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Monitoring the Mode of Action of Antibiotics Using Raman Spectroscopy: Investigating Subinhibitory Effects of Amikacin on *Pseudomonas aeruginosa*

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During the last 20 years the rate at which new antimicrobial agents are produced has decreased dramatically, with concomitant increase in the number of pathogens that are becoming multidrug resistant. Together these have created a patient healthcare risk and this is of great concern. A crucial aspect for the discovery of new antibiotics is the development of new techniques that allow rapid and accurate characterization of the mode of action of the pharmacophore. In this work UV resonance Raman (UVRR) spectroscopy has been developed to monitor the concentration effect of antibiotics on bacterial cells. UVRR was conducted at 244 nm and spectra were collected in typically 60 s. Supervised multivariate analysis and 2D correlation spectroscopy were used to evaluate whether the UVRR spectra contained valuable information that could be used to study the mode of action of antibiotics. The clustering pattern in the discriminant factors space correlated directly to the concentration of amikacin, and partial least squares (PLS) regression analysis of the UVRR spectra was able to predict the concentration of amikacin to which bacterial cells had been exposed. 2D correlation spectroscopy contour maps indicated that spectral changes due to the presence of amikacin in the growth media occur according to the known mode of action of the studied antibiotic. Therefore, we conclude that UVRR spectroscopy, when coupled with chemometrics and 2D correlation spectroscopy, constitutes a powerful approach for the development and screening of new antibiotics.

The modern era of antimicrobial chemotherapy began in 1929 with Fleming's discovery of penicillin and with Domagk's discovery in 1935 of synthetic chemicals with broad antimicrobial activity.[1] The use of these antimicrobials and others that were soon discovered has resulted in a major decline in the incidence of life-threatening infections in the developed world. However, the inappropriate use of these drugs seems to be seriously threatening our ability to cope with various common infections. The frequency with which antibiotics are currently prescribed, the use of broad-spectrum antibacterial agents, and the ability in some countries to purchase antibiotics over the counter have resulted in the continuous alarming rise of multidrug resistance among many pathogens. Indeed, resistant organisms to Fleming's penicillin arose within a few years of its widespread use. As a result, certain bacteria that were thought to be under control are starting to be seriously life threatening, especially to those who are immunosuppressed, and include *Staphylococcus aureus* and enterococci that are resistant to all known antibiotics, including vancomycin;[2,3] pseudomonads, which are increasingly acquired in hospitals; and, perhaps most worryingly, the increase in multidrug resistant *Mycobacterium tuberculosis*.[4,5]

Some initiatives to prevent emergence of resistance have concentrated on reducing the indiscriminate use of antibiotics in medicine and agriculture.[6] Others are trying to limit the use of broad-spectrum antibacterial agents. Still, these efforts will only delay the inevitable build up of resistance. Experts seem to agree that the only solution is to continue developing new drugs.[7] However, despite the critical need for new antibiotics, the development of these is declining; pharmaceutical companies seem to have cut their research on antimicrobial drugs, which has resulted in a 56% decrease in new antibacterial agents over the past 20 years.[8] Furthermore, only two novel modes of action have been discovered in the last 6 years, the only new antibiotics being a combination of, or subtle modifications of, previously existing ones.[8] To stimulate antimicrobial research, novel approaches are needed that can rapidly and reliably allow full understanding of mode of action.

Recently, to aid management of infection, many researchers have spent much effort in developing new physicochemical methods for whole-organism fingerprinting. As a result, techniques such as mass spectrometry, FT-IR spectroscopy, and Raman microspectroscopy (with excitation in the visible, near-infrared and ultraviolet)[9−13] have been proven very successful in rapidly discriminating and identifying microorganisms. The next step

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forward in antimicrobial research may be to explore whether these techniques could also be exploited for studying the mode of action of antibiotics.

In the present study, UV resonance Raman (UVRR) spectroscopy (with excitation wavelength of 244 nm) was investigated as a novel approach for the screening of new antibacterial agents. The nature of the UV resonance Raman effect introduces selectivity of UV-absorbing molecules.[14] Hence, excitation of bacteria at wavelengths between 222 and 257 nm has been shown to result in spectra dominated by the Raman bands of nucleic acids and aromatic amino acids.[15−18] Common targets of antimicrobial drugs are protein and nucleic acid synthesis related processes; thus, it is not unreasonable to expect that the UVRR spectra may yield valuable data to study the mode of action of a wide variety of antibiotics. Therefore, one of the aims of this study is to explore this possibility.

MATERIALS AND METHODS

Antibiotics and Microorganisms. *Pseudomonas aeruginosa* PAO1 was used as a model organism; this was chosen because *P. aeruginosa* has the ability to become drug resistant and is increasingly being associated with hospital-acquired infections. A commercial preparation of amikacin, an antibiotic belonging to the aminoglycosides group that is in clinical use, was purchased from Sigma Chemical Co.

P. aeruginosa PAO1 minimum inhibitory concentration (MIC) to amikacin was estimated with diffusion gradient plates[19] after 18 h incubation period and was found to be 6.5 mg/L. Bacterial cells were grown in the presence of different subinhibitory concentrations of amikacin (0 to 6 mg/L), incubated at 37 °C for 18 h, and were harvested from axenic (pure) cultivations on LabM blood agar base plates (without blood). Cells were cultured on 72 individual Petri dishes to give three biological replicates of the biomass grown in the presence of each antibiotic concentration (0, 0.25 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 5, 5.25, 5.5, 5.75, and 6 mg/L). The biomass was carefully removed from the Petri dishes, washed with sterile distilled water, and resuspended in 400 μL of sterile distilled water. An aliquot (15 μL) of the suspension was then spotted onto a CaF_2 substrate and air-dried at room temperature to form a uniform semitransparent film suitable for UVRR measurements. UVRR spectra were collected immediately after sample preparation to avoid sample degradation in the desiccated state.

UV Resonance Raman Spectroscopy. Raman spectra were collected as described elsewhere.[13] Briefly, Raman scattering was exited with a UV 244 nm frequency-doubled argon ion laser (Lexel Laser Products-Cambridge Laser Laboratories) with the power at the sampling point typically at ~0.5 mW. Bacterial samples that were dried onto CaF_2 substrates were spun under the laser beam to prevent sample photodegradation. Spectra were collected for 1 min. Spectral resolution was ~8 cm^{-1}. After calibration with diamond, the wavenumber accuracy is estimated to be ±1 cm^{-1}. All data were exported from the GRAMs WiRE software used to control the spectrometer into Matlab (The Mathworks, Inc. Natick, MA) for data analysis.

Data Analysis. Spectral preprocessing included baseline removal and a denoising routine. The baseline of each spectrum was approximated by a fifth-order polynomial, and this polynomial contribution was subtracted from each raw spectrum, resulting in flat baseline spectra.[20] Spectra were smoothed using a Savitzki−Golay (polynomial) smoothing filter with an 11-point-wide (approximately 44 cm^{-1}) window and a second polynomial.[13] To account for cell density variation in the sample film, the data were then normalized so that the sum of photons from 650 to 1800 cm^{-1} = 1.

To investigate the relationship between the UVRR bacterial spectra and the effect of the antimicrobial agent on the *P. aeruginosa* cells, multivariate statistical methods were used and these included cluster analysis, linear regression, and 2D correlation analyses.

Initially cluster analysis was carried out in two steps. (1) Principal components analysis (PCA)[21,22] was employed to reduce the dimensionality of the UVRR data while preserving most of the variance. PCA was performed using the NIPALS algorithm.[23] (2) Discriminant factor analysis (DFA) was then used to discriminate between groups on the basis of the retained principal components (PCs) and the a priori knowledge of which spectra were biological replicates (i.e., the three spectra from the three cultures for each antibiotic concentration).

Partial least squares (PLS) regression,[24] a quantitative spectral decomposition technique generally used for predictive linear modeling, was employed for modeling the relationship between the spectral features of *P. aeruginosa* and the conditions of growth, viz. the variable concentration of amikacin. PLS was performed as detailed in ref 25 following the computations given in ref 24.

Multivariate statistical methods are valuable and popular methods for the analysis of spectral data from biological systems; nonetheless, these methods cannot provide information regarding the dynamic spectral changes due to an external perturbation of the system. In this respect, generalized two-dimensional (2D) correlation spectroscopy,[26] a cross-correlation method, has already been found to be especially useful for monitoring dynamic fluctuations of spectral signals, and many applications have been

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reported. Those include composition-induced structural changes,[27] temperature-dependent spectral variations,[28−30] and concentration-dependent spectral changes,[31] as well as 2D near-infrared (NIR) and mid-infrared heterospectral correlation analysis on protein structure.[32]

Consequently, to study the bacterial changes reflected in the UVRR spectra as the concentration of antibiotic is increased, 2D UVRR correlation spectra were constructed. The generalized two-dimensional correlation method described by Noda[26] was used, and calculations were performed in Matlab using code downloaded from http://sci-tech.ksc.kwansei.ac.jp/~ozaki/Main-eng.htm. The one-dimensional spectra used for the 2D correlation analysis are the average of the three biological replicates for each antimicrobial exposure. The average baseline corrected spectrum of bacterial cells not exposed to antibiotic was used as a reference.

The synchronous 2D correlation spectra are characterized by autopeaks along the diagonal and cross-peaks off the diagonal for bands that exhibit (at least partially) correlated (similarities in) dynamic behavior. In turn, the asynchronous 2D correlation spectrum contains only cross-peaks that reveal out of phase (dissimilar) behavior of two bands. Peaks that do not change their intensities throughout the series will not appear in the 2D spectra. According to the correlation rules established by Noda,[26] in a synchronous spectrum, a positive cross-peak at (ν1, ν2) will indicate that the intensity changes at these two wavenumbers (Raman shifts) are in the same direction. A positive asynchronous cross-peak at (ν1,ν2) will indicate that the spectral change at ν1 occurs earlier and at lower antibiotic concentration compared to ν2. A negative asynchronous cross-peak indicates the opposite. In the correlation contour maps, shaded regions indicate negative correlation intensities.

RESULTS

Figure 1 shows typical spectra of *Pseudomonas aeruginosa* cells that have been grown in both the absence and presence of antibiotic. [Note that bacterial spectra were collected by rotating the sample (see Materials and Methods), and therefore, the Raman spectra depicted in Figure 1 are likely to represent the spatial averaging of the cells rather that the spectra of a single cell.] The main bands in UVRR bacterial spectra are mainly due to the vibrations of nucleic acid and protein components of the cells and, as a result, spectra from bacteria belonging to different genera and species are very similar. Thus, as expected, the spectra presented here, are in good agreement with previously published spectra.[13,33,34] Accordingly, spectra shown in Figure 1 (and certainly all the spectra acquired) appear to be *qualitatively* very similar. However, on closer inspection, *quantitative* differences can be

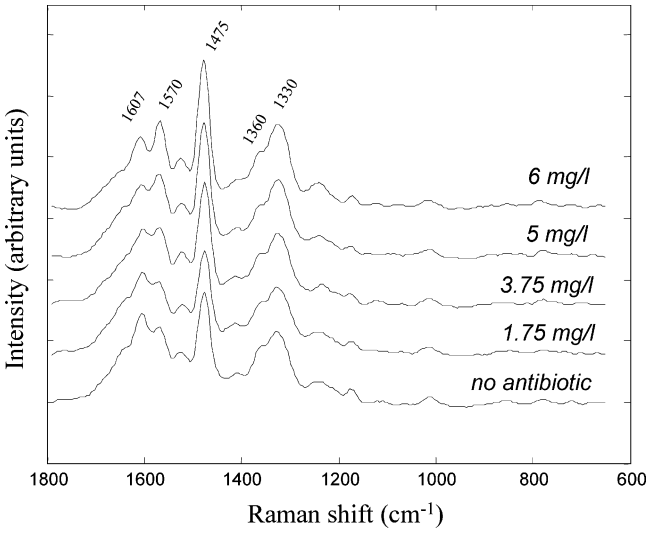

Figure 1. Typical UVRR spectra from *P. aeruginosa* PAO1 grown in the presence of various amikacin concentrations. The spectra are processed as detailed in the text.

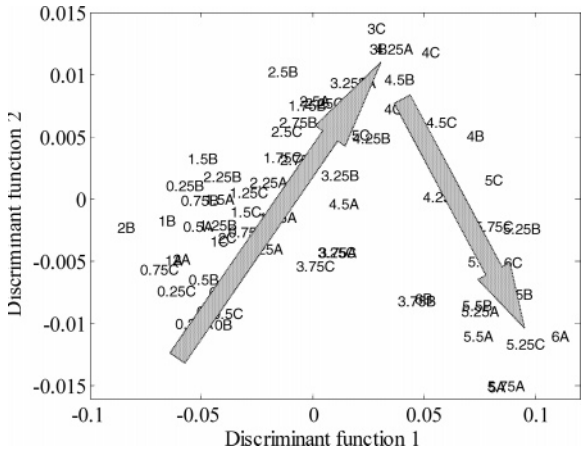

Figure 2. PC−DFA plot showing the relationships between the spectra of the bacterial cells exposed to different concentrations of antibiotic. 10 PCs that contained 90% of the explained variance were used to construct the ordination plot. The arrow is drawn as a visual guide and relates to the concentration of the antibiotic. A, B, and C are three repeat experiments.

seen, note particularly the changes in the 1600-cm^{-1} region, which can be ascribed to tryptophan and tyrosine.[13,17,34] We attempted to collect an amikacin spectrum at the concentrations used in this work (0−6 mg/L); however, no UVRR spectrum was recovered and so its contribution was considered negligible (data not shown).

Initial analysis of the bacterial spectra was carried out by PC−DFA as described above, where the first 10 PCs (which accounted for 90.0% of the explained variance) were used by the DFA algorithm, with knowledge of the biological replicates. The resultant PC−DFA ordination plot is shown in Figure 2. As can be clearly seen, the spectra of cells exposed to low concentrations of antibiotic cluster in the same region (lower left corner), and as the concentration of antibiotic increases, the cluster spreads from left to right, as depicted in Figure 2. Note that the first discriminant function is extracted to explain the largest difference between groups; it is therefore encouraging that this relates directly to the concentration of amikacin. Note that the "inflexion" approximately coincides with the subinhibitory concentrations

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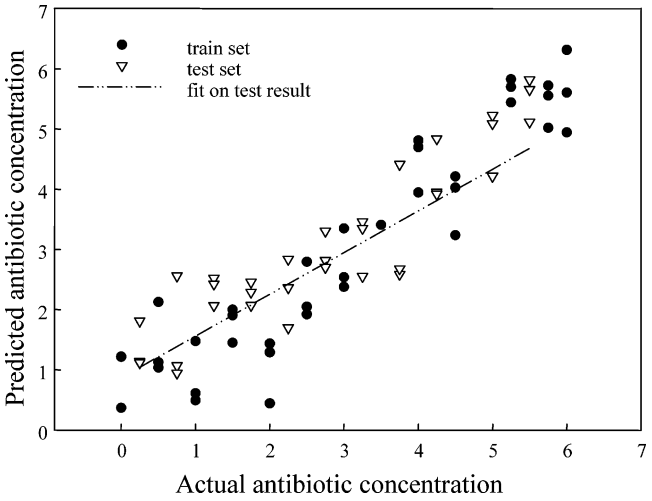

Figure 3. Estimates from PLS versus the true concentration of antibiotic present in the growth media. The calculated fit on the test set results in $y = 0.69x + 0.87$ with $R^2 = 0.78$. Root mean square errors of 0.6 and 0.5 were found for the calibration and validation, respectively.

3.75−4 mg/L of amikacin. It seems that the discriminant analysis is able to indicate a prominent change in the effect of the antibiotic on the bacterial cells at concentrations above 4 mg/L. Interestingly, the MIC at 12 h was estimated to be 4 mg/L.

This result strongly suggests that these UVRR spectra contain biochemical information that will allow for the correlation of pertinent bacterial spectral features with the concentration of amikacin present in the media. Consequently, supervised learning analyses using PLS regression was used to quantify the level of amikacin. Data were split into a training (or calibration) set and a test (or validation) set. The training set contained the replicate spectra of bacteria grown in the presence of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5.25, 5.75, and 6 mg/L of antibiotic, while the test set contained UVRR spectra from cells grown under the influence of 0.25, 0.75, 1.25, 1.75, 2.25, 2.75, 3.25, 3.75, 4.25, 5, and 5.5 mg/L of the antimicrobial agent. PLS regression was calibrated with the training set and cross-validated with the test set. It was found that the best model (i.e. lowest prediction error in the test set) occurred when three latent variables were used. The plot of the PLS estimates versus the known concentration (Figure 3) shows relatively good predictions for both the training data and the test set.

To determine whether a particular spectral region is used for the discrimination and quantification models, the PC−DFA and PLS loadings were calculated and plotted against Raman shift (Figure 4). It appeared that the PC−DFA loadings predominantly used the Raman peak at 1607 cm^{-1}, which is negatively correlated to the antibiotic concentration. By contrast, the PLS loadings showed many variables that were positively and negatively correlated with amikacin concentration. While no single band was preferentially selected, on closer inspection the band at ~1607 cm^{-1} was negatively correlated, as also shown by the PC−DFA loadings. As discussed above, this band can be attributed to the protein content in the cells;[13,17,34] however, PLS and PC−DFA cannot readily give details on the *dynamic* nature of the spectral changes over the full antibiotic range studied.

Therefore, to explore further the potential of UVRR spectroscopy to monitor the mode of action of amikacin, 2D correlation

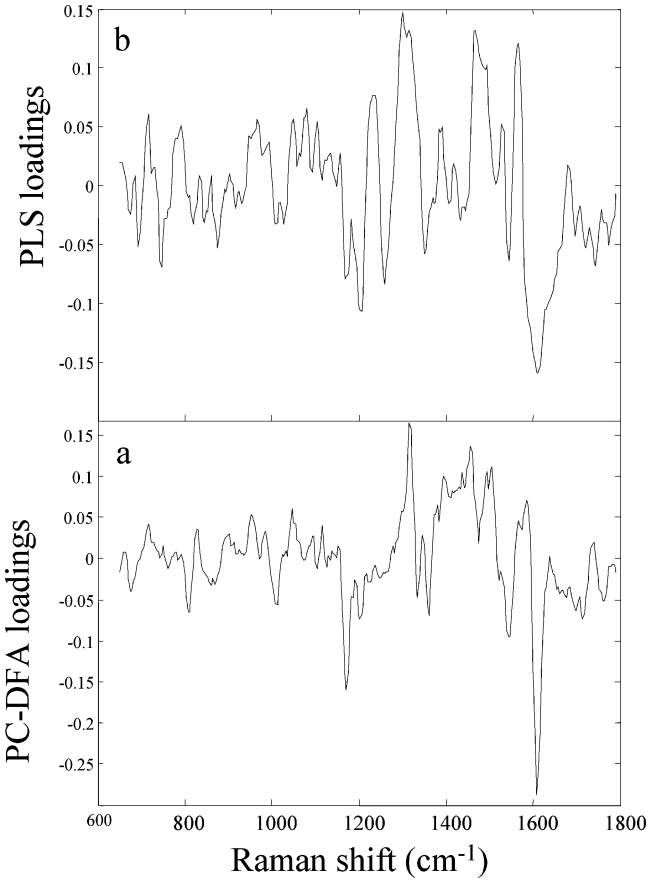

Figure 4. Loadings plots for (a) PC−DFA and (b) PLS.

spectra were calculated. In the synchronous spectrum (Figure 5a), three autopeaks are observed at ~1607, 1475, and 1325 cm^{-1}, indicating that the intensities of these bands change with the increase of amikacin concentration. The band at ~1607 cm^{-1} has been attributed to the vibrational mode of the amino acids tyrosine and tryptophan; thus, it can be considered a measure of the protein content of the cells. By contrast, the band at ~1475 cm^{-1} may be associated to the nucleic acid composition of the cells, since it is typically attributed to vibrations of the nucleotide bases adenine and guanine.[13,17,34] The band at ~1325 cm^{-1} is mainly due to adenine, but it also has a small contribution from tyrosine.[13,17,34]

Several cross-peaks appeared in the synchronous contour map. The shape of the autopeak at ~1607 cm^{-1} may indicate that there is a cross-peak at (1600, 1640) cm^{-1}. The region around 1640 cm^{-1} corresponds to the shoulder of the more prominent protein band at 1607 cm^{-1} and results from weak contribution from thymine,[13] tryptophan,[35] and the amide I;[36] its change in intensity may only reflect the fact that the protein peak (~1607 cm^{-1}) decreases dramatically with the increase of amikacin. Correlation analysis in the spectral region comprised around 1475 and 1600 cm^{-1} resulted in two negative cross-peaks at (1475, 1640) and (1475, 1620−1590) cm^{-1} and a positive one at (1475, 1570) cm^{-1}. The ~1620−1590 range corresponds to the spectral region around the protein band and the ~1570 cm^{-1} peak has been attributed to adenosine and guanine.[13,17,34]

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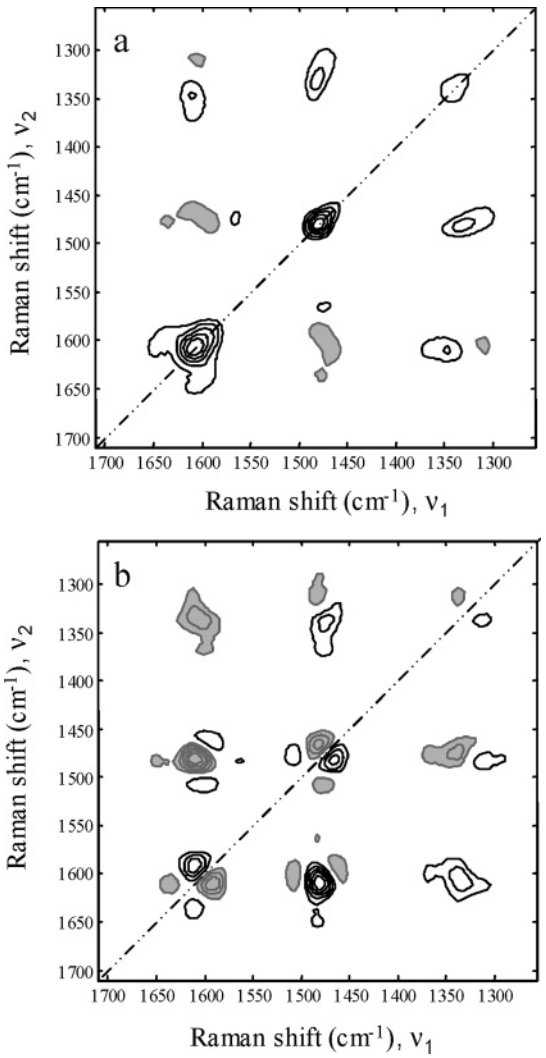

Figure 5. (a) Synchronous and (b) asynchronous correlation spectra for the bacterial cells exposed to antimicrobial concentrations ranging from 0 to 6 mg/L. The shaded area indicates negative intensity.

In addition, three other cross-peaks can be seen in the 2D correlation spectrum: (1303, 1475), (1303, 1607), and (1350, 1607) cm^{-1}. Raman shifts at ~1303 and 1350 cm^{-1} do not appear in the one-dimensional spectra as bands; rather, they both are around the edges of the prominent band at ~1325 cm^{-1}. Interestingly, they may be attributed to adenosine[37,38] with weak contributions from tyrosine and tryptophan,[13,36] respectively. The sign of the observed cross-peaks indicated that the bands at ~1303, 1325, 1570, and 1475 cm^{-1} increase together while the band at 1350 cm^{-1} and the shoulder at ~1640 cm^{-1} decrease with the band at 1607 cm^{-1}. Therefore, the 2D correlation synchronous spectra seems to indicate that, as the concentration of amikacin increases, the peaks ascribed to the protein content of the cell (~1350 and 1607 cm^{-1}) decrease, whereas the nucleic acid related bands (~1303, 1570, and 1475 cm^{-1}) increase.

In Figure 5b the asynchronous plot is shown. Two distinctive cross-peak arrangements that correspond to the synchronous peaks at ~1607 and 1475 cm^{-1} can be observed close to the diagonal, possibly revealing either a shift of one band or the correlated behavior of two heavily overlapped bands.[39,40] The pattern seen around the 1600 cm^{-1} region may indicate a shift, and close visual inspection of the one-dimensional spectra corroborated that, as the concentration of amikacin was increased, the peak at ~1607 cm^{-1} not only decreased in intensity but also shifted to higher wavenumbers (approximately by 3 cm^{-1}).

In contrast, close visual inspection of the one-dimensional spectra did not indicate a shift of the band at ~1475 cm^{-1} (data not shown). It has been suggested that, when it is not clear whether a pattern of this characteristics is due to shift or heavy overlapping of bands, the second derivative may be helpful;[39,40] thus, when the second derivative spectrum shows one peak, the pattern indicates a band shift, and two bands in the second derivative spectrum indicate the existence of two overlapping bands. However, if the band resulting from overlapping bands is wide enough (~50 cm^{-1}), the second derivative yields only one peak and it is not possible to clearly decide whether the observed pattern is indicating shift or overlapping.[39,40] In the case of the asynchronous cross-peaks observed at ~1475 cm^{-1}, no definite answer was obtained from visual inspection or from second derivative analysis (data not shown). Given that this band is thought to be composed of guanine and adenosine,[13,17,34] it may be that the observed pattern in the asynchronous plot is due to different rate intensity changes of these two bands. In addition, the cross-peaks in the asynchronous spectra indicate that intensity changes of the main observed bands most likely occur in the following order: 1475 cm^{-1} occurs before 1325, and 1350 cm^{-1} before 1607 cm^{-1}.

DISCUSSION AND CONCLUSION

Aminoglycosides are well-known antibacterial agents whose mechanism of action has been studied extensively. It has been reported that the initial action of these antibiotics is to create fissures in the outer membrane,[41,42] although their major target is the bacterial ribosomal RNA. Binding to the rRNA results in inhibition of translation and amino acid misincorporation; [43,44] thus, aminoglycosides inhibit protein synthesis. Thus, the action of amikacin has a preferential effect on the protein content and also on the nucleic acid content of the cell, and considering the nature of the UVRR spectra, it was hoped that the mode of action of amikacin could be monitored.

Multivariate analyses on the UVRR spectra presented have clearly demonstrated that these types of data indeed contain valuable information for the study of antimicrobial agents. In particular, it has been shown that PC−DFA related well with the concentration of amikacin and that the loadings plot showed that the prominent spectral feature is the protein band at 1607 cm^{-1}. By contrast, when PLS was used to estimate the concentration of amikacin that *P. aeruginosa* was exposed to, the spectral information used was spread over the entire spectral space, although the

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tyrosine and tryptophan band was negatively correlated to amikacin level. As described above the main target of amikacin is protein synthesis; therefore, it is perhaps not surprising that both PC−DFA and PLS use the prominent protein region to build the discriminatory and quantification models.

Two-dimensional correlation analysis indicated that, as the concentration of amikacin was increased, the protein-related peaks decreased, whereas the intensity of nucleic acid bands increases. Again this fact correlated well with the mode of action of amikacin. As the protein synthesis is inhibited, the nucleic acid content of the cell increases, and as a result, the intensity of protein and nucleic bands changes in opposite directions. Furthermore, the overall increase of the nucleic acid bands intensity may be partly due to less absorption of scattered light by tryptophan and tyrosine.[17] Contour maps have also revealed spectral features not readily noticeable in the conventional one-dimensional spectra; those are the shift of the ~1607 cm^{-1} band and the change of intensity at different rates of the heavily overlapped bands from adenosine and guanosine. The shift of the protein band is consistent with the fact that, as less protein is being synthesized and amino acids are being misincorporated into proteins, the molecular environment of the aromatic amino acids changes, resulting in a subtle wavenumber shift of their major band. We have also shown that the 2D correlation method can probe the specific order of spectral changes taking place as the concentration of amikacin is varied, and it seems that the nucleic acid bands were affected at lower concentrations than the protein-related peaks, which again is correlated to the action of amikacin.

The work presented here clearly demonstrates that UV resonance Raman, together with appropriate data analysis techniques, can be used as a tool for quantitatively characterizing the mode of action of amikacin, and the results are entirely consistent with its known mode of action. In the future we wish to extend this study and screen more antibiotics and microorganisms. In conclusion, we believe that UVRR, when combined with chemometrics and 2D correlation spectroscopy, presents itself as a powerful method for the characterization of new antimicrobial agents.

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