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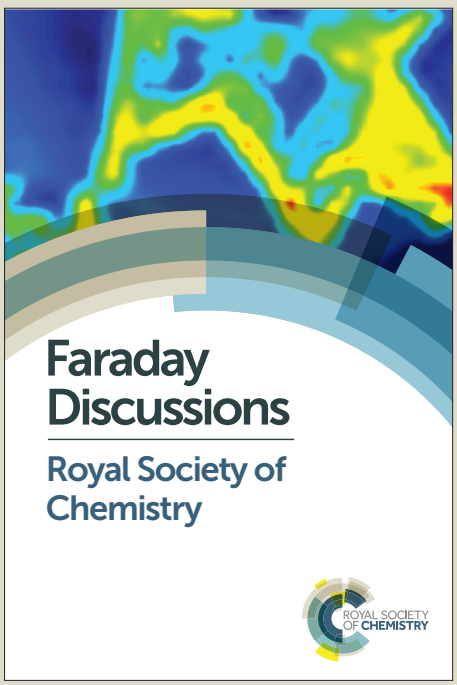

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Sheath Flow SERS for Chemical Profiling in Urine

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Abstract.

The molecular specificity and sensitivity of surface enhanced Raman scattering (SERS) makes it an attractive method for biomedical diagnostics. Here we present results demonstrating the utility and complications for SERS characterization in urine. The chemical fingerprint characteristic of Raman spectra suggests use as a label free diagnostic; however, the complex composition of biological fluids presents a tremendous challenge. In particular, the limited number of surface sites and competing absorption tend to mask the presence of analytes in solution, particularly when the solution contains multiple analytes. To address these problems and characterize biological fluids we have demonstrated a sheath-flow interface for SERS detection. This sheath-flow SERS interface uses hydrodynamic focusing to confine analyte molecules eluting out of a column onto a planar SERS substrate where the molecules are detected by their intrinsic SERS signal. In this report we compare direct detection of benzoylecgonine in urine using DSERS with chemical profiling by capillary zone electrophoresis and sheath-flow SERS detection. The SERS spectrum from the observed migration peaks can identify benzoylecgonine and other distinct spectra are also observed, suggesting improved chemical diagnostics in urine. With over 2000 reported compounds in urine, identification of each of the detected species is an enormous task. Nonetheless, these samples provide a benchmark to establish the potential clinical utility of sheath-flow SERS detection.

Introduction.

Biomedical diagnostics increasingly rely on multiplex indicators to identify health and disease in patients. While measuring cholesterol provides one data point for monitoring cardiovascular disease, it is more common to include additional risk factors to assess a patient's condition. Progressively, systems biology approaches are being investigated to evaluate how changes in multiple indicators correlate to the overall changes in different systems.[1] The field of metabolomics, for example, uses differences in more than 40,000 known metabolites to assess changes in biochemical pathways relevant to health and disease.[2] While a large number of metabolites have been reported, typical assays only identify about 10-20% of the known metabolites in a given sample. In a recent comprehensive study examining urine, which is reported to contain 2651 metabolites, only 445 metabolites were identified.[4] The challenge of metabolite identification is tremendous, as recently noted that less than 2% of the recorded mass spectra in metabolomics experiments can be attributed to a specific metabolite.[3] Improved diagnostics with increased identification power could have tremendous impact.

Currently, the technology available for metabolomics largely consists of nuclear magnetic resonance (NMR) and mass spectrometry (MS).[5-8] These methods take advantage of distinctly different chemical properties to identify metabolites. NMR offers universal identification arising from the deconvolution of NMR peaks associated with the nuclear spins of individual metabolites. The downside to NMR is that samples can require millimolar concentrations and milliliter volumes, making detection of many biological samples difficult.[6] The increased sensitivity of MS has made it an attractive detection technology, as it is capable of characterizing nanomolar concentrations and nanoliter volumes.[1] Even yoctomolar (10^{-24} M) metabolite detection, comparable to single molecule fluorescence, was demonstrated by

nanostructure initiated MS.[9, 10] While LC-MS has proven quite capable of detecting low concentrations of analyte, there are certain classes of molecules that present challenges, such as structural isomers and poorly ionizing molecules. Some of these molecules are identifiable using chemical ionization or complex sample derivatization and gas chromatography (GC).[1] Commercial metabolomics services utilize NMR and MS for assays. A study comparing the 3 commercial metabolite panels used to analyze blood detected a total of 482 metabolites.[11] The NMR based panel identified 24 metabolites (including 4 unique) and MS methods identified the rest. Though NMR spectra can be deconvoluted without using a separation technique, simplifying sample analysis, the sensitivity of MS generally provides increased identification. Orthogonal and chemically specific detection methods with sensitivity comparable to LC-MS would provide additional diagnostic information.

Raman spectroscopy, particularly surface enhanced Raman spectroscopy (SERS), is an attractive option for biomedical diagnostics.[12] Similar to NMR, the signals observed in SERS arise from the structure of the molecule and are thus chemically specific. Also, very low concentrations of molecules can be detected by SERS at levels comparable to MS detection. The sensitivity and chemical specificity of SERS suggest an appealing approach for biochemical profiling. In fact, many classes of biologically relevant molecules that are difficult to discern by MS are readily discriminated by their Raman spectrum. Examples of SERS based metabolomics include the work that identified and quantified nicotine and two of its major metabolites,[13] that separated and identified pyrimidine and purine bases following liquid chromatography,[14] and that detected methamphetamines in urine using Au nanorods and a portable Raman detector.[15]

There are also a number of challenges associated with using SERS for biochemical profiling. Mass transport of analytes to the enhancing nanostructures is one challenge.[16] We

recently demonstrated that, in solution, only molecules that at least temporarily adsorbed to the nanostructures were detected by SERS.[17] This result is consistent with the fact that most high sensitivity SERS assays either use nanoparticles in solution, where mixing is more effective, or deposition and evaporation onto a substrate, which confines the molecules to the surface. In both of these approaches, spot to spot variation in the nanostructures can alter the observed SERS signal, complicating identification. Because SERS requires analytes to interact with the nanostructures, the number of available surface sites can also limit detection. In these cases, molecules at significantly higher concentrations can inhibit detection of low concentration species of interest. In many ways, this challenge is similar to MS detection and matrix ion suppression.

In SERS, the nature and organization of the nanostructures also affects the detected signal. It has been shown that the nature of the plasmonic hot spot can have a profound impact on the observed SERS signal.[18] Ideally, the same plasmonic construct is used for the detection of all molecules. This can be challenging with colloidal nanoparticles, but is more straightforward for planar SERS substrates. However, the transport issues discussed above have to be addressed, which has promoted the use of colloids thus far. Even in a microliter drop of solution on a substrate, it requires time for molecules to diffuse to the surface and the molecules can also diffuse away. In general, this transport problem has limited the utility of substrate based sensing assays.

Recently, our laboratory demonstrated the use of hydrodynamic focusing to improve transport and the efficiency of SERS detection in solution on planar substrates.[19] By confining analyte molecules near the SERS substrate, sheath-flow SERS shows more than 1000x improvement in the limit of detection. Additionally, the directed sample delivery minimized the

amount of sample necessary to less than 0.1 microliters. An unexpected but useful aspect of the sheath-flow SERS detector is that low concentrations of analyte do not foul the surface, which enables repeated detection. The advantages realized with sheath-flow SERS make substrate based sensing competitive with colloidal assays. The sheath-flow SERS detector has been successfully coupled with capillary zone electrophoresis for the detection of amino acid[20] and peptide mixtures,[21] as well as dye molecules[22] and lipid vesicles.[23]

In this report, we examine the ability of sheath-flow SERS to identify metabolites in urine. The chemical complexity of urine introduces substantial matrix effects and represents an important test case for establishing the clinical relevance of sheath-flow SERS for metabolomics. Specifically, we examine the ability to identify benzoylecgonine (BZE), the primary metabolite of cocaine that can be detected in urine following drug use. We demonstrate two approaches, direct detection of BZE in urine as well as identifying BZE in a CZE separation of a urine sample. In both cases, BZE can be identified without the use of sophisticated chemometrics.

Experimental.

Materials and Reagents. Certified drug free urine was obtained from UTAK Laboratories. The urine was diluted in borate buffer at a pH of 9.4 to a 2:1 dilution of buffer to urine. Reference benzoylecgonine (BZE) dissolved at 1 mg/mL in methanol was obtained from Sigma Aldrich.

Sheath-Flow SERS Detector. A previously described sheath-flow SERS detector was used to focus the analyte to the surface.[19, 22] Borate buffer was used as the sheath fluid for all studies. The continuous flow studies used a sheath flow of 150 μL/min and when coupling with CZE a sheath flow of 15 μL/min was used. These flow rates were previously determined to be most

successful for the sample capillary flow rates used. In continuous sample flow experiments, a pressure driven flow rate of approximately 4 μL/min was used. The electrokinetic flow rate in CZE experiments was measured to be 0.2-0.5 μL/min. The capillary used had an inner diameter of 72 μm and an outer diameter of 143 μm. The capillary was 30 cm long for the steady state experiments and 50 cm long for the CZE experiments. The capillary and SERS substrate were cleaned by washing with 0.01 M NaOH, nanopure water, and borate buffer between studies. All SERS spectra were obtained using sheath-flow unless otherwise specified.

Raman Measurements. The home-built Raman microscope,[23] equipped with a 660 nm diode laser and a cylindrical lens for line focus excitation, and the Ag SERS surfaces used have both been previously described.[24] The line focus is diffraction limited in width at approximately 1 μm, and the width is estimated to be approximately 60 μm in width,[23] similar to the inner diameter of the sample capillary. The excitation laser was focused onto the SERS flow cell using a 40x water-immersion objective. The excitation power measured at the output of the objective was 3-4 mW. Spectra were recorded in a kinetic series with acquisition times of 200-250 ms and a varying number of spectral acquisitions.

Capillary Zone Electrophoresis. A Spellman CZE1000R power supply provided a constant voltage of 15 kV to a Pt electrode in the injection block, where it sits in the solution of borate buffer along with one end of the sample capillary. A two second pressure injection (ca. 130 nL) was used to deliver analyte into the capillary preceding the start of CZE run. The system was grounded from the outlet port of the flow cell.

Analysis. Continuous sample flow experiments were analyzed using the DSERS method, as described by Scott and Carron, in order to separate SERS signals from background Raman signals.[25] In DSERS, the fluctuations associated with SERS detection can be extracted from the steady-state background, recognizing that the SERS signals contribute more substantially to the standard deviation than does the invariant signal response. All data analysis was performed in MatLab.

Results and Discussion.

Figure 1 shows the time-dependent response observed from the sheath-flow detection of urine spiked with 1 μg/mL of BZE continuously pumped over the SERS substrate. The observed SERS spectrum is observed with a high degree of continuity. Small variations in intensity are observed, but no clear fluctuations in the Raman shift of the acquired signals are observed. Experiments using unspiked urine show the same overall features. Figure 2A plots the average spectrum observed from 4000 acquisitions of urine both with and without the addition of BZE. From the observed signal it is not clear that BZE is detected.

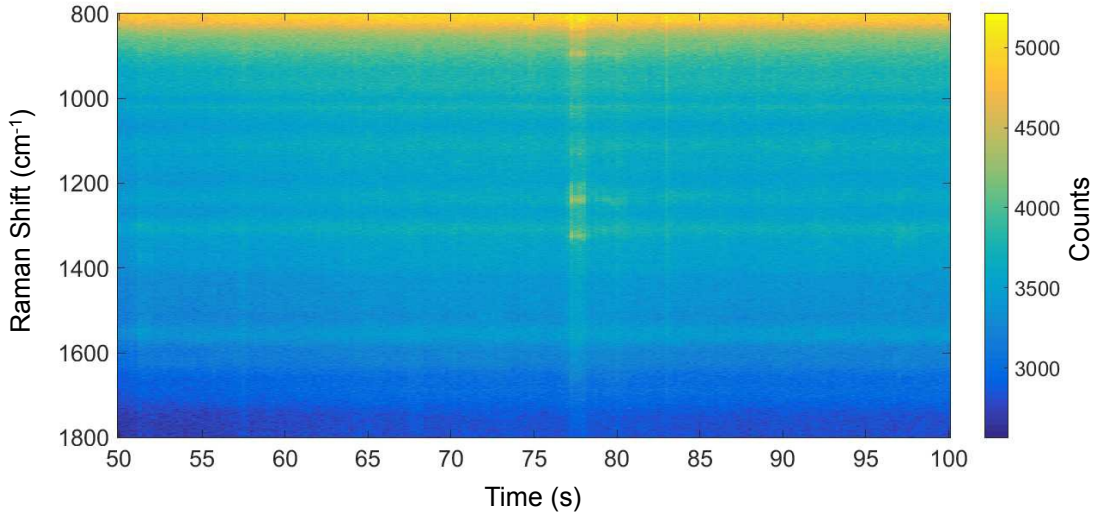

Figure 1. Heatmap of the SERS signal of urine spiked with 1 μg/mL BZE. Under sheath-flow confinement, the sample was measured with 250 ms acquistions over 100 s. The acquisitions

between 50 – 100 s when the analyte eluted are shown. The signal is largely invariant over the 200 spectra plotted.

Recent work by Carron and colleagues suggests that weak signals observed on a strong background Raman signal can be extracted using DSERS.[25] In their work, Carron and colleagues showed that fluctuations associated with the transient association of molecules in SERS hotspots add significantly more to the standard deviation of the spectrum than does the constant SERS background. Equation 1 describes the general DSERS process:

$$\text{DSERS} = \sigma_{\text{total}} - A \times I_{\text{average}} \qquad \text{Eq. 1}$$

where σ_{total} is the standard deviation of the spectra, A is a scaling coefficient and I_{average} is the mean intensity over multiple acquisitions. The difference in the standard deviation spectrum and the mean spectrum can be used to pull out SERS signals as weak as 1 part in 1000.

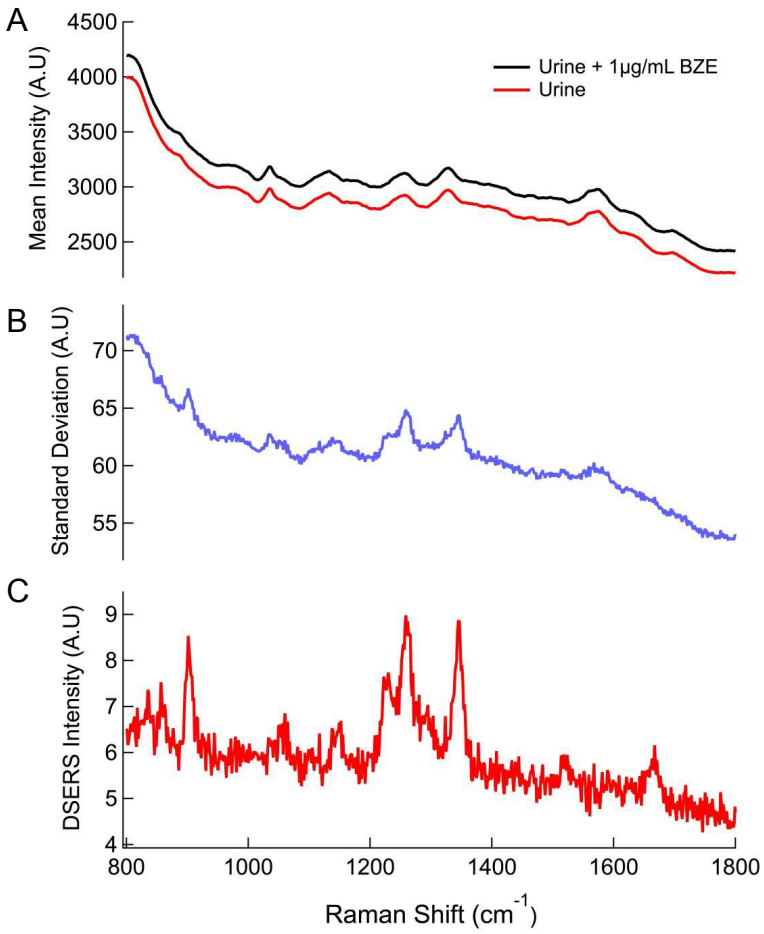

Figure 2. The DSERS analysis of urine spiked with 1 μ g/mL of BZE is shown. (A) The mean SERS spectra obtained from 200 separate acquisitions of Urine (red) and urine spiked with BZE (black) are shown. (B) The standard deviation of the signal of urine spiked with BZE shows deviations attributable to BZE peaks more than the average urine background. (C) The DSERS spectrum obtained from A and B shows peaks characteristic of BZE.

Figure 2 uses the DSERS approach to examine the data plotted in Figure 1. In Figure 2A, it is difficult to discern the differences in the observed spectra in the absence and presence of BZE. The observed peaks are similar to those observed from uric acid in controlled SERS experiments. Uric acid is found at high concentrations in urine and is known to complicate detection of trace species. This suggests that uric acid is preferentially adsorbed to the Ag substrate, preventing the BZE from contributing significantly. When the BZE spectra are

averaged over long periods without the BZE on the surface, the BZE signal is difficult to observe. This is also true in the DSERS analysis. We previously showed a similar effect for the transient detection of lipid vesicles, where the interaction with the surface lasted on the order of 100 ms and signal acquisitions of several seconds did not provide detectable signals.[26] In Figure 2C, we observe that a clear spectrum can be obtained from DSERS analysis.

Figure 3A shows the reference spectrum from BZE on our SERS substrate. In this experiment, the BZE in methanol was deposited onto the SERS substrate and the methanol was allowed to evaporate. The BZE reference spectrum was background subtracted, using a difference over sum model, where the background was measured by taking spectra from a portion of the SERS substrate where the analyte was not present. Figure 3B shows the DSERS result from both the BZE spiked and unspiked urine. The DSERS spectrum of blank urine shows a number of small peaks attributable to any of the 2000+ components found in urine. Interestingly, the DSERS spectrum of the BZE spiked sample shows a spectrum with a high degree of similarity to the reference spectrum in Figure 3A.

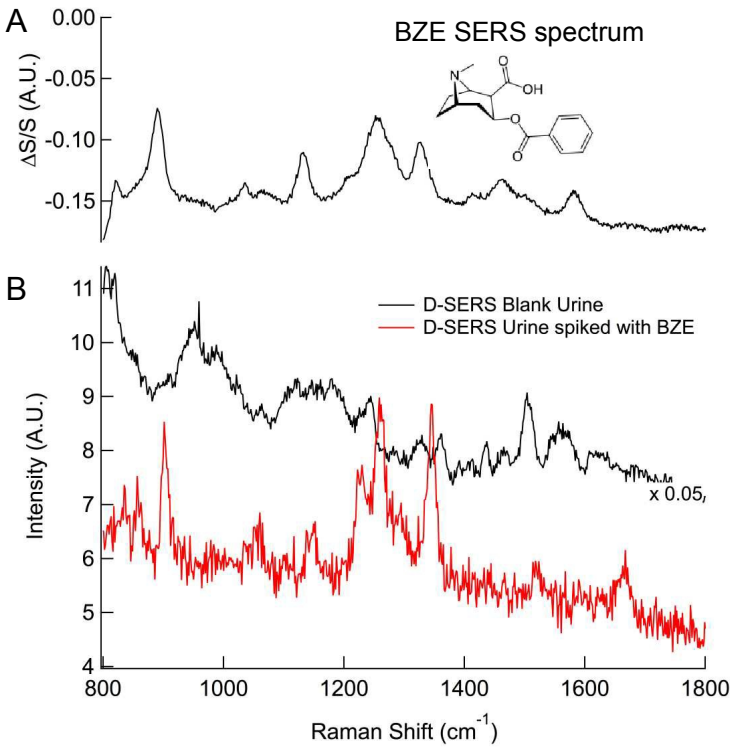

Figure 3. A) The reference spectrum of BZE deposited on our SERS subsrate is shown. The spectrum was obtained by depositing a drop BZE in methanol onto the surface and allowing the methanol to dry. B) The DSERS results from blank and BZE spiked urine are shown. The spectra in B are offset for clarity.

Figure 3 clearly demonstrated that DSERS in combination with sheath-flow SERS can detect the cocaine metabolite in urine. This result is consistent with previous results showing that methamphetamines can be detected in urine using DSERS.[15] It is quite amazing that the spectrum is so clearly detected. This may correspond to the concentration (1 μg/mL) of BZE added; however, urine is known to naturally contain mg/mL concentrations of other compounds such as uric acid. The strong BZE signal may be associated with the carboxyl and amine functional groups, which would be expected to interact strongly with a positively charged silver surface. The role of sheath-flow in this experiment is not obvious, but it enables ready

comparison of blank and analyte-containing urine on the same substrate. This may enable further quantitative analysis.

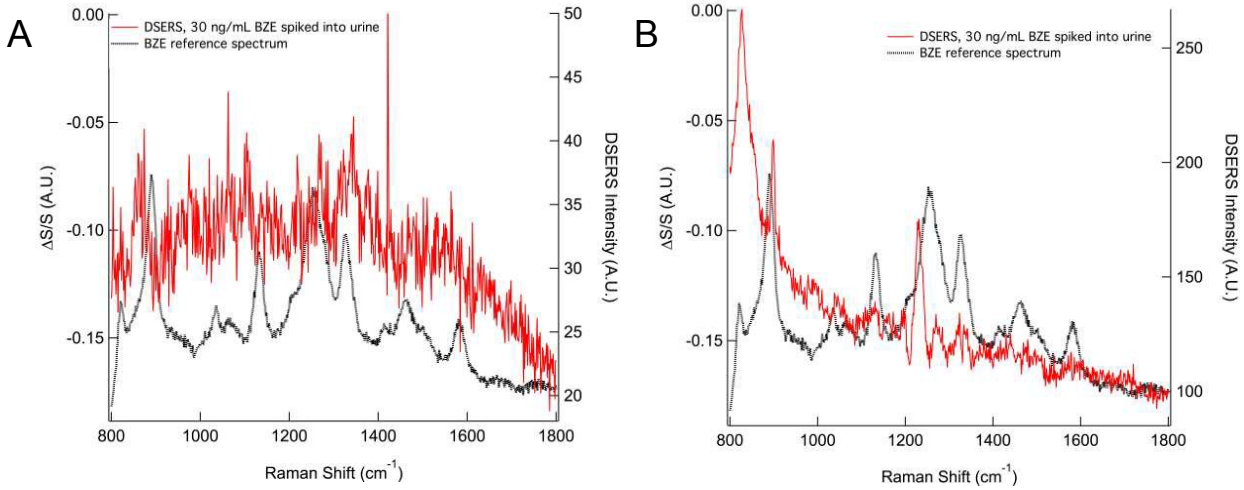

Figure 4. The DSERS analysis of urine spiked with 30 ng/mL (100 nM) of BZE are shown in A and B. The two panels represent replicate measurements. The intensity of the observed features varies, but the frequencies are consistent with the reference spectra shown in black. It should be noted that peaks below 1000 cm^{-1} Raman shift can be obscured by the presence of oxide on the Ag SERS substrate.

Figure 4 shows DSERS results obtained from 30 ng/mL of BZE spiked into a urine sample and sampled using the sheath-flow SERS flow cell. The peaks observed in Figure 4A are near the limit of detection, but show agreement with the more concentrated sample in Figure 3. Figure 4B shows several peaks with increased intensity. The variation observed at low concentration may be attribute to detection of molecules in different hot-spots, a known challenge in SERS analysis. The differences in intensity also highlight reproducibility issues known in quantitative SERS analysis. Detection in hotspots at low concentrations is often indicative of stochastic sampling, where individual molecules may exhibit spectral differences. Use of an internal standard or other approach may address the problem of quantification at higher concentrations. The detection of 30 ng/mL of BZE is similar to the environmental

exposure level and suggests that sheath-flow facilitates transport and improves detection capabilities.

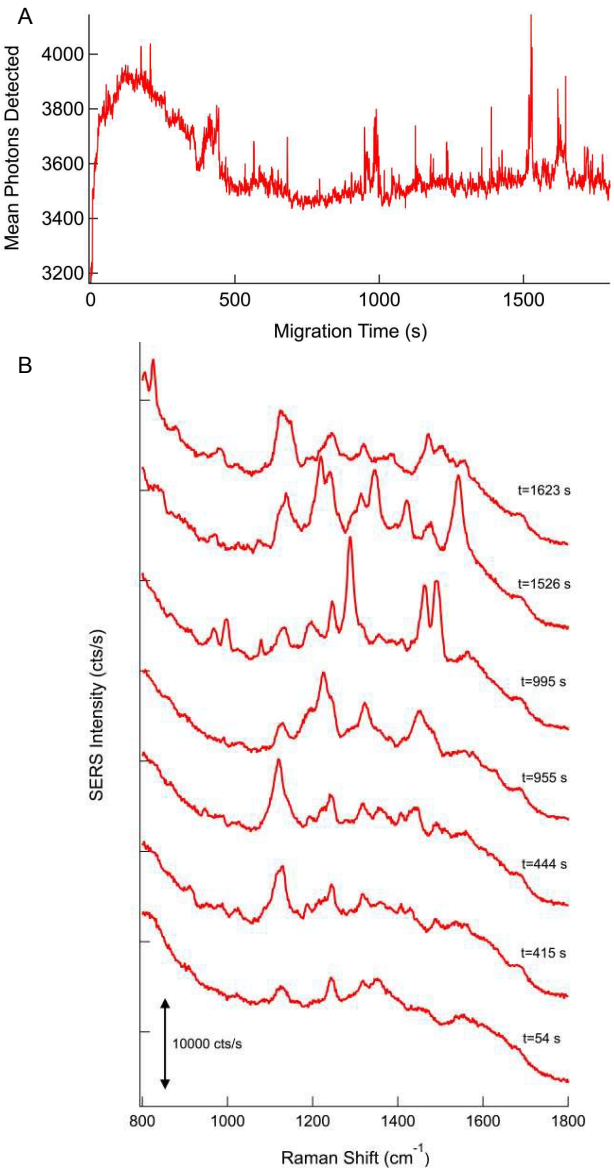

Figure 5. A) The CZE-SERS electropherogram is plotted by averaging the total number of photons detected during each 200 ms acquisition at each time in the CZE separation. B) the SERS spectra acquired at each time point indicated are shown. The SERS spectra are the average of the 5 spectra at the indicated time ± 0.5 s.

While the DSERS detection of BZE presents a straightforward, targeted metabolite diagnostic, it would be beneficial to detect other analytes for metabolic profiling that would enable pathway analysis. As discussed above, this is typically done using LC-MS methodology. To test the utility of sheath flow SERS for metabolic profiling in urine, we performed CZE-SERS on a BZE spiked urine sample.

Figure 5A shows the mean detected photons as a function of migration time. The CZE run lasts approximately 30 minutes and consists of a large number of spikes, which each correlate to specific Raman signals. In Figure 5B, the Raman spectra acquired at the specified times indicate that distinct compounds are being detected throughout the CZE separation.

In contrast to MS characterization, the observed SERS signal is difficult to attribute to specific molecules. Equally confounding, co-migrating analytes may exhibit a composite SERS signal provided that both species can equally adsorb to the SERS substrate. As discussed above, the analyte with the strongest interaction to the surface will be preferentially adsorbed and detected. This is consistent with our previous results published regarding a mixture of amino acids methionine, serine, and arginine, where the observed SERS spectrum was dominated by the signal from methionine.[23]

A significant advantage of sheath-flow SERS is that the detected compounds do not readily foul the surface. Thus, sequentially eluting compounds can be interrogated at the same spot on the SERS substrate. Furthermore, it has been shown that, in some cases, closely migrating compounds can be discerned from each other. In a CZE separation of 8 peptides, the peaks attributed to each analyte were baseline resolved. Performing an equivalent separation with MS detection showed the peptides co-eluted.[21] The peak narrowing in CZE was attributed to Langmuir effects associated with SERS detection; however, it also suggests that narrow

features detected in the urine SERS electropherogram may be associated with low concentration species. In our studies of amino acid and protein mixtures, a spot focus (~ 1 μm diameter) was used for detection. This limited the number of hot spots, or molecules, detected and likely corresponds to a very low numbers (possibly 1) of molecules giving rise to the observed signal. The line focus used in the present work increases the number of molecules sampled, which should increase signal to noise, but also increases the possibility of different molecules being detected in each spectrum. Clearly good separations are still necessary for identifying complex mixtures.

To definitively assign the observed spectra to compounds of interest, statistical algorithms are needed. Figure 6 uses the Pearson's correlation coefficient determined between every spectrum in the CZE-SERS trace against the reference spectrum for BZE. Pearson's correlation coefficient determines the degree of correlation between two spectra on a scale from -1 to 1. A coefficient of 1 indicates a strong positive correlation, while a value of -1 means the signals anti-correlate. A score of 0 means there is no correlation. The background correlation appears to result for the background associated with the SERS substrate. The highest correlation in our experiment is observed at the migration time of 955 s. Figure 6B shows the the spectrum at t=955 s with the reference BZE spectrum (Figure 3A) superimposed. There is some variation in the peak at 1275 cm^{-1}, which we attribute to solvo-chromatic effects observed in SERS reference spectra. There is a change in pH between the reference and the CZE run associated with the basic running buffer, or there may be co-migrating compounds that affect the spectrum. The strong agreement suggests this is the migration peak associated with BZE.

There is some evidence of uric acid throughout the separation, which may arise from adsorption in sequential runs. In the CZE-SERS experiments, it appeared that improved

detection was achieved after the first run, suggesting some form of conditioning may be necessary for detection on the surface. This effect requires further study into the mechanism of sheath-flow SERS.

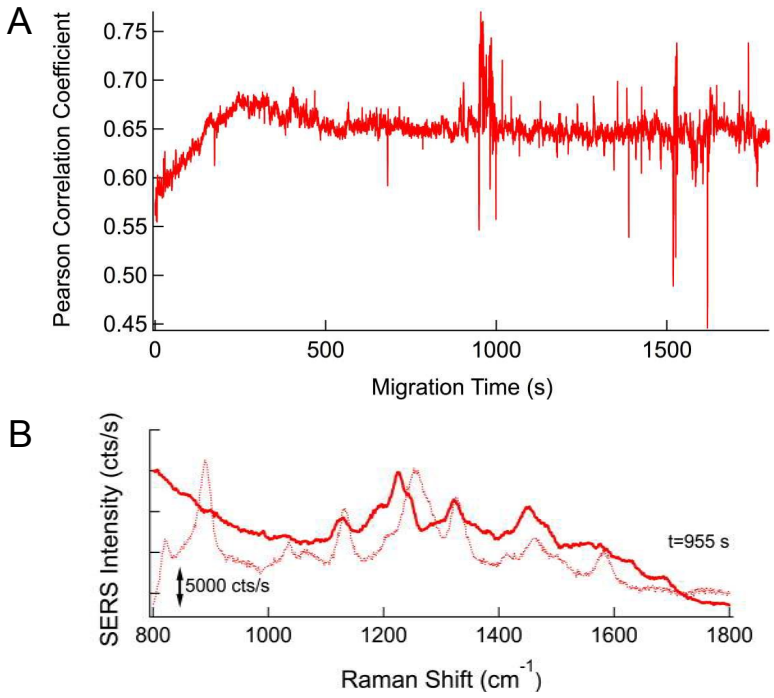

Figure 6. A) The Pearson's correlation coefficient between each spectrum in the CZE-SERS run and the reference SERS spectrum of BZE is plotted. B) The spectrum showing the highest correlation coefficient is plotted (solid) along with the reference spectrum of BZE (dotted), showing the strong agreement.

This approach of using library spectra is similar to methods used with GC-MS for identification of volatile compounds based on fragmentation patterns. Still in its infancy, SERS has all the challenges of MS for identification, and as mentioned above only ~2% of MS spectra can be attributed to a specific metabolite. The importance of sheath-flow SERS detection is that different analytes are clearly observed during the CE-separation and that these analytes show agreement with reference spectra of the compound.

The sheath-flow SERS detector has the potential for in-line use prior to, or concurrently with, MS detection. Again, the amount of sample needed for SERS is quite small. This may enable SERS elucidation of features detected by MS. One direct application would be the discrimination of structural isomers, which typically requires sample derivatization in MS analysis. The fact that SERS samples require no pretreatment and can also be analyzed by MS may enable improved coverage of the metabolome in future experiments.

One common challenge to SERS detection is the irreproducibility of the signals. Indeed, the SERS spectrum of BZE taken from aggregated colloids and our planar substrate show some spectral differences. This is typically attributed to the heterogeneous nature of the plasmonic environment associated with the different SERS assays. This presents a challenge to the development of spectral libraries and is something that must be taken into consideration going forward.

Conclusions.

Sheath-flow SERS is capable of detecting and identifying specific metabolites in complex biological samples, such as urine. We have successfully detected BZE in urine at 30 ng/mL, which is relevant to clinical diagnostics. The importance of adsorption in the SERS process is both facilitated and remediated using this sheath-flow SERS detector. The sheath-flow promotes transport and adsorption to the surface for detection, but also promotes desorption and subsequent detection of other compounds in the CZE experiment. The development of library spectra will be important for the identification of multiple diverse analytes. Signals observed in CZE-SERS suggest that complementary characterization may enable a more

complete coverage of the metabolome and will facilitate systems biology approaches to clinical diagnostics.

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