Improvements in the Generation of Quasi-Continuous, Tunable Ultraviolet Excitation for Raman Spectroscopy: Applications to Drug/Nucleotide Interactions*

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We demonstrate the generation of quasi-continuous ultraviolet excitation at wavelengths shorter than 240 nm using the frequency-doubled output of a synchronously pumped picosecond dye laser operating in the region of 420 nm. Owing to the relatively low peak power but high average power of the system, we obtain high-quality resonance Raman spectra at wavelengths as short as 209 nm. We apply the source to the study of the binding of anticancer drugs to DNA. In particular, we compare the binding of cis-diaminedichloroplatinum (II) (cisplatin) and cis-diammine-1, 1-cyclobutane dicarboxylate platinum (II) (carboplatin) to certain mononucleotides.

Index Headings: Lasers, picosecond; Nucleic acids; Raman spectroscopy; spectroscopic techniques.

INTRODUCTION

Resonance Raman spectroscopy with ultraviolet excitation has been demonstrated to be a valuable method for the study of biological molecules.¹ Ultraviolet resonance Raman spectroscopy (UVRR) offers improved sensitivity and selectivity, as has been shown in investigations of such biological systems as DNA,²,³ hemoglobin,⁴ and aromatic amino acids.⁵ Following the pioneering work of Ziegler and Hudson,⁶ the extension of laser sources into the deep UV region has opened new areas of study. For example, in DNA it is now possible to probe a number of different electronic transitions of the purine and pyrimidine bases.⁷,⁸ Pronounced changes in band intensities are observed, permitting further characterization of these electronic transitions.

We are using UVRR to investigate the interaction of anticancer drugs with DNA. In particular, certain coordination complexes of heavy metals have been applied to the treatment of cancer, as the metal centers are known to initiate chemotherapeutic activity by binding to DNA within cells. A variety of analytical techniques have been used to contribute to the understanding of the binding process of heavy metals to nucleotides, including NMR spectroscopy,⁹,¹⁰ circular dichroism,¹¹ differential-pulse polarography,¹² and x-ray diffraction.¹³ Because of the significant structural information that Raman spectroscopy provides and the high sensitivity available from resonance enhancement, UVRR has proven to be a valuable method for exploring complexation reactions between heavy metals, such as platinum, and nucleic acids. In particular, the widely used antitumor drug cis-diaminedichloroplatinum (II) (cisplatin) is known to bind preferentially to the N7 position of guanine residues along the major groove of DNA.¹⁴,¹⁵ The perturbations to the vibrational motions that arise from the binding process have been studied with UVRR. These investigations have confirmed that N7 of GMP is the preferred binding site and have established the order of reactivity in competitive reactions between the four nucleoside monophosphates with cisplatin as being GMP > AMP > CMP > UMP with Raman difference spectrophotometric measurements.¹⁶

In addition to its anticancer properties, cisplatin is cytotoxic to normal DNA, which has limited its clinical application. Efforts to synthesize analogues of cisplatin having similar or better chemotherapeutic abilities but reduced toxicities have led to the development of second-generation drugs including cis-diammine-1,1-cyclobutane dicarboxylate platinum (II) (carboplatin). Carboplatin has proven to be as effective as cisplatin against ovarian and testicular tumors, but is less toxic (i.e., nephrotoxicity and nausea are decreased).¹⁷,¹⁸ It also appears to be active against certain cancers that have minimal response to cisplatin,¹⁹ as well as tumors that may have become resistant to cisplatin.¹⁹ Carboplatin has recently been approved by the FDA. It is of both fundamental and practical interest to determine the molecular level interactions that cause the biological activity of carboplatin, and to compare and contrast its activity with that of cisplatin.

A typical UVRR experiment involves the use of a high-power, low-repetition-rate laser to generate the deep UV excitation.²⁰,²¹ High-power pulsed sources are not ideal for the Raman experiment; photochemical transients and saturation effects limit the average power that can impinge on the sample.²²,²³ Alternatively, the frequency-doubled, cavity-dumped, synchronously pumped dye laser has proven to be a convenient source of quasi-continuous, tunable, UV excitation for Raman spectroscopy.²⁴ The advantages of this high-repetition-rate (MHz) system are the relatively low peak power to avoid photochemical transients and saturation effects and the ability to focus the source tightly and collect the resultant scatter efficiently. However, the relatively low peak pow-

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† We dedicate this paper to the memory of Professor R. Stuart Tobias, a true gentleman/scholar, whose creativity provided the original motivation for this work.
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APPLIED SPECTROSCOPY
er of the system limits the power at the second harmonic and restricts the wavelength regions that are accessible. We have demonstrated that these limitation can be significantly improved by amplifying the picosecond pulses at MHz repetition rates using a cavity-dumped argon-ion laser. The increased peak power increases the average power available and it improves the wavelength coverage by providing for more efficient nonlinear frequency conversion. Using this system we have demonstrated the generation of quasi-continuous, tunable excitation in the 200-nm region with application to Raman spectroscopy. We used sum frequency mixing in beta barium borate ($\beta$-BaB$_2$O$_4$, BBO) to combine the fundamental and second harmonic from an MHz amplified synchronously pumped dye laser to generate UV radiation. While this arrangement provides a UV source down to 197 nm, it has the disadvantages of a limited tuning range and relatively low laser powers, because it depends on the cube of the laser intensity ($I^3$). The $I^3$ dependence also results in greater fluctuation in the average power of the output. It would be desirable to have a quasi-continuous source for UVRR that does not have these limitations. In this paper, we demonstrate the generation of quasi-continuous, tunable UV radiation in the region of 210 nm using the frequency-doubled output of a tunable synchronously pumped dye laser operating in the region of 420 nm. We obtain the UVRR spectra at ~209 nm of nucleotide monophosphates in the presence of carboplatin and cisplatin.

EXPERIMENTAL

We have previously described the basic design of our MHz laser system. In the present work, we have made several modifications to the system. A cw mode-locked Nd:YAG laser (Coherent, Inc., Model Antares 76s) is frequency doubled and tripled to pump a synchronously pumped cavity-dumped dye laser (Coherent, Inc., Model 702-2). The output power at 532 nm is ~3 W and at 355 nm is ~900 mW. Using either 532-nm or 355-nm excitation we can obtain tunable radiation from ~400 nm to ~950 nm. For dyes operating in the region > ~540 nm, we can amplify the output at MHz repetition rates using the cavity-dumped argon-ion laser and a six-pass amplifier stage. We have used a similar system to obtain UV resonance Raman spectra with excitation > 270 nm. In this work we concentrate on the region from ~410 to ~480 nm that is available from using the dye stilbene 420 (Exciton Corp.) pumped by the 355-nm excitation. The repetition rate of the dye laser is adjustable, and for quasi-continuous UVRR, we typically operate at 3.8 MHz. The energy per pulse is less at this higher repetition rate, but the average power, including the average SHG power, is higher. The generation of second harmonic radiation is done by using angle-tuned BBO for the region from ~210 to 240 nm. The output beam from the blue dye laser is focused with a 25-mm quartz lens into a BBO crystal (Metastat Advanced Optical Materials, 80° cut). The crystal angle is adjusted by mounting the BBO in a rotating stage. The output beam is recollimated and the second harmonic is separated from the fundamental using a quartz Pellin-Broca prism. The average UV power is ~1 mW, which has proven to be sufficient for acquiring high-quality Raman spectra. We use spinning sample cells for biological materials in order to avoid photodegradation of samples with prolonged exposure to UV radiation. The excitation beam impinging upon the sample is incident at ~45° to the collection axis. The spinning sample cells are 1-cm-diameter quartz cells fabricated in our glass shop. The experimental configuration for collection of UV Raman data is shown in Fig. 1.

Our detection system consists of a single 0.64-m spectrograph (Instruments SA, Model THR 640) and a CCD optical multichannel detector (Photometrics, Model CC200, CE200, and CH210, with a Thomson-CSF 384 × 576 chip). With a 2400 g/mm grating, our effective spectral resolution is ~10 cm⁻¹ (10 to 20 μm). For the UV Raman data presented here, collection times were ~50 min total. The wavenumber shift was calibrated with the use of the Raman spectra of acetonitrile and acetone. Aqueous solutions of nucleotide samples (i.e., adenosine-5'-monophosphate, cytidine-5'-monophosphate, guanosine-5'-monophosphate, thymidine-5'-monophosphate, and uridine-5'-monophosphate, from Sigma) were prepared by dissolving crystalline samples. The pH of each solution was approximately 7. A 0.3 M Na₂SO₄ internal standard was utilized in analyses. Sulfate has a strong Raman line at 981 cm⁻¹, which is marked in the figures with an asterisk. The cisplatin was acquired from Aldrich, and the Bristol-Myers Company provided the carboplatin (Paraplatin®). The complexion reactions between cisplatin and carboplatin and the mononucleotides were initiated by combining equal amounts of each (i.e., a drug-to-nucleotide ratio of 1:0). For cisplatin/nucleotide reactions, approximately 7 days were required for the binding process to go to completion, as indicated by changes in characteristic nucleotide bands. The reactions were carried out in the dark at room temperature, with periodic stirring. The cisplatin products are stable at room temperature for up to a month. Carboplatin/nucleotide products, however, are seen to decompose over a period of days.

RESULTS AND DISCUSSION

In Fig. 2 we show the UV excited resonance Raman spectra of AMP, CMP, UMP, and TMP. The excitation
wavelength is 209 nm and nucleotide concentrations are 5 mM. In order to obtain these data we only required approximately one milliliter of solution. While such a small volume was not critical for these particular samples, it demonstrates that the experimental methods used here will be applicable even for samples that are difficult to prepare in large quantities (e.g., synthetic oligonucleotides or natural DNA samples). These spectra show acceptable resolution and S/N, demonstrating the capacity of our laser system for acquiring quality Raman spectra of small amounts of material.

The Raman bands observed represent vibrational motions of base portions of the nucleotides, as outlined in Table I. These modes of vibration have been discussed in detail by others. With excitation at 209 nm we are in resonance with the second strongly allowed $\pi^* \rightarrow \pi$ transition in each of the bases.

It is important to emphasize that the frequencies of the Raman bands often shift only slightly with chemical interaction. However, the intensities of the bands (i.e., via resonance enhancement) are significantly perturbed by these same interactions. This effect was the original basis for Raman hypochromism, even under nonresonance conditions. Therefore, intensity changes can often provide better “markers” for the chemical interactions than absolute frequencies, depending on the system. For example, in Fig. 3, we compare the spectra of CMP with and without the presence of cisplatin. These data show a large decrease in the intensity of the 1535-cm$^{-1}$ band of CMP in the presence of cisplatin. This band has been attributed to an $e_{2g}$ mode of benzene, and platinumation at the N3 site of the CMP pyrimidine ring is expected to affect this motion significantly. Furthermore, changes in the relative intensities and frequency shifts of the 1298-, 1214-, and 1060-cm$^{-1}$ bands are observed upon cisplatin binding. These data are representative of the types of spectral changes that occur with drug/nucleotide interactions.

Figure 4 displays representative UVRR spectra of GMP with and without the presence of cisplatin and carboplatin. As can be seen from these spectra, significant changes in band shape and intensity are observed upon binding of these anticancer drugs. For example, the interaction of cisplatin with GMP results in a lowering in intensity of the bands at 1586 cm$^{-1}$ and 1686 cm$^{-1}$. The 1586-cm$^{-1}$ band is associated with an $e_{2g}$ mode of benzene (i.e., a ring deformation), while the 1686-cm$^{-1}$ band is...
TABLE I. Resonance enhanced Raman bands of nucleotide bases.

<table>
<thead>
<tr>
<th>Raman band (cm⁻¹)</th>
<th>Approximate assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP 1585</td>
<td>ε₂ mode of benzene²</td>
</tr>
<tr>
<td>1493</td>
<td>C₆-NH₃⁺, C₂=N₃, C₄-N₉</td>
</tr>
<tr>
<td>1428</td>
<td>Symmetric stretch of 5-membered ring</td>
</tr>
<tr>
<td>1377</td>
<td>Glycoside N-C stretching mode</td>
</tr>
<tr>
<td>1340</td>
<td>C₅=C₇ str. (ε₂ mode of benzene)²</td>
</tr>
<tr>
<td>1317</td>
<td>ε₁ mode of benzene</td>
</tr>
<tr>
<td>1220, 1185, 1045</td>
<td>Assignments uncertain</td>
</tr>
<tr>
<td>CMP 1652</td>
<td>C₂=O str.; C₅=C₆ stretching mode⁴</td>
</tr>
<tr>
<td>1619</td>
<td>C₅=C₆ stretching mode</td>
</tr>
<tr>
<td>1535</td>
<td>ε₂ mode of benzene</td>
</tr>
<tr>
<td>1298</td>
<td>Assignments uncertain</td>
</tr>
<tr>
<td>1214</td>
<td>Glycoside N-C str.</td>
</tr>
<tr>
<td>1060</td>
<td>Assignments uncertain</td>
</tr>
<tr>
<td>GMP 1686</td>
<td>C=O str.; C₅=C₆ out-of-phase str.</td>
</tr>
<tr>
<td>1586</td>
<td>ε₂ modes of benzene</td>
</tr>
<tr>
<td>1490</td>
<td>Kekule vibration of benzene (b₂₁)</td>
</tr>
<tr>
<td>1308</td>
<td>Assignments uncertain</td>
</tr>
<tr>
<td>1180</td>
<td>C₅=N₇ out-of-phase str. of 5-membered ring</td>
</tr>
<tr>
<td>1076</td>
<td>Assignments uncertain</td>
</tr>
<tr>
<td>TMP 1646</td>
<td>C₅=C₆ str.; C₄=O str.</td>
</tr>
<tr>
<td>1470</td>
<td>Out-of-phase ring str., coupled to C₂=O</td>
</tr>
<tr>
<td>1400</td>
<td>Out-of-phase ring str., coupled to C₄=O</td>
</tr>
<tr>
<td>1174, 1042</td>
<td>Assignments uncertain</td>
</tr>
<tr>
<td>UMP 1686</td>
<td>C₂=O str.</td>
</tr>
<tr>
<td>1633</td>
<td>C₅=C₆ str. (ε₂ mode of benzene)</td>
</tr>
<tr>
<td>1474</td>
<td>ε₂ mode of benzene</td>
</tr>
<tr>
<td>1402</td>
<td>Assignments uncertain</td>
</tr>
<tr>
<td>1189</td>
<td>C₅H₅C₆H₁₈O² out-of-phase bending</td>
</tr>
<tr>
<td>1057</td>
<td>Assignments uncertain</td>
</tr>
</tbody>
</table>

* Assignments tabulated from Ref. 1, Vol. 2, pp. 109-79, and Refs. 11, 12, 37, and 40.
* From Ref. 37.
* Nishamura et al. favor the C₅=C₆ str. assignment (Ref. 37).
* This band may also be attributed to a C₂-N (amino) str. + a N₁-H bend (Ref. 40).

predominantly due to a C=O stretching mode with an out-of-phase contribution from C₅=C₆ stretching. These changes are consistent with the binding of cisplatin to the N₇ position on GMP. Another pronounced change in the GMP spectrum following cisplatin binding centers around the 1368-cm⁻¹ band. Although normal mode calculations do not yet explain this particular band, it is likely that it reflects stretching of the C₅=N₇ bond in the purine ring of GMP because of the shift in frequency and the significant increase in intensity that occurs. The band at 1180 cm⁻¹ can be assigned to antisymmetric stretching of the five-membered ring of the purine base, and the 1180-cm⁻¹ and 1076-cm⁻¹ bands change only in terms of relative intensity. We also observe a relatively strong band at 1219 cm⁻¹. The assignment of this band is not certain; the assignments of the bands in the 1100-1450-cm⁻¹ region have not been clearly established.

The GMP/carboplatin spectrum also shows a decrease in 1686- and 1586-cm⁻¹ band intensities, as well as increased bandwidth for these bands. The 1368-cm⁻¹ band decreases in intensity and shifts to 1345 cm⁻¹. The relative intensities of the 1180- and 1076-cm⁻¹ bands reverse upon interaction of GMP with carboplatin, and the 1076-cm⁻¹ band shifts to 1052-cm⁻¹. In general, however, the changes in the GMP/carboplatin spectrum are less pronounced in comparison to the GMP/cisplatin results. That is, the changes in the GMP/carboplatin spectrum appear to be consistent with the type of bonding that occurs in cisplatin, but the extent of the interaction is not as strong. It should be noted that we have found nucleotide/carboplatin mixtures to be fairly unstable, and it is possible that significant decomposition occurs before we are able to monitor adequately the alterations in GMP structure induced by carboplatin. We are presently characterizing the changes in the spectra of the carboplatin complexes as a function of time.

Finally, in Fig. 5, we present the Raman spectrum of a 16 μM solution of the oligonucleotide d(GGCCG GCC) with and without the presence of cisplatin. We want to emphasize again the fact that we required only 1 mL of sample in order to obtain these data. The interpretation of these data is consistent with the binding of cisplatin to N₇ of GMP. This can be seen by the frequency shift in the 1178-cm⁻¹ band, which is attributable to a stretch in the five-membered ring of guanine. For d(GGCCGG CC), we also see perturbations of the cytosine portion of the molecule. The 792-cm⁻¹ band, which represents a
pyrimidine ring breathing vibration of CMP, loses considerable intensity with the binding of cisplatin. (Some of the intensity of this band can be attributed to the sulfate standard.) Since CMP bands do not shift with the addition of cisplatin, it is not anticipated that binding will occur on the cytosine residue. This result is expected, as the N3 of CMP is involved in base pairing in oligonucleotides such as d(GGCCGGCC) and is therefore less available for Pt binding. Although the number of observable Raman bands due to d(GGCCGGCC) is small at these concentrations, these spectra demonstrate the extremely low concentrations our Raman apparatus is capable of detecting. For many biological systems it is problematic to secure large quantities of sample owing to difficulties in sample preparation or to the cost of the sample. The apparatus described here offers the possibility for obtaining useful spectra from such systems.

CONCLUSIONS

In this work, we have demonstrated improvements in the frequency-doubled, synchronously pumped dye laser system. We have increased the wavelength range of the system by frequency doubling the output from a blue dye laser into the region of ~210 nm. We have used the system to obtain high-quality resonance Raman spectra of biological materials at low concentrations and with small volumes of sample (~1 mL). We have presented preliminary results concerning the alterations in nucleotide structure caused by interactions with the antineoplastic drug, carboplatin, as evidenced by changes in UVRR spectra. More work regarding the kinetics of the carboplatin/nucleotide interactions remains to be done. We note that the laser system described here can be applied to many other biological and chemical systems, including proteins.10,14 And we can take advantage of the fact that the system is pulsed to obtain picosecond transient spectra (absorption or Raman) using the ~210 nm excitation as either the pump or the probe.14,42

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36. W. L. Weaver, K. Iwata, and T. L. Gustafson, unpublished data.
42. R. L. Benson and T. L. Gustafson, unpublished data.