Resonance Raman Spectroscopy at 257 nm Excitation of Normal and Malignant Cultured Breast and Cervical Cells

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The sensitivity and selectivity of UV-excited resonance Raman spectroscopy indicate that this technique may be useful in studying certain biochemical changes in cells, especially changes in DNA that occur during the development of cancer. To determine whether this technique can distinguish normal from malignant cells, we have measured UV resonance Raman spectra at 257.26 nm excitation of suspensions of normal and malignant cultured breast and cervical cells. Samples were excited with the use of an intracavity doubled argon-ion laser, and the spectra were recorded with a single grating spectrograph and a liquid nitrogen-cooled charge-coupled device. Cell spectra obtained closely resembled that of DNA, with peaks around 1330, 1480, and 1580 cm^{-1}, due to the nucleotide bases. In addition to these, the uracil base in RNA provides a peak at 1230 cm^{-1}. Strong tryptophan and tyrosine contributions appear in the 1520–1670 cm^{-1} range. The ratios of Raman spectral peaks 1480/1614 cm^{-1} and 1480/1540 cm^{-1}, which are sensitive to the concentration of nucleic acids relative to cell proteins, were found to be higher in malignant cells than in normal cells. Normal and malignant cells could also be differentiated by using the ratio at 1330/1480 cm^{-1}. This difference may be the result of decreased hypochromism due to changes in stacking of the purine bases. Changes in relative amounts of RNA may also contribute to this ratio. The results of this pilot study indicate that there may be significant differences in the UV resonance Raman spectra of normal and cancerous cells. These differences may be related to changes in nucleotide/protein concentrations in the cell, as well as changes in the vibrational structure of the nucleic acids associated with the malignant cell phenotype.

Index Headings: **UV resonance Raman spectroscopy; Cultured cells; Breast cancer; Cervical cancer.**

INTRODUCTION

Raman spectroscopy is increasingly being applied as a tool in understanding and diagnosing disease. Recent reviews summarize its application in the histochemical analysis of biological tissues[1] and in the detection of cancers and precancers.[2] When the Raman excitation wavelength corresponds to an absorption peak of a chemical group of interest, the Raman scattering from that group is enhanced by several orders of magnitude. This resonance enhancement adds increased sensitivity and selectivity to Raman spectroscopy.

In biological samples, excitation in the ultraviolet range from 200 to 260 nm selectively enhances the Raman signal from aromatic amino acids and the purine and pyrimidine bases of nucleic acids and proteins. UV resonance Raman spectroscopy (UVRRS) has been used extensively to probe the structure of these molecules[3] and their interaction with other compounds. UVRRS has been found to be sensitive to changes in the nucleotide base conformation, hydrogen-bonding state, and base stacking in polynucleotides[4] and DNA.[5] For example, in the polynucleotide poly(A–T), binding of the histone protein SPKK results in decreased hypochromism, i.e., increased UVRRS intensity of adenine peaks with respect to thymine peaks. There is also a change in the ratios of intensities of adenine peaks; specifically, the ratio of peaks at 1336/1481 cm^{-1} increases on binding SPKK.[4]

The sensitivity and selectivity of UVRRS indicate that this technique may be useful in studying changes in cell biochemistry, especially changes in DNA, associated with precancerous conditions and the development of cancer. For example, it has been found that there is an increase in the presence of OH-adducts to the DNA purine bases in precancerous and cancerous breast tissue, measured by using mass spectrometry.[6] Detecting and tracking the presence of this and other DNA adducts may be a useful marker for investigating cancer progression and for evaluating the success of treatment.[6,7] IR absorption spectroscopy is also sensitive to the these changes.[6] However, because of interference with water absorption, the DNA must be carefully extracted from the cells to be examined and dried before IR spectra can be recorded. UVRRS, on the other hand, through selective enhancement, isolates nucleic acids optically. This factor may permit the recording of UVRR spectra of nucleic acids directly from suspensions of whole cells, eliminating the need to isolate them chemically. Thus UVRRS may be useful as a rapid and relatively simpler method to monitor DNA adducts and other precancerous changes in cells.

UVRRS of whole cells, first reported in 1983, revealed that the UVRRS of a monkey kidney cell line at 257 nm excitation was very similar to that of DNA.[8] Several studies explored the use of UVRRS for bacterial taxonomy[9,10] and monitoring antibiotic efficacy.[11] These were recently reviewed.[12] It was found that UVRR spectra of bacteria at wavelengths from 200 to 231 nm were contributed to mostly by aromatic amino acids, such as tyrosine and tryptophan, and at 242 nm and 251 nm by the nucleic acids. With 242 nm excitation, the ratio of nucleotide peaks at 1530 cm^{-1}, due to cytosine, and 1485 cm^{-1}, due primarily to guanidine and adenine, was found to correlate with the A–T/G–C ratio in the cells. The ratio of peaks at 1485 or 1575 cm^{-1} due to nucleic acids, and at 1616 cm^{-1} due to protein aromatic amino acids, was

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found to be sensitive to growth phase, with increased nucleic acid peak intensity relative to protein peaks when the cell cultures were in the log growth phase compared to the stationary phase. Ratios of protein peaks, such as 1556/1616 cm^{-1}, and ratios of nucleic acid peaks, were found to be relatively independent of media and growth-phase factors.

With the use of continuous-wave (CW) excitation and more sensitive detectors, attempts have been made to couple UVRRS with microscopy to obtain single-cell spectra. Spectra have been obtained from a group of 20–50 bacteria cells in phosphate buffer solution (PBS) on a microscope slide with 257 nm excitation.[13] The spectra were identical to those of bacteria in bulk samples. UVRRS has also been used to obtain spectra *in vivo* from the DNA of a single cultured human mammary cell,[14] also at 257 nm.

We have developed a UVRRS instrument that combines a CW laser source and a highly efficient collection and detection system to characterize the UVRRS of normal and malignant human epithelial cell suspensions. We present the UV resonance Raman spectra at 257 nm excitation of normal and malignant cultured breast and cervical cells. We have identified specific ratios of nucleic acid peaks and protein peaks that differ significantly between normal and malignant cells. The clearest differences are the increased nucleotide-to-protein UVRRS ratio in malignant cells. A more subtle change observed is the increase in the UVRRS intensity in malignant cells of the purine peak at 1330 cm^{-1} with respect to overall nucleotides present, possibly due to changes in base-stacking of the purine bases. These results indicate that UVRRS of whole cell suspensions may be a useful tool in the study and monitoring of changes in cells associated with cancer.

METHODS

Samples. Breast epithelial cell suspensions were provided by the laboratory of Professor Kimberly Kline at the University of Texas at Austin. Cell lines used were MDA-MB435 (late-stage malignancy), MCF-7 McGuire (early-stage malignancy), and MCF-10A (nonmalignant immortalized cells).

Normal cervical cells (CrEc-Ec 4665) were from primary cultured normal cervical epithelium (mixed squamous and columnar cells) and were obtained from Clonetics Corporation. Cells were shipped in media at room temperature and then replated and grown in the laboratory of Professor Reuben Lotan at the University of Texas M. D. Anderson Cancer Center in Houston. Cervical cancer cells were SiHa and HeLa cell lines originally obtained from the American Type Cell Collection and maintained in the above laboratory. Spectra were recorded from two samples of MDA-MB435 cells, three samples of MCF-7 McGuire cells, two samples of MCF-10A cells, one sample of CrEc-Ec 4665 cells, one sample of SiHa cells, and one sample of HeLa cells.

Cells were harvested by trypsinization and washed three times in PBS and resuspended in PBS at concentrations of $2–6\times10^6$/mL. All cell samples were harvested while in the logarithmic phase of growth. Cells were tested for viability before UVRR spectra were recorded by using the trypan blue exclusion test. Viability of samples was 80% on average, measured immediately before each scan. Viability was measured on a test sample as a function of time after resuspension in PBS and as a function of UV exposure time and intensity, and no change in viability was observed at the limits used in this study. Spectra were recorded within 2–5 h after harvesting.

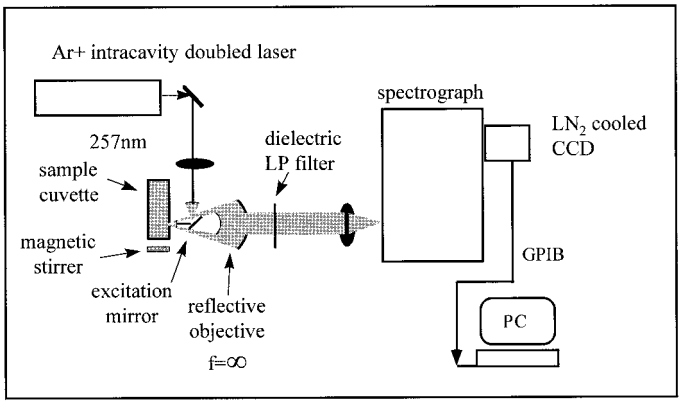

FIG. 1. Experimental setup used to record UVRR spectra.

Nucleotides and amino acids were obtained from Sigma-Aldrich Corporation, and suspensions were made in PBS and stored at room temperature. Sample concentrations were 2.5 mg/mL for calf thymus DNA, 15 mM for tryptophan, and 45 mM for tyrosine, and 14.2 mM for dUMP.

Instrumentation. The experimental setup is shown in Fig. 1. The excitation source is a FReD 300 Ar^+ laser (Coherent Corporation) with an intercavity beta barium borate (BBO) doubling crystal. The excitation power at the sample was 4–5 mW. Exposure time was 20 min for four samples (two breast normals, two breast early-stage) and 10 min for the other samples. The excitation beam spot was focused on the sample with a 60 μm full width at half-maximum (FWHM) beam diameter. Samples were held in a 1 × 1 cm fused-silica cuvette. In a step to avoid Raman scattering and fluorescence from the quartz cuvette, a small hole approximately 1.5 mm in diameter was made in the cuvette wall to create a window through which the spectra were recorded. The hole was small enough for the sample to be held in the cuvette by hydrostatic forces. Samples were stirred with a magnetic micro-stirrer at 900 rpm.

Scattered light was collected and collimated by using a reflecting objective with an effective numerical aperture (NA) of 0.5 (Ealing Electro-Optics). A second fused-silica lens was used to focus the collected light onto the entrance slit of a 500 mm spectrograph (Chromex Corp) with an aperture ratio of *f*/8.0. A 2400 groove/mm grating and 50 μm slit were used, resulting in a spectral resolution of 13 cm^{-1} at 257 nm. The detector used was a liquid nitrogen-cooled charge-coupled device (CCD) with UV-enhanced coating (Princeton Instruments). Raleigh scattering from the sample was blocked with an optical density (OD) greater than 3 with the use of custom dielectric filters (Barr Associates), which allowed transmission of more than 50% in the range of 600 to 2000 cm^{-1}.

Signal Processing. Spectra for all samples were first median filtered (filter width = 7 cm^{-1}) to remove narrow-

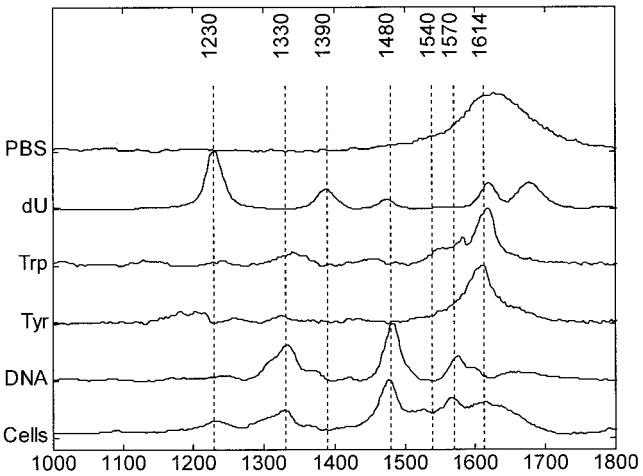

FIG. 2. UVRR spectra of cells and cell constituents. Symbols: Cells (early-stage malignant breast cells), Tyr (tyrosine), Trp (tryptophan), dU (deoxyribouridine monophosphate), PBS (phosphate buffer solution).

bandwidth "cosmic ray" peaks. Next, very broad spectral peaks and the baseline were removed by using an automated routine that calculated and subtracted the moving average of the minimum value function of the spectra within a 140 cm^{-1} window. Wavenumber calibration was performed by using a linear fit to three known peaks (314, 459, 2249 cm^{-1}) of a 1:1 carbon tetrachloride/acetonitrile standard solution. Spectra were then normalized to the peak at 1480 cm^{-1}.

RESULTS AND DISCUSSION

Spectra for a typical cell sample (early-stage breast malignant), DNA, dUMP, tryptophan, tyrosine, and PBS are shown in Fig. 2. Cell spectra closely resemble those of DNA, in addition to the uracil RNA peak at 1230 cm^{-1}. Tryptophan and tyrosine make strong contributions in the 1520–1670 cm^{-1} range with peaks at 1614 cm^{-1}, and weaker contributions in the range of 1360–1420 cm^{-1}. PBS exhibits a strong broad-band water contribution centered around 1630 cm^{-1}. There are no peaks present that are associated with fused silica, which has weak peaks around 1050 and 1180 cm^{-1} in addition to stronger peaks below 1000 cm^{-1}.

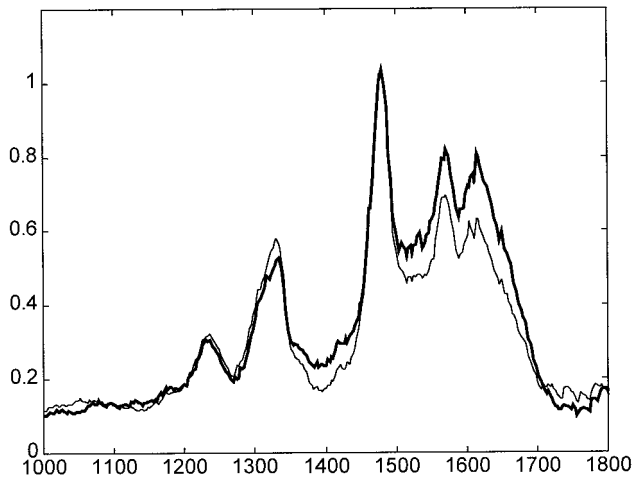

FIG. 3. UVRR spectra of normal and malignant breast cells.

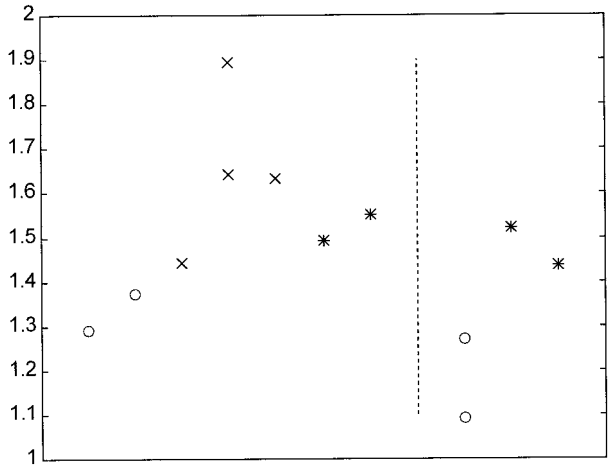

FIG. 4. Ratio of UVRRS peaks at 1480/1614 cm^{-1}. Symbols: (o) normal breast or cervical cells; (x) early-stage malignant breast cells; (*) late-stage malignant breast cells or cervical cancer cells. Vertically aligned symbols represent repeat measurements of the same sample. The first (from the left) cervical malignant sample symbol is for the SiHa cells, and the second for the HeLa cells.

Typical normal and early-stage breast sample spectra are shown in Fig. 3, both normalized to the nucleotide peak at 1480 cm^{-1}. With respect to this peak, the normal cell spectrum is more intense in the 1500–1700 cm^{-1} range and in the 1330–1440 cm^{-1} range. The malignant spectrum is greater around the 1330 cm^{-1} peak.

A plot of the ratio of the highest nucleotide peak and the highest protein peak, 1480/1614 cm^{-1}, is shown for all samples in Fig. 4. All malignant samples showed a higher value of this ratio than the normal samples. The ratio at 1480/1540 cm^{-1} is shown in Fig. 5. In the region around 1540 cm^{-1}, nucleotide contributions are very low, while there is noticeable protein contribution; thus this ratio represents another measure of nucleotide to protein concentrations. This ratio also shows a clear increase in malignant cells.

The UVRRS peak ratio at 1330/1480 cm^{-1} is plotted in Fig. 6. This ratio is known to be sensitive to changes in nucleotide base stacking.[4] For the breast cells, this ratio

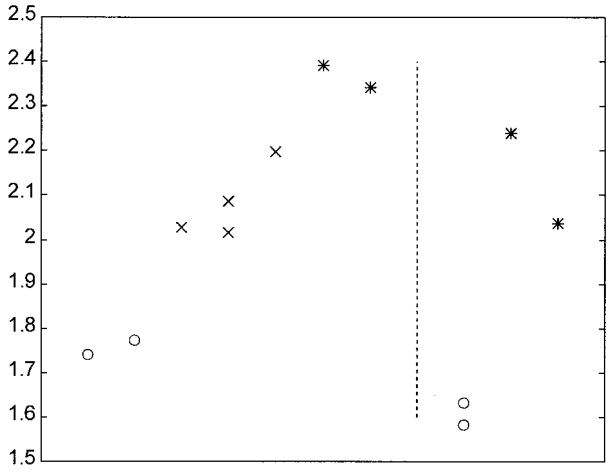

FIG. 5. Ratio of UVRRS peaks at 1480/1540 cm^{-1}. Symbols to the left of the dotted line are for breast cell samples; those to the right are for cervical cell samples. Symbols: same as those in Fig. 4.

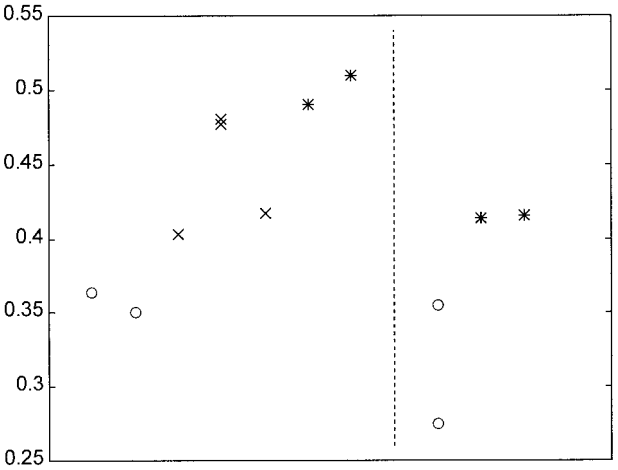

FIG. 6. Ratio of UVRRS peaks at 1330/1480 cm^{-1}. Symbols: same as those in Fig. 4.

showed a clear increase in malignant cells over nonmalignant cells. This trend, to a lesser extent, was also present in the cervical cell samples. The 1480/1614 cm^{-1} and 1480/1540 cm^{-1} ratios indicate an increase in the nucleic acid-to-protein ratio, as expected in malignant cells.[15] The peak at 1614 cm^{-1} is mostly due to tryptophan and tyrosine, but there is also some contribution from the nucleotides, especially uracil. The 1540 cm^{-1} region, on the other hand, is almost entirely due to proteins. This result may explain why the latter ratio showed a more clear differentiation between normal and malignant cells.

The UVRRS peak ratio at 1330/1480 cm^{-1} in Fig. 6 shows an increase in malignant breast cells. The trend with this ratio was also present to a lesser extent in the cervical cell samples. The purine bases, adenine and guanine, contribute mostly to both peaks, with very little contributions from the pyrimidines at this excitation wavelength (data not shown). Decreased base stacking of the adenine nucleotide has been shown to increase the intensity of UVRRS peaks of this base overall, due to decreased hypochromism, and to increase the ratio of intensities at 1330/1480 cm^{-1}.[4] However, the relationship of the change in this ratio to changes in nucleotides associated with malignancy is not clear.

CONCLUSION

Differences were observed between the UVRR spectra of nonmalignant and malignant breast cell lines with the use of 257 nm excitation. The clearest differences are consistent with an increase in the DNA/protein ratio in malignant cells, as expected. Also observed is a change in the purine UVRR scattering consistent with decreased hypochromism due to changes in the base stacking of the purine bases. These changes were also observed in normal and malignant cervical cells. The results of this pilot study indicate the possibility of the application of UVRRS in cytological screening for cancer, and as a tool in monitoring the presence of changes in DNA and RNA associated with increased cancer risk. It should be noted that, due to the small number of samples used and the extent of differences found, these conclusions must be considered to be of a preliminary nature. Further studies that incorporate additional samples and other excitation wavelengths are needed. The 240–245 nm excitation range is of particular interest. Also, an examination of a wider spectral range, from 200 to 3500 cm^{-1}, may provide further insight.

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