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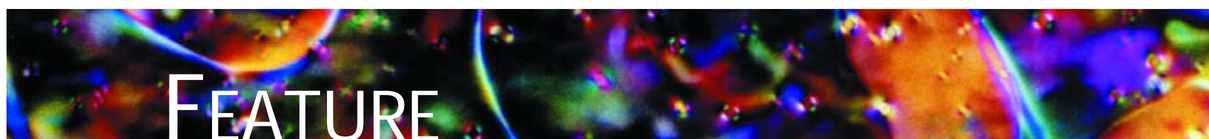

Fran Adar, Andrew Whitley, and Ruth Geiger

A Raman Microprobe Optimized for Microarrays and Multiple-Well Plates

Abstract

The Raman microscope, which provides detailed molecular and/or crystallinity information with 1-µm spatial resolution, has been adapted for automated studies of microarrays and multiple-well plates. The system can provide identification of microsamples and information on binding. In combination with Ag, and Au colloids, sensitivity approaches single-molecule detection. Application areas include lab-on-a-chip (for reactions, separations, etc.), solid-phase synthesis, genomics/gene chips, proteomics (protein expression), and protein interactions (protein–protein, enzyme-substrate, hormone–receptor, antigen–antibody, protein–drug, etc.). Sample positioning is automated, and acquisition/data treatment protocols are stored for future use. In combination with three-tiered access, examination of large sample arrays can be automated for high-throughput screening.

Key Words

Raman, microarrays, multiple-well plates, high-throughput screening, molecular information

Raman spectroscopy is a probe of molecular vibrations that is often compared to infrared absorption. There are, however, some significant differences between the techniques that provide important advantages for examining biological systems.

Description of the Raman effect and spatial resolution provided

The Raman effect was first reported in 1928.[1] Raman spectra are generated when a laser beam is scattered by a sample. The laser beam is monochromatic (every photon has the same energy) before the scattering event, but after the event, the energy of some of the photons is shifted following the creation of molecular vibrations during the scattering process. It is the spectrum of the scattered light that corresponds to vibrational transitions in the sample, and this vibrational spectrum provides chemical information on the sample. As such, a Raman spectrum is analogous to an infrared spectrum, but the chemical structures to which it is most sensitive tend to be different (see below).

The commonly used laser beams have wavelengths between 1.06 µm and 244 nm. These laser beams can be focused to diffraction-limited spots with sizes approximately equal to the wavelength of the light. IR beams, in contrast, cannot be focused to better than 10–20 µm because of the intrinsic wavelengths of the light.

Sensitivity of Raman versus IR

Raman and IR spectroscopies tend to be complementary; that is, if vibrations of particular functional groups are intense in one phenomenon, they are weak in the other. In general, polar vibrations tend to be quite intense absorbers of IR light but weak Raman scatterers; this would include the important carbonyl (>C=O) and hydroxyl groups. Symmetric stretching bands of aromatic groups, sulfur–sulfur stretches, and carbon double bonds tend to be strong in the Raman spectra.

Raman solvents and substrates

An important consequence of this is the ability to observe Raman spectra in aqueous solutions because the water background is weak and the spectral features are broad. It is also not problematic to examine samples mounted on standard glass microscope slides. The glass spectrum is also very weak and broad. When the spectrum of interest is also weak, and the glass presents an unacceptable background, the glass can be metal coated (Ag, Au, or Al) before the sample is mounted. In contrast, IR microscopy must be done on special substrates, often halide salts that are hygroscopic.

Polymorphy

Raman spectra reflect not only molecular vibrations, but effects of the crystalline phase as well. That is, if

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there is more than one crystalline polymorph of a particular molecular species, its spectrum will be different. Not only are polymorphs distinguishable, but pseudopolymorphs (where the differences are in the degree of hydration) are distinguishable as well.

While this capability is of some academic interest, it is very important in the pharmaceutical industry. Curiously, many drugs exhibit multiple crystalline forms that are

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sometimes difficult to control. Temperature, humidity, and pressure can induce conversion. The crystalline form can affect bioactivity of the final product (uptake and dissolution rates), dissolution and reactivity of intermediates, and shelf-life. These physical/chemical properties are consequently used to provide patent protection.

Raman microprobe

The Raman microprobe was developed more than 25 years ago in Northern France by Prof. Michael Delhaye and Prof. Paul Dhamelincourt as a molecular analog to Castaing's electron/elemental microscope.[2] It was recognized at the time that a pencil-narrow laser beam could be effectively focused to a diffraction-limited spot whose size would be determined by the wavelength of the beam, and the numerical aperture (NA) of the focusing objective. For a variety of reasons, the Raman microprobe remained a research tool without widespread analytical usage until the 1990s. During the late 80s and early 90s, various technological breakthroughs enabled a significant reduction in the size and complexity of the instruments, a reduction in utility requirements, and an increase of at least two orders of magnitude in sensitivity.[3] A technique that had previously been of academic interest now became a tool with practical potential to solve current analytical problems.

Application of Raman spectroscopy in biomedical sciences

For the reader interested in knowing how the technology has been successfully applied to materials of interest to biological and pharmaceutical sciences, the authors refer to several chapters in the recent texts entitled ***Infrared and Raman Spectroscopy of Biomolecules,***[4] ***Analytical Applications of Raman Spectroscopy,***[5] and ***Handbook of Raman Spectroscopy.***[6] These chapters document the spectral behavior of the various classes of biomaterials of interest such as proteins, lipids, nucleic acids, carbohydrates, and pharmaceuticals.

Raman microprobe interface with biotechnology

The explosion in biotechnology over the past decade has been accompanied by requirements for high-throughput screening techniques to analyze sets of hundreds or thousands of samples. While Raman measurements have not been the first choice for many of these applications, there are certainly cases in which the wealth of information present in a Raman spectrum could be of use. It is the purpose of this article to review these applications, and to describe how Raman could provide unique information.

Biotechnology

The goals of the biotechnology revolution include the determination of the causes of the disease state and the consequential development of medical interventions. In order to accomplish these goals, the molecular basis of the disease is identified, providing a target for intervention. Using a chemical theme, many possible molecular structures are designed and synthesized, forming a chemical library. Using high-throughput screening of the many compounds, good leads are identified and then optimized. This process has been described as combinatorial chemistry for drug discovery. Preclinical studies, clinical trials, and FDA approval follow the identification and production of promising drug candidates.

The elucidation of the human genome has also created needs for high-throughput screening. In the fields of genomics and proteomics, large numbers of DNA oligomers and proteins need to be analyzed. Genotype variations can account directly for some human disease, for the genetic tendency to contract certain diseases, and for individual variability in response to drug agents. Gene expression of proteins (when, where, and for what purpose) and posttranslational modification (cleavage, refolding, addition of phosphate, carbonate, carbohydrate, etc.) can also be disease markers that may be screened. Thus, for

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example, it would be simple to identify the presence of a carbonate or phosphate in a protein, and careful measurement of its position and/or line shape could also enable differentiation between similar species. Another possible application would be the differentiation between sulfhydryl groups (–SH) and disulfide groups (–S–S–), which are often involved in converting a protein from an inactive to an active form (insulin is a prime example).

Binding studies can also provide information. In addition to detection of nucleic acid hybridization implicit in genomics, protein–nucleic acid interactions, protein–protein binding, hormone–receptor binding, antigen–antibody binding, and protein–drug binding can characterize many metabolic states. Note that antibody studies have been done for years using gold colloids whose presence can, coincidentally, greatly enhance Raman signals (see discussion of surface-enhanced Raman scattering below).

Parallel arrays of samples can be assembled in a number of ways. One of the most ingenious breakthroughs to occur in recent years is the lab-on-a-chip. In 1998, a group at the University of Michigan (Ann Arbor, MI) reported a nanoliter device based on microfabricated fluidic channels and fluorescence detectors in which DNA and reagents were introduced and electronic signals corresponding to genetic information were output.[7] Several corporations have either introduced lab-on-a-chip systems or have re-

Figure 1 *Instrument sampling mechanism.*

ported using such systems for high-throughput parallel synthesis of single compounds in an addressable format.

The power of a Raman microprobe with its high spatial resolution provides the ability to detect detailed differences in molecular composition and/or conformation for microsamples. The ease with which it can be used as a detector for lab-on-a-chip experiments or procedures makes it a valuable choice in the arsenal of detectors for these arrays. During synthesis, Raman can be used to confirm structure and establish purity; during bioscreening, it can help identify lead compounds and their structural elements that contribute to the bioeffect.

The DNA chip is essentially a microarray of known DNA oligomers providing highly parallel and efficient genetic analysis when exposed to a population of DNA oligomers from an individual or organism under investigation. The goal is to identify single-nucleotide polymorphisms (SNPs) that can be signatures, for instance, of different biological traits, including disease susceptibility or antibiotic resistance of microorganisms. The identification of the oligomer on the array that forms a DNA hybrid with the unknown helps to identify the genotype. Until now, the identification has been with fluorescent probes and gold nanoparticle tags in combination with melting experiments.[8]

The gold nanoparticle, by coincidence, produces enormous enhancements of Raman signals (6–12 orders of magnitude), and this technology may be of huge potential for other chemical systems. Michael Natan, previously at Penn State (University Park, PA) but currently Chief Technical Officer at **Surromed** (Mountain View, CA), has been exploring the use of colloidal gold aggregates for surface-enhanced Raman scattering (SERS) detection of biomolecules.[9] He has proposed and demonstrated a variety of SERS active substrates and colloidal conjugates that can be integrated in high-throughput detectors. For example, proteins or small molecules on protein microarrays[10] can be conjugated to metal colloids for high-throughput screening by SERS. The advantage of detecting the SERS signal rather than the color of the colloid is in the detailed spectral information that is provided. The SERS signal can reflect details of molecular conformation that would not be detectable colorimetrically. Several groups have demonstrated SERS activity in biologically relevant samples.[11–15]

Raman multiple-plate reader

The Multiwell Plate Reader was developed as a result of a collaboration between **JY Horiba, S.A.** (Lille, France), and **BASF** (Ludwigshafen, Germany) for following reaction chemistry in a 96-well plate. The instrument is based on a standard LabRam. The BX40 microscope (**Olympus Corp.,** Tokyo, Japan) was replaced by a BX30. The quality control version of the LabSpec software (delivered for more than five years to the computer industry) enables definition of measurement parameters and data treatment for reporting. *Figure 1* shows the sampling part of the instrument, with the multiple-well plate loaded on the motorized *XY* stage. In order to accommodate the large sample volumes, the optics were optimized to probe a 10–50 μm^3 volume with the use of a low-numerical-aperture, low-magnification lens. To ensure that the sample position relative to the objective is always correct, the position of the lens is controlled by the motorized *Z* adjustment, using an algorithm based on information from the television camera monitor; ±3 mm travel is provided to compensate for the anticipated unequal filling of the wells. This capability is well adapted to the examination of combinatorial chemistry beads used in the production of pharmaceuticals as well.

The software has three different access levels that are password protected. The engineer/supervisor can select any of the options. The manual level can access some of the options, and the production level can only initiate a measurement according to previously developed protocols. Features of the software include:

- Preprogrammed masks for selection of wells
- Preprogrammed fitting procedures for user-defined analytical algorithms
- Configuration file defining the type of well plate, sample index, product name, and acquisition parameters
- Data output in the form of an Excel spreadsheet (**Microsoft Corp.,** Redmond, WA).

One can envision a sample set in which a rapid fluorescence measurement could be used for prescreening, after which (slower) Raman measurements would be implemented to provide detailed structural information. A software algorithm designed to identify wells of interest from the fluorescence signal would then provide the information for the mask selection of the wells for Raman analysis.

In a model study, a 48-well plate was filled with ethanol/water solution of caffeine—1.5% caffeine in 20% ethanol in water. The goal was to screen the concentration and generate a statistical estimate of the precision of the measurement. Measurements were made with a 70-mW double Nd:YAG laser, λ = 532 nm (power measured at the sample). The total measurement time for the 48 wells, including autofocus at each well, was 8 min (≤10 sec/well).

Measurement statistics could be derived using the following protocols: 1) one measurement per well; or 2) multiple measurements per well, treated as the sum, individually, or both.

The signal-to-noise ratio is calculated for every measurement, and if the noise is out of range, the entire measurement series is terminated with an error message. The results of the measurements and the calculation algorithm are reported in an Excel table. An out-of-range value will also result in termination. The reported error statistics include the mean, standard deviation, and standard deviation as a percentage of the mean.

Figure 2 shows typical spectra of 20% ethanol in water without (bottom trace) and with 1.0% caffeine (top trace). In this case, the calculation algorithm was designed to

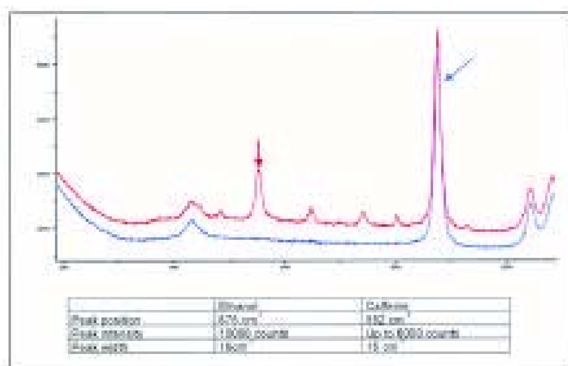

Figure 2 *Typical spectra of 20% ethanol in water with 10% caffeine (top trace) and without caffeine (bottom trace).*

Table 1

Raman predictions of caffeine concentrations

			Standard Solutions			
1.	Sample	1	2	3	4	5
2.	$C_{(weight\ \%)}$	0	0.5	1.0	1.5	2.0
3.	$I_{(caff.)}/I_{(eth.)}$	0	0.058	0.108	0.170	0.235
4.	$C_{(calc\ by\ Raman\ \%)}$	0	0.56	0.98	1.49	2.08
			Random Solutions			
	Sample	1	2	3	4	
	$C_{(weight\ \%)}$	0.69	1.16	1.40	1.89	
	$C_{(calc\ by\ Raman\ )}$	0.65	1.13	1.39	1.90	

provide the caffeine concentration from the intensities of the ethanol and caffeine bands. The relevant spectral parameters from which the concentrations are to be derived are tabulated as well.

By making a series of measurements on samples with varying caffeine concentrations, a relationship between the Raman intensity ratio (caffeine band to ethanol band) and the caffeine concentration can be derived (lines 1 and 2, *Table 1*). This relationship can be applied to different measurements in order to confirm the precision of the technology (line 3, Table 1). Tests on samples with random concentrations yielded the results shown at the bottom of Table 1.

Raman microarray analyzer

The standard LabRam or LabRam Infinity would be used for high-throughput screening analysis of a microarray where the goal is to probe sample volumes on the order of 1–5 μm^3. In this case, instead of focusing the beam in a multiple-well plate where the samples probed are tens to thousands of cubic micrometers in volume (1 mL = 10^{12} μm^3), the maximum spatial resolution enables probing samples on the order of 1–5 um^3. Either the LabRam or LabRam Infinity would provide 1-µm spatial resolution.

Examples in which this technology would be useful are nucleic acid or protein microarrays. In addition, cell growth in bioreactors is now being reported. At the National American Chemical Society Meeting held in San Diego, CA (April 1–5, 2001), a group in the UCSD Microscale Tissue Engineering Laboratory (San Diego, CA) reported the growth of hepatocytes in a micro-electric mechanical systems (MEMS) structure, a porous silicon bioreactor in which wells of the correct size enabled growth of functionally normal individual cells. This work is described in a publication listed on the group's Web site.[16]

All of the software capabilities described for the Multiwell Plate Analyzer are available for characterizing microscopic amounts of material in microarrays.

Conclusions

The Multiwell Plate Reader and MicroArray Raman Analyzer offer a new dimension for characterizing large numbers of samples in high-throughput screening. The chemical information provided by Raman spectra recorded from microscopic volumes will enable a more detailed characterization of biotechnology materials than is currently available.

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