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New Substrate for Raman, Infrared, and Mass Spectroscopic Chemical Analysis

This article discusses a novel sample deposition method and a new substrate for microanalysis, together with some applications for protein and pharmaceutical analysis.

Raman spectroscopy is a powerful tool for the identification and quantitation of biological materials because of its high chemical/structural information content, low interference from water and minimal sample preparation requirements. However, the low intensity for Raman scattering has traditionally precluded its use in microanalysis, as relatively high concentration (>1 mM) and large quantities of analyte (> 1 µg) are typically required. The intensity of Raman scattering may be increased by resonance and/or surface-enhancement, but this is done at the cost of more restricted applicability and the introduction of spectral variations, increased background and greater susceptibility to photochemical damage.

Research in our laboratory at Purdue University has focused on overcoming the limitations of low sensitivity to tap into the tremendous power of Raman spectroscopy. This has led to development of a novel substrate and sample deposition method, Drop Coated Deposition Raman spectroscopy, or DCDR, that facilitates microanalysis of biological and pharmaceutical compounds using normal Raman scattering (1) and is compatible with other analytical methods including light microscopy, infrared (IR) spectroscopy and Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry.

Our best DCDR substrate is now commercially available as a SpectRIM™ Slide, produced by Tienta Sciences, Inc. The SpectRIM™ Slide consists of a 3 x 1 inch plastic carrier with an optically flat stainless steel plate coated with an ultra-thin (<50 nm) hydrophobic layer. The SpectRIM™ Slide substrates are chemically inert and highly solvophobic, making them ideal for measuring small quantities of material, especially from dilute solutions in polar solvents (such as water or other liquids commonly used for chromatographic separation). The ultra-thin hydrophobic layer displays no appreciable Raman, IR or fluorescence signal and so facilitates essentially zero-background measurements of analyte spectra. When a liquid microdrop is applied to the substrate it beads up and dries to a smaller diameter, thus concentrating the analyte deposit. The slides are compatible with standard microscope stages allowing samples to be easily analyzed with optical microscopy, reflectance infrared (IR) and Raman spectroscopy.

We have demonstrated that DCDR may be used to obtain high quality normal Raman spectra from small volumes (down to sub-nL) of dilute protein solutions (down to sub-µM concentrations) for spectral identification and chromatographic detection. High quality DCDR spectra can be obtained from less than 1 fmol of protein probed non-destructively within a 10 mW Helium-Neon (633 nm) Raman excitation laser beam (either from manually deposited micro-drops of µL volume or from ink-jet microprinted protein spots of nL volume containing less than a total of 20 fmol of total deposited protein). This represents over a 1000-fold increase in the sensitivity of Raman for analysis of protein solutions, relative to the best normal Raman results obtained prior to DCDR (1). The exquisite chemical structure sensitivity of DCDR is exemplified by the spectroscopic classification (identification) of three natural insulin variants—human, bovine and porcine (2). These proteins, which differ by between only 1 and 3 amino-acid residues, can be identified with 100% accuracy from their Raman spectra, as illustrated in **F1**. These DCDR measurements were performed on solutions obtained from reverse-phase high performance liquid chromatography (RP-HPLC) eluent fractions, either before or after lyophilization. These and other such results (3-6) demonstrated that DCDR has the sensitivity, both in terms of detection limit and in terms of chemical information content, to distinguish proteins of very similar structure under conditions that are compatible with biomedical research and diagnostics.

Since Raman spectral features represent molecular vibrational normal modes, rather than mass or electronic differences, DCDR offers information which is complimentary to other protein analysis methods. To date, the DCDR method has been used to demonstrate use of Raman spectroscopy for protein analysis, including detection of protein conformational changes, peptide phosphorylation and disulfide bond reduction as well as the identification of different equal mass glycans and quantitation for glycan and peptide mixtures.

A key feature of the SpectRIM™ Slides is that they are designed to be an integrated tool for protein analysis using both optical methods, such as those described above, as well as non-optical methods such as liquid chromatography and MALDI mass spectrometry. For example, the Raman and MALDI-TOF spectra shown in **F2** were collected from the same protein spot on the slide. This was the first reported demonstration that both normal Raman and MALDI-TOF spectra can be obtained from the same protein sample.

Since IR spectroscopy is more sensitive than Raman, IR may be used to rapidly collect spectral images and chemical maps of deposited protein arrays spots. Thus, IR can be used to rapidly identify proteins of interest in spots derived from different chromatographic fraction or combinatorial library samples. Such IR spectral images can also rapidly reveal hot

spots where high concentrations of protein are located within a deposition region. For example, **F3** shows an interfero-metric image and selected IR spectra derived from a single ink-jet microprinted spot showing that protein is located primarily in the outer ring of the spot (containing a total of 20 fmol of protein).

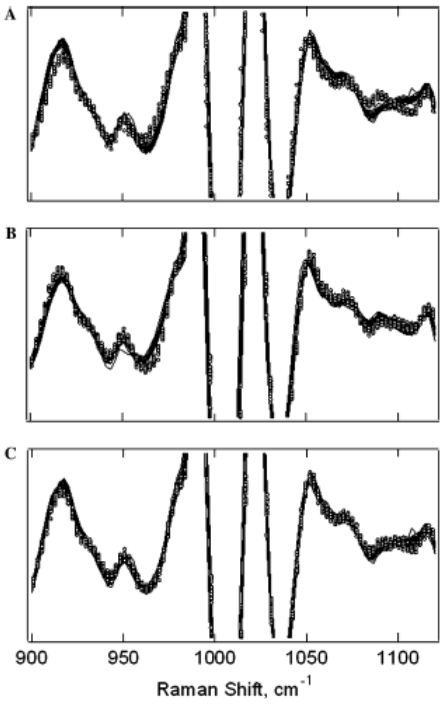
The versatility of the SpectRIM™ Slide facilitates its integration into a complete protein analysis scheme. For example, chromatographic fractions or aliquots down to nL volume can be deposited on the slide. IR spectroscopy can then be used to identify fractions (spots) containing proteins of interest. The identity of the protein(s) in each spot can then be analyzed in greater detail using Raman and/or MALDI mass spectrometry.

In addition to its proteomic applications, Raman spectroscopy can also be used in studies of DNA. In such experiments, even dilute solutions of DNA can be analyzed by using the SpectRIM™ Slide. For example, **F4** shows a white light optical image of *Bacillus megateurium* DNA deposit on the SpectRIM™ slide and its Raman spectrum.

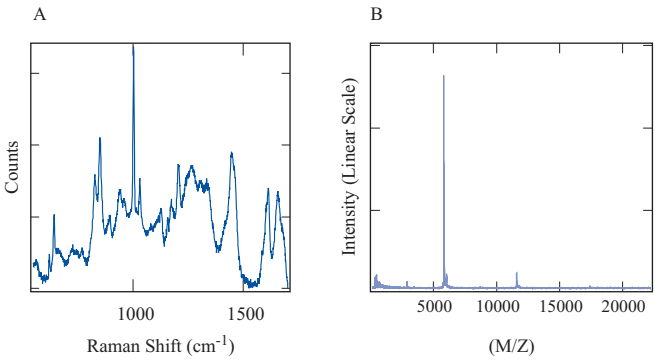
The SpectRIM™ Slides can also be used in identification of pharmaceuticals. This can be of particular importance in drug development, process monitoring and in detecting counterfeit drugs. In such applications the extraction methods used to separate drug active ingredients from tablet matrices and concentrate the dried extracts can be problematic. This is especially true when using traditional glass slides and/or IR transmission windows, such as BaF_2, because on glass slides the dried material tends to disperse rather than dry down into a concentrated area. As a result, larger volumes are traditionally needed to build up a significant concentration of the analyte of interest for detection using IR and Raman spectroscopy. The SpectRIM™ Slide's ability to produce a more highly concentrated evaporation deposit allows for deposition of much smaller amounts of solvent and analyte. Typically the deposit on the SpectRIM™ Slide is 10 to 20 times more concentrated (1/10 to 1/20 the size) than that produced on a glass slide or IR window. For example, **F5** contains photomicrographs of a sample (lorazepam) dried on SpectRIM™ versus a standard glass slide, as well as the Raman and IR spectra. Ultimately, use of this new type of sampling substrate allows for extraction and concentration of a low-dose drug substance as well as other materials such as inks, adhesives and explosives. Indeed, the SpectRIM™ Slides were used successfully to identify different solutions of explosives as illustrated in **F6**. Thus, we anticipate that SpectRIM™ Slides and DCDR technology will have tremendous value not only in biomedical proteomic diagnostics, but also in applications ranging from drug discovery to process monitoring and forensics.

DCDR may be used to obtain high-quality normal (nonenhanced) Raman spectra from femtomole quantities of protein derived from solutions of micromolar concentration. The resulting spectra are highly reproducible and essentially identical to those obtained from higher concentration protein solutions. We have demonstrated compatibility of SpectRIM™ Slides with proteomic sensing by performing (i) the first combined Raman and MALDI-TOF measurements on the same sample, (ii) the first normal Raman measurement of protein derived from a HPLC fraction, and (iii) the first normal Raman measurement of protein deposited using an ink-jet microprinter. Additional improvement in detection limit and throughput (speed) are expected with further optimization of

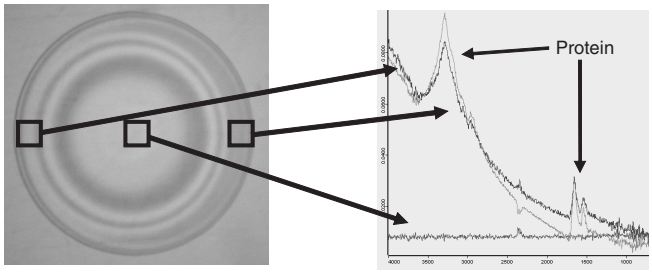
F1. Superposition of 30 individual NSD Raman spectra of insulin variants: (A) Human (black) and bovine (gray), (B) bovine (black) and porcine (gray), and (C) human (black) and porcine (gray).



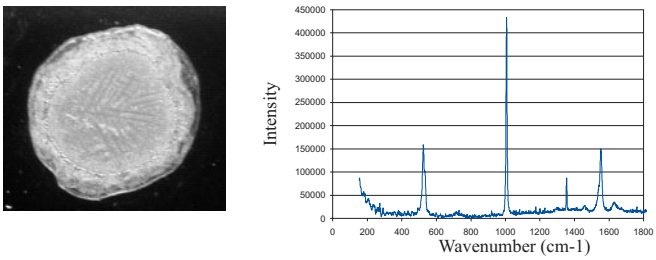
F2. Raman spectrum (A) and MALDI-TOF spectrum (B) obtained sequentially from the deposition of 1 µL of 10 µM insulin solution. The integration time for the Raman spectrum was 200 s with a laser power of 12 mW. The MALDI-TOF spectrum was the sum of 100 accumulative acquisitions.



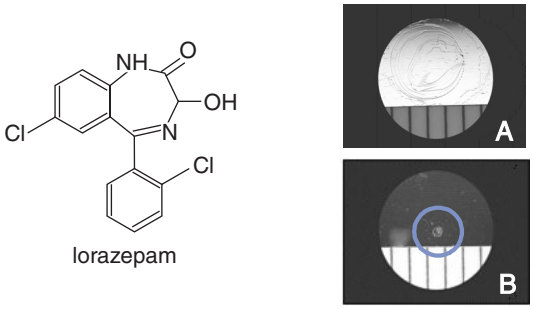
F3. The left panel is a photomicrograph of a protein deposit. The right panel shows three reflection infrared spectra that indicate that the protein is located in the outer ring of the deposit and not on the inside of the deposit. This data was collected at Bruker Inc.



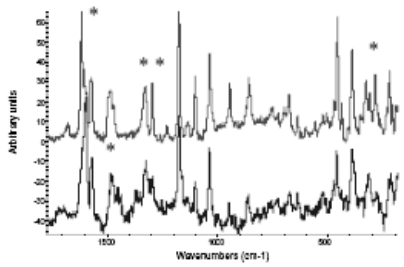
F4. White light optical image of the *Bacillus megaterium* DNA deposit on the SpectRIM slide (left). The image was obtained using a Nikon Light optical microscope with a Sony CCD high-resolution video camera. Raman spectrum of a deposit (8 µg) of *Bacillus megaterium* on the SpectRIM™ slide (right).



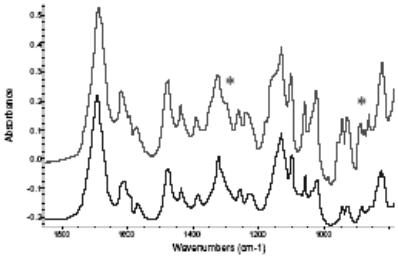
F5. Photomicrograph of a 2 µl droplet of a lorazepam standard dried on (A) a glass slide and (B) on SpectRIM™. The scale lines represent 1mm.



Raman spectrum of a 3 µg deposit of lorazepam standard (upper spectrum) compared to the Raman spectrum of 1.5 µg deposit of lorazepam extract from a 0.5 mg lorazepam tablet (lower spectrum). These spectra were recorded on a LabRAM HR-IR using a 632 nm laser, 950 grooves/mm grating, 100X objective, 10 sec integration time averaged twice, and 200 µm slit width.



Infrared spectrum of a 3 µg deposit of lorazepam standard (upper spectrum) compared to the infrared spectrum of 1.5 µg deposit of lorazepam extract from a 0.5 mg lorazepam tablet (lower spectrum). These spectra were recorded on a LabRam HR-IR using ATR objective, at 4 cm^{-1} resolution and 128 co-additions.



the optical detection and sample deposition procedures. Since the intensity of a normal Raman spectrum is proportional to the surface density of the analyte on the substrate, it is desirable to confine the deposited sample to the smallest possible area to maximize the Raman signal-to-noise ratio. A confocal micro-Raman system can achieve lateral and depth resolution on the order of 1 µm. Such a small collection volume is estimated to contain less than 0.1 fmol of protein (less than 1 pg of a 10 000-Da protein), which may be taken as an estimate of the protein detection limit achievable using DCDR on SpectRIM™ Slides.

The potential importance of DCDR as a new tool for proteomics and pharmaceuticals derives both from its demonstrated detection limit and from the fact that Raman spectroscopy yields information not readily obtainable from current proteomic separation and detection methods. For example, although mass spectroscopy is a tremendously powerful analytical tool, it is primarily sensitive to composition and primary structure, while Raman contains information about secondary (e.g., α-helix and β-sheet content, etc.), tertiary (folding), and quaternary (binding) structure of molecules and molecular aggregates. Furthermore, although fluorescence-based detection of DNA and protein binding is more sensitive than Raman, fluorescence spectral features are much broader than Raman features and thus have a lower chemical information-carrying capacity. Most important, fluorescence tagging requires significant additional preprocessing time and cost. The chemical structure sensitivity of Raman means that it could be used as a stand-alone method for identifying and detecting changes in biomolecules without the need for any tagging or chemical modification. Our demonstration of combined DCDR/MALDI-TOF provides a mechanism for correlating Raman and mass spectral information. In addition, our demonstration of detection of microprinted protein spots opens the door to future applications of Raman sensing in array-based detection systems.

References

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F6.

Compound	Solvent
2,4,6-trinitrotoluene (TNT)	acetonitrile
Pentaerythritoltetranitrate (PETN)	acetonitrile
Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	acetonitrile
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazine (HMX)	acetonitrile
Triacetone triperoxide (TATP)	acetonitrile
Hexamethylenetriperoxidediamine (HMTD)	acetonitrile
Methyl-2,4,6-trinitrophenylnitramine (Tetryl)	acetonitrile
Ethylene glycol dinitrate (EGDN)	acetonitrile
Triaminetrinitrobenzene (TATB)	acetonitrile
3-nitro-1,2,4-triazol-5-one (NTO)	acetonitrile
CI-20	acetonitrile
Nitrocellulose (NC)	acetonitrile
1,3,3-trinitroazetidine (TNAZ)	acetonitrile
Nitroglycerin (NG)	methanol
Picric acid (PA)	methanol
Ammonium nitrate (AN)	water
Ammonium perchlorate (AP)	water
Ammonium dinitramide (ADN)	water
Potassium nitrate	water
Potassium perchlorate	water

Stock solutions of explosives prepared at a concentration of 1mg/mg, studied for the application of SpectRIM™ in detection of explosives. Samples deposited on Tienta Sciences SpectRIM™ Slide and their respective Raman spectra.