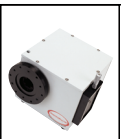

BALF Research with Raman Spectroscopy

© 2000 Roper Scientific, Inc. All rights reserved.

The development of extremely sensitive CCD detectors and holographic-notch filters is fueling a resurgence in the field of Raman spectroscopy, particularly in the study of organic structures. Traditionally, using Raman spectroscopy to investigate living systems was hampered in two ways: its inability to detect very low-level signals ($<10^{-9}/10^{-12}$) from the excitation source, and the high stray-light levels in monochromators that masked low-frequency components in proximity to excitation wavelengths. With the more powerful and precise equipment available today, Raman spectroscopy has overcome these problems and is well suited even for the study of complex organic compounds. This note describes the landmark use of Raman vibrational spectroscopy to compare bronchoalveolar lavage fluid (BALF) of normal and alveolar proteinosis (AP) lungs.

BALF RESEARCH WITH

Introduction

Raman spectroscopy is a powerful and widely used technique for identifying and quantifying materials based upon their molecular and crystal-lattice bond vibrations. It is extremely sensitive, noninvasive, and unaffected by aqueous matrices. Modern technology has taken this technique even further. High-performance CCD cameras, spectrometers, and filters deliver single-photon sensitivity, precisely focused high-energy-wavelength emissions, and greatly reduced stray light or radiation, thereby bringing a unique level of flexibility into experimentation.

Southern BioGene, located in Birmingham, Alabama, recognized the potential of using Raman spectroscopy for studying the intramolecular and intermolecular interactions involved in a bilayer lipoprotein matrix. In his September 1999 article, "Structural Changes in the Lipoprotein Complex of Bronchoalveolar Lavage Fluid Detected by Raman Spectroscopy" (*Spectroscopy*, vol. 14, #9, 21-24), Nihal S. De Silva discusses their groundbreaking use of this technique to measure spectra from bronchoalveolar lavage fluid (BALF) obtained from normal and alveolar proteinosis (AP) patient lungs. Their research, described in the following sections, shows that with the right equipment, Raman spectroscopy is an effective means for studying complex living systems.

Application Background

The surfactant lipoprotein system of the lung is made up of roughly 90% lipids (the majority of which are phospholipids) and 5 to 10% specific proteins (the apoproteins SP-A, SP-B, SP-C, and SP-D). The cells secrete this mixture of lipids and proteins into the alveoli and respiratory air passages, where it is transformed into the tubular myelin lattice (TML). The TML forms a coating film that reduces the surface tension of pulmonary fluids and contributes to the elastic properties of pulmonary tissue, thereby limiting collapse during inhalation and exhalation.

The goal at Southern BioGene was to investigate how the biochemical structure of the lipid-protein complex facilitates host defense and surface-tension reduction at a molecular level in the TML. The development of replacement surfactants for respiratory therapy depends on understanding this process. Southern BioGene examined the lipoprotein complex of TML and multilamellar structures, paying particular attention to the interaction of the lung apoproteins SP-A, SP-B, and SP-C with defined phospholipids. Using Raman spectra obtained with the equipment and methods detailed below, they compared the BALF from normal lungs to that of AP patients to investigate possible differences in constituent apoproteins and phospholipid makeup.

Research Equipment and Methods

Southern BioGene has put together a state-of-the-art registration system based on the Acton Research SpectraPro® 150 f/4 spectrograph, with a reciprocal linear dispersion of 5 nm/mm for 1200-g/mm grating, and a thermoelectrically cooled 1024 x 256 CCD camera (the Acton Research SpectruMM:GS256 detector). The CCD has a 7 x 25-mm imaging area with 24 x 24-μm pixels. By using a set of gratings with different groove densities (600 and 1200 g/mm), they were able to cover both 60- and 125-nm spectral windows. A grating with a low groove density was utilized to get a full Raman spectrum at ≤2600 wavenumbers relative to the excitation wavelength. To analyze different regions of the Raman spectrum with a high spectral resolution (≤4 cm^{-1}), a grating with a high groove density was used. A Pentium-based personal computer controlled the spectrograph and the CCD and collected and analyzed the experimental data.

The laser-excitation source was a TEM_{00} linearly polarized HeNe laser ($\lambda = 632.8$ nm) with 10-mW output power (Uniphase, San Jose, CA). For sample excitation, the team from Southern BioGene placed the laser 5 m from the sample and used first-order reflection of the laser beam from an 1800-g/mm diffraction grating. Several apertures were used to control the beam path. These steps were taken to clean up the spectrum of the HeNe laser and to prevent spectral lines of He and Ne gas discharge in the laser tube from

entering the spectrograph. As a result, the spectral region of interest was virtually clear of parasitic lines. The output power of the first-order reflected beam was about 3 mW at 632.8 nm. Thermal effects during sample excitation were avoided by using low-power excitation. This also prevented thermal damage of the sample.

Southern BioGene investigated two BALF samples collected from AP patients and healthy subjects at the University Medical Center Hospital in Birmingham, Alabama. The samples were extracted from the lungs with 0.9% NaCl (saline) and stored at -20°C until use. Samples were thawed at 4°C for 2 hours to allow compaction of the multiple layers. Immediately before analysis, each sample was carefully mounted in the Raman setup. The cuvette was positioned as shown in **Figure 1** to prevent both the stray reflection of light from the cuvette's top wall and the unwanted Raman signal from the water layer.

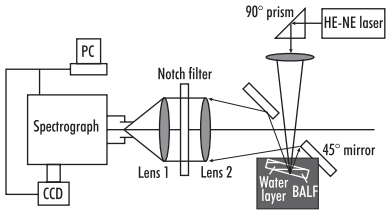

***Figure 1**. Schematic drawing of Southern BioGene's experimental arrangement to measure Raman spectra of BALF.*

The laser beam was focused into the sample by a 500-mm lens through a hole in a 45° mirror. Using a back-scattered scheme to collect the Raman signal, the scattered radiation was reflected by the mirror and then focused by a two-lens objective (f/1 = 50 mm, f/2 = 75 mm) onto the input slit of the spectrograph. A holographic-supernotch filter with an optical density of >6 at 632.8 nm (Kaiser Optical Systems, Ann Arbor, MI) was employed to prevent the excitation wavelength from entering the spectrograph. The spectral bandwidth of the supernotch filter was ~350 cm^{-1}. Southern BioGene also used a high-power alexandrite laser tunable in the 730 to 810-nm range, an ideal range for Raman excitation of biological systems. Excitation by alexandrite laser with I_{ex} = 785 nm exhibited sufficient signal-to-noise gain of the Raman spectra.

Research Results

The data collected and analyzed by Southern BioGene were the results of careful subtraction of BALF extractant (0.9% NaCl in distilled water) and quartz (cuvette wall) Raman spectra. The specific frequency changes in the spectra of AP and normal BALF samples are readily apparent in **Figures 2 and 3**.

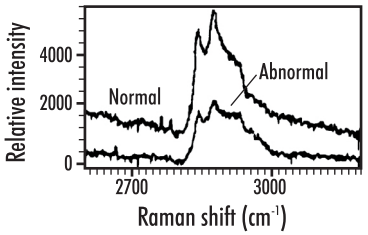

***Figure 2**. Raman high-frequency spectra. Normal is BALF from control patients and abnormal is BALF from AP patients.*

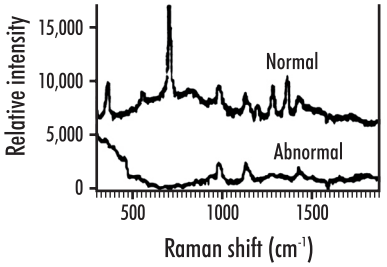

***Figure 3**. Raman mid-frequency spectra. Normal is BALF from control patients and abnormal is BALF from AP patients.*

The high-frequency area of the Raman spectra contains the lipid acyl chain methylene and methyl C-H stretching modes. The Raman band 2850 cm^{-1} refers to the acyl chain methylene's (CH_2) symmetric stretching vibrations, whereas 2880 cm^{-1} refers to its asymmetric stretching vibrations. The 2935-cm^{-1} feature represents a complex interval that contains spectral components from Fermi resonance interactions involving the chain methylene moieties, and separately the C-H symmetric stretching modes of the lipids' chain methyl termini. The spectral intensity ratio $m = I_{2850}/I_{2880}$ shows pure lateral chain-chain interactions. The intensity ratio $n = I_{2939}/I_{2880}$ reflects chain-chain interactions as well, but these also contain contributions from intrachain trans/gauche isomerizations. Southern BioGene's results show that the *m* value is the same (0.83) for both the AP and normal samples, whereas the *n* values are significantly different (0.78 and 0.56, respectively). De Silva believes this indicates a similar lateral chain-chain ordering for the samples, but a different intrachain structure and, presumably, a different degree of perturbation in the various portions of the bilayer.

In the mid-frequency region, the absence of Raman lines 380-, 730-, 1220-, 1300-, and 1380 cm^{-1} from the AP BALF sample indicates a difference in constituent structure of diseased and normal lungs. De Silva theorizes that this could be due to the absence of functional groups specific for peptides and amino acids rather than for lipids. These data support the findings of Southern BioGene's earlier electron microscopy studies comparing the BALF structures of normal and AP patients. Based on these results, their conclusion is that the TML from AP patients contains no membrane interdigitization and is poorly developed in comparison to normal lungs.

Southern BioGene's use of Raman spectroscopy to show structural changes in constituent apoproteins and phospholipids that occur in the BALF of AP patients is an important step in understanding the molecular structure of TML. Now that the technology exists, the biggest roadblock remaining is simply the relatively few Raman spectra of BALF studies that have been performed. According to De Silva, Southern BioGene is in the process of adding to these preliminary results to obtain meaningful and reproducible data for more detailed conclusions. As researchers like those at Southern BioGene continue to use Raman spectra to study complex living systems, correlative data will begin to amass. This in turn will fuel the development and success of future drug and therapy design.

ROPER SCIENTIFIC

Components for Raman Spectroscopy

In addition to the spectrograph and CCD detector used in the research above, Roper Scientific™ offers a wide range of high-performance spectrometers, detectors, and spectroscopy accessories designed for Raman. The Acton Research SpectraPro monochromators and spectrographs feature automated multiple-grating turrets and a 32-bit microprocessor scanning controller with automated self-calibration. The digital cameras combine exclusive front- or back-illuminated CCDs, deep-depletion silicon, ultra-low-noise amplifiers, and a choice of thermoelectric or cryogenic cooling to provide unparalleled sensitivity. Roper Scientific also offers complete analytical Raman packages. The Acton Research SpectruMM™ for Raman incorporates the SpectraPro 300i spectrometer and a SpectruMM™ GS CCD. It is supplied with an InPhotonics probe for use with a 785-nm laser. The SpectruMM for Raman system can be outfitted with other versions of the standard probe for 514-, 532-, and 670-nm laser-excitation wavelengths. It can also be configured using the SpectraPro 500i spectrograph for high-resolution requirements with shorter wavelength lasers, and either SpectruMM HP or SpectruMM LN series CCDs for weak or low-concentration Raman samples. The SpectruMM for Raman system comes standard with Acton Research SpectraSense™ spectral-acquisition and data-treatment software. A full line of sources and sampling accessories are also available from Roper Scientific.

Contact **Roper Scientific, Inc.** for more information:

530 Main Street Acton, MA 01720
tel: 978.263.3584
fax: 978.263.5086
email: mail@acton-research.com
web: www.roperscientific.com

3440 East Britannia Drive Tucson, AZ 85706
tel: 520.889.9933
fax: 520.573.1944
email: info@roperscientific.com
web: www.roperscientific.com

3660 Quakerbridge Road Trenton, NJ 08619
tel: 609.587.9797
fax: 609.587.1970
email: info@roperscientific.com
web: www.roperscientific.com

ROPER SCIENTIFIC™
ACTON RESEARCH

Rev A0