a parent \textit{E. coli} strain and two gene deletion mutants affecting two
enzymes in the purine metabolic pathway were acquired [72]. The effect of deleting the gene encoding adenine deaminase (ade), the enzyme converting adenine to hypoxanthine, is relatively small but measurable, on the SERS signature of the ade-missing mutant. However, the effect of deleting adenosine deaminase (add), the enzyme that converts adenosine to inosine, had a much larger effect on the SERS spectrum than the ade deletion, resulting in the appearance of about 10 times more adenine in the extracellular metabolome.

Furthermore, we showed that by adding to the washed bacterial cells the enzymatic cofactor, diphosphate (diphosphoric acid), which is required for the reversible reaction of AMP to adenine and GMP to guanine by the purine metabolic enzyme, adenine phosphoribosyltransferase as pictured below (Scheme 1), we could control the size and appearance rate of the SERS spectrum of S. aureus [72].

Washing the S. aureus cells with a 1 mM solution of sodium pyrophosphate, forming diphosphate by hydrolysis, an intense prompt SERS spectrum identical to the SERS spectrum normally observed at 30–60 min was observed as soon as the cells were placed on the SERS substrate. These data are further confirmation that the source of the SERS spectrum of S. aureus is predominantly resulting from the metabolic degradation of AMP and GMP. The enzymatic conversion of AMP and GMP to adenine and guanine has been accelerated by the cellular uptake of the excess diphosphate and thus accounts for the faster rate of adenine appearance in the cell’s exogenous metabolome. The gene deletion and diphosphate experiments further help confirm that the bacterial SERS spectrum is dependent on the absence or presence of key enzymes in the purine metabolic pathway.

All of the evidence summarized above unequivocally establishes that the 785 nm excited SERS spectra of bacterial cells, excluding C. trachomatis, are dominated by the reproducible SERS contributions of the free purine nucleobases: adenine, hypoxanthine, xanthine, guanine, and uric acid and AMP, guanosine, and NADH/NAD+. We attribute these molecular SERS signals to result from the bacterial cell stress response to the no-nutrient, water only environment that the bacterial cells are placed in during sample washing and signal acquisition. In these starvation conditions, it is known that the stringent response of bacterial cells is stimulated resulting in suspension of cell growth and the shutting down of the most energy-dependent de novo biosynthesis pathways [119,120]. The cell adopts this survival strategy in order to enable bacteria

\[ \text{AMP} + \text{Diphosphate} \rightarrow \text{PRPP} + \text{Adenine} \]

**SCHEME 1** AMP-adenine conversion catalyzed by adenine phosphoribosyltransferase.
to persist in stressful environments, reorganizing metabolic activity for both maintenance and survival until conditions potentially become favorable again for the resumption of growth [121,122]. Recent HPLC [121], LC-MS [123], and ESI-TOF-MS [122] studies have reported increased concentrations of various purines when bacteria are subjected to starvation conditions and their immediate uptake once nutrients are supplied. These results are consistent with the appearance of the bacterial SERS spectra where spectra are obtained after cells have been enriched from nutrient-rich environments and repeatedly washed in pure water prior to signal acquisition. Furthermore, the enzymes responsible for the conversion of the purine XMP nucleotides to the base X, such as adenine, guanine, and hypoxanthine phosphoribosyltransferases, are located at the cell membrane and not only facilitate the $X \leftrightarrow XMP$ conversion but also transport these reactants and products across the cell membrane [124–126]. Hence, this explains why the SERS intensities are generally the most intense for the excitation of spatial regions closest to the bacterial cell wall regions. Nucleotide degradation during starvation is necessary for cell viability [127]. We speculate that these purines appear in the exogenous metabolome as a result of the metabolic degradation of nucleic acids and nucleotides, such as RNA, ATP, GTP, and other nucleotide-containing molecules for two reasons: They provide a low-cost energy source for cell maintenance and survival during the low nutrient conditions, and they serve as an instant nitrogen source for the bacterial community once a carbon source or other required nutrients are available. In preliminary experiments we have already seen that following bacterial starvation for about 2 h, the steady-state SERS spectrum intensity vanishes after the addition of glucose. Such SERS-based metabolic studies are ongoing in our laboratory.

Although not used in our laboratory, bacterial SERS spectra excited at 514.5/532 nm have also been reported. However, they are strikingly similar to each other and do not resemble the 785 nm SERS spectra of bacteria [88,89,93,95,100,108,110,128]. The 514.5/532 nm excited bacterial spectra are nearly equivalent to SERS spectra of FAD [93,128]. FAD has an electronic absorption in the 500–350 nm region; hence at 514.5 nm, this molecule will be electronically and plasmonically enhanced due to this near electronic resonance [110]. Purine absorptions are further in the UV; hence the 514.5 nm signature of bacterial SERS is dominated by this single molecule, which provides little basis for diagnostic purposes.

### 7.4 Chemical and Biochemical Origins of SERS Spectra of STD Pathogens

The SERS spectrum of the chlamydia etiologic pathogen *C. trachomatis* on both Au and Ag substrates (Figs. 3 and 10) is completely different than all other 785 nm excited bacterial SERS spectra in comparison with spectra displayed in Figs. 1, 4, 5, and 6 that reveal and, more specific to STD diagnostics, very
FIG. 10  SERS spectra of *C. trachomatis* supernatant wash, cells only, avidin and human serum albumin (HAS) on Au and Ag SERS substrates.
different than the SERS spectrum of *N. gonorrhoeae* the bacterium causing gonorrhea. Although both *Ct* and *Ng* are Gram-negative bacteria, the molecular and biochemical origins of this difference are readily understandable in terms of the activity of their purine metabolic pathways which are diagrammatically summarized in Fig. 11. Excellent fits to the observed SERS spectrum of *N. gonorrhoeae* are shown in Fig. 4 and reveal that the SERS spectrum is due to adenine, guanine, and time-dependent contribution of NADH or NAD⁺ [60]. (The SERS spectra of NADH and NAD⁺ are nearly identical.) The appearance of these free bases is fully consistent with the purine degradation enzymes present in this organism (Fig. 11). In striking contrast, *C. trachomatis* has none of the required enzymes for the appearance of any purine bases consistent with their absence from the *Ct* SERS spectrum and the nearly parasitic nature of this organism. As indicated above, it is an obligate intracellular organism that is reliant on a eukaryotic host cell to complete its biosynthetic and metabolic life cycle [58]. Hence none of the molecular species are available for the purine-based SERS spectra from these cells that characterize the often coinfecting *N. gonorrhoeae* and found for all other bacteria that we have investigated.

**Fig. 10** illustrates another anomaly regarding the *C. trachomatis* SERS signal and the molecular origins of its characteristic SERS signature. As shown in

![Diagram of purine metabolic pathways for *N. gonorrhoeae* and *C. trachomatis*](image)

**FIG. 11** Purine metabolic pathways given by the KEGG database for *N. gonorrhoeae* and *C. trachomatis*. None of the enzymes required for purine degradation to free bases are present in *C. trachomatis*. 
the top half of this figure, no Ct SERS spectrum is observed in the bacterial wash supernatant, on either Au or Ag, unlike all other bacterial SERS signals (see Fig. 6D and E) and consistent with the absence of active purine metabolic pathways. Instead, we have determined that the observed SERS spectrum on Au nanoparticle substrates is just that of aggregated proteins on the outer cell surface of C. trachomatis cells. As shown in Fig. 10, by analogy with the SERS spectra of other proteins, we have discovered that the signature of aggregated proteins on Au is the appearance of three very broad features (100–200 cm$^{-1}$) centered at 850, 1150, and 1550 cm$^{-1}$. These high concentrations of cell surface proteins are essential to the special life cycle of this parasitic organism [59]. Interestingly, on Ag substrates, however, a very different protein signature is found. The broad aggregated bands are not evident and discrete; more typically shaped vibrational bands assignable to individual amino acids and other specific molecular level moieties are observed (Fig. 10). For example, phenylalanine, tryptophan, C$\equiv$S stretch, and CO$_2$ and CH$_2$ deformation bending vibrational modes can be readily identified [60]. We attribute this protein metal dependence to the more perturbative effect of Ag on protein structures. However the results summarized here demonstrate that the distinguishability of these two STD causative agents may be readily accomplished by SERS spectral signatures primarily due the very different activity of the purine metabolic pathways in these two species: N. gonorrhoeae and C. trachomatis.

8 FORENSICS

8.1 Introduction

There is a continuing need to exploit new and developing technologies in order to ensure that the best and most accurate outcomes of a criminal investigation are achieved. Among the most important forensic evidence that can be collected at a crime scene are body fluids such as blood, semen, vaginal fluid, and saliva. Detecting and identifying these fluids helps identify the perpetrator and victim and aids in understanding the events, including the timeline, of a crime. An ideal detection and identification platform for these fluids is confirmatory, rapid, portable for on-site capabilities, easy-to-use, highly sensitive for detection of trace amounts, and specific. Furthermore, nondestructive and/or highly sensitive techniques are desirable for forensic identification because they leave sufficient amounts of trace evidence for other detection platforms.

More specifically, the National Institute of Justice Forensic Science Working Group [31] has recently expressed interest in technologies that can (1) simultaneously detect location and identify biological materials/fluid type with minimal destruction to evidence at crime scenes or from evidence taken from crime scenes; (2) identify biological material that is invisible to the eye (with or without the aid of alternate light sources), in sufficient quantity for subsequent DNA analysis; (3) identify the original body fluid type at the time of genetic
analysis; (4) determine species of a biological stain; and (5) allow rapid and accurate preliminary testing at a crime scene that can have the ability to guide investigators prior to traditional confirmatory laboratory testing. The development of a SERS-based platform for body fluid identification and detection has the capability to address each of these advancements in forensic science.

8.2 SERS Detection and Identification of Human Body Fluids

We have been able to show that SERS can be effectively used to detect and identify dried stains of different human body fluids [129]. As shown in Fig. 12, SERS spectra of 24 h dried stains of human blood, vaginal fluid, semen, saliva, and urine can provide good-quality, reproducible characteristic SERS signatures that result in rapid body fluid identification. In order to acquire these spectra 1 μL of water or saline solution is dropped on the dried stain and then pulled back into the pipet and placed on the SERS substrate. Some subsequent variations of this simple sample “extraction” procedure are discussed further below. The solid lines correspond to the averaged spectra from two donors each (30 spectra) on our Au substrates. Signal acquisition time for each spectrum is about 10 s excited by <1 mW at 785 nm. The shaded regions of the body fluid spectra (Fig. 12) are the standard deviations at each wave number for the averaged normalized spectra and indicate the excellent degree of reproducibility obtained for the SERS spectra of these dried body fluids. In addition to the high

![SERS spectra of dried human body fluids. Shaded regions correspond to standard deviation of 60 spectra (two donors each body fluid type).](image-url)
quality of these data, no fluorescence backgrounds are observed unlike the background emission reported by normal Raman of some dried body fluids [30,130,131].

Distinct spectral differences are clearly evident in Fig. 12 for these different body fluid types. In order to develop a quantitative procedure for body fluid classification and identification, a PLS-DA procedure employing our “barcode” procedure [36], that is, SERS spectra are converted to a series of “ones” or “zeros” based on the sign of the second derivative at each wave number, was carried out. This methodology resulted in a highly successful body fluid identification of body fluids. The 300 SERS spectra of human body fluids that contributed to the averaged spectra displayed in Fig. 12 were classified by this PLS-DA treatment with 98% sensitivity and 99.5% specificity as given by standard cross validation procedures [129]. Thus, the introduction of this optical approach for crime scene investigations seems very promising.

For applications in forensic science, it is crucial to evaluate the effects of individual variability on the ability to identify a specific body fluid type. Although some donor variability was identified for the SERS spectra of body we have studied, we find that the donor variability within our sample pools was less than the differences between body fluid types. The use of our barcode-based multivariate classification procedures helps to provide robustness to these statistical identification approaches since it emphasizes where a characteristic peak is as compared to its relative intensity [36]. Hence spectral variability to changes in the relative amount of a component in a complex mixture is not as important for identification purposes as the absence or presence of a particular component. The principal molecular components that we have identified contributing to the SERS spectra of the four key body fluids of interest on Au nanoparticle covered substrates, prepared by the water or saline solution extraction protocol, are the following [129]:

- **Blood**—uric acid, hypoxanthine, HSA, hemoglobin
- **Semen**—protein, hypoxanthine, xanthine
- **Saliva**—phenylalanine, protein, thiocyanate (SCN⁻)
- **Vaginal fluid**—protein (HSA), hypoxanthine, adenine

Comparisons with the SERS spectra of these compounds reveal their dominance in the complex biological mixtures. These SERS spectra are very different than their normal, non-SERS 785 nm excited Raman spectra [30,130–135] and highlight how the SERS surfaces serve to simplify the Raman spectra of multicomponent biological mixtures. This same point is made evident by the above comparisons of SERS spectra with mass spectrometry analysis of bacterial metabolome [72].

As an example of how this library of SERS spectra and methodology would be used by forensic investigators to identify of an unknown suspected human body fluid stain is shown in Fig. 13. The classification of a potential dried body fluid is determined by group membership via this PLS-DA classification
procedure in the body fluid library. To illustrate, the previously determined classification procedure was challenged with 60 spectra of dried semen stains from two donors not used to create this classification model including human blood, vaginal fluid, saliva, and urine in addition to semen. The PLA-DA results (Fig. 13) show that the “unknown” spectra, indicated by the blue data points on the far right-hand side in the classification panels, match the library semen SERS spectra. Fifty-eight out of 60 spectra were correctly identified as semen, and the resulting analysis of 360 SERS spectra exhibited an overall analytic sensitivity of 96.7% and specificity of 99.2%. These excellent results are achieved nearly instantaneously after spectral acquisition that requires only minutes after the body stain is recognized.

Aside from the ability to rapidly distinguish different complex body fluid stains, another important attribute that SERS offers is limit of detection sensitivity. In one demonstration of this sensitivity, SERS spectra of a dried (24 h) 1 μL diluted whole blood stain were obtained as a function of dilution (Fig. 14) [136]. This sample type, for example, mimics a crime scene “cleanup.” As described above, 1 μL of water is dropped on the diluted stain and then recaptured by the pipet and placed on the SERS substrate. At the highest dilutions, no red color can be seen by eye, but such samples result in strong SERS spectra as shown in Fig. 14. Under these conditions, the SERS spectra are completely dominated by the characteristic red blood cell SERS signature due to hemoglobin [31,137]. As shown excellent S/N spectra are readily observed for dried blood sample diluted by more than 10,000 times. Furthermore, at these dilutions, no normal Raman spectrum can be observed. RSID strip test (immunochromatographic assay), typically the most sensitive method for human blood

**FIG. 13** PLS-DA classification model tested the ability of this SERS platform to correctly identify dried semen stains. Fifty-eight out of 60 SERS spectra were correctly identified by this procedure and represented by the group of spectra on the far right-hand side of these panels.
identification, can detect 1 μL whole blood [138]. These results demonstrate that SERS can produce an identifiable signature with <100 pL blood (total volume) or in other words a volume of blood that is $10^4$ times less than one of the most sensitive current forensic techniques for blood identification.

Another theme illustrated above can be exploited for SERS-based forensic investigations of trace amounts of human body fluids. The SERS spectra of blood on Au and Ag substrates offer very different signatures. In contrast to the blood SERS spectrum on Au substrates, a redox reaction occurs for hemoglobin on Ag substrates that results in a product that has a strong characteristic SERS spectrum that is very distinct from the robust hemoglobin on Au SERS signature [136]. Use of these two SERS substrates can be used as further test for the presence of hemoglobin. We noted above that proteins can offer very different SERS spectra on Au and Ag substrates as well. For body fluids such as vaginal fluid and semen in particular, where proteins make a significant spectral contribution, the use of Ag substrates relative to Au can enhance spectroscopic recognition but reduce the contribution of the very broad features due to protein aggregation on Au substrates [139].

Since each body fluid offers a unique spectrum, resolution of body fluid mixtures may be accomplished via SERS. For example, due to the distinct and characteristic SCN$^-$ component of saliva, 1% of saliva in vaginal fluid can be detected by SERS. Such body fluid mixture resolution may be especially
important for evidence evaluation in sexual assault cases. Finally we note that SERS spectra of human blood stains may be readily identified relative to other species. This is largely attributable to the stronger presence of thiocyanate in the human blood stain.

Thus, our initial work on the use of SERS for rapid detection and identification of trace amounts of body fluids indicates that a SERS-based methodology has the potential to be transformative in forensic science. The results briefly summarized here already indicate that SERS can be used for trace body fluid identification in a time frame of minutes and at sensitivity levels that for some fluids are beyond what is currently available for forensic scientist. When combined with a portable Raman microscope such a single platform capability offers a much-needed solution for technological improvements sought by the forensic science community [140].

9 CONCLUSION

The results presented here demonstrate that SERS-based diagnostic platforms have the capacity to offer rapid, growth-free detection and identification of bacterial cells enriched from human body fluids. UTI, STD, and bacteremic diagnostics via SERS have the potential to meet the unmet biomedical need for rapid, specific identification of viable bacterial cells enriched from human body fluids. Perhaps most impressively, the ability to detect an enriched bacterial pathogen in urine, for example, and match its SERS spectrum with that of a library strain with a predetermined drug susceptibility profile as discussed here has the potential to provide physicians with antibiotic-specific information in an unprecedented time frame of approximately 1 h as compared with 48–72 h. These results underscore that the detection sensitivity and specificity is available from SERS for the implementation of SERS-based approaches for rapid diagnostics once best, low-cost strategies and solutions for sample enrichments and preparation are achieved.

On the molecular level, conclusive evidence, based on isotopic substitution studies, bacterial supernatant analysis, spectral fitting, and experiments with defined bacterial mutants and purine catalytic pathway perturbations, clarifies the molecular and biochemical origins of the SERS spectra of bacterial cells excited at 785 nm. The SERS spectra of vegetative bacterial cells harvested from culture media or body fluids and washed in water result from the purine bases appearing at the outer layer of bacterial cells and in the extracellular metabolome. They result from the degradation of nucleotides that occurs as part of the bacterial starvation response. The unique SERS bacterial signatures are due to the different amounts of these purine components in this extracellular region and can be understood in terms of the different enzymes that are present or functional for a given organism. This result is in contrast to the long-held assumption that structural cell wall components, more specifically the peptidoglycan layer components, NAG, NAM, lipids, and proteins make the dominant contribution
to the observed SERS spectra at 785 nm and account for their unique characteristic vibrational signature. Only *C. trachomatis*, owing to its unique parasitic lifestyle, exhibits a cell wall SERS signature. These bacterial molecular level assignments demonstrate the potential of SERS for monitoring real-time exogenous metabolomics in living cells and its more general use to address questions in systems biology.

Finally, results are summarized here that demonstrate that the introduction of SERS into the forensic science community has the potential to be developed into a transformative single platform tool for the rapid identification of trace amounts of body fluids at crime scenes and in forensic laboratory settings. Efforts in these areas remain ongoing in our laboratory.

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