Solving the biological sample problem: Detection limits in small samples of 4-thio thymidine by resonance Raman spectroscopy

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Introduction

A potential problem in applying spectroscopic approaches to protein-DNA binding is the fact that proteins and DNA both have large numbers of similar chromophores, the bases of the DNA, and the side chains and backbone of the protein. This problem can be circumvented by using base analogues as spectroscopic probes (see Figure 1). The family of sulphur substituted bases offers an attractive approach to studying protein-DNA binding. The thymidine analogue 4-thio-dT (4S-dT) has been characterised by Resonance Raman Spectroscopy in isolation, in single stranded oligonucleotides, as part of a DNA duplex and as a protein-bound duplex.1) These initial studies, carried out at the Central Laser Facility, showed that 4S-dT is a sensitive Resonance Raman probe of the state of the DNA duplex. Specifically bending of the DNA on protein binding lead to peak shifts in the RR spectrum that correlate with the likely weakening of hydrogen bonding. Thus this study demonstrated that a specific structural transition in the DNA can be observed and correlated with structural information. Moreover there is the potential to use the nature and magnitude of the peak shifts to directly validate computational models.

Figure 1. Structures of thymidine, the natural component of DNA (left), 4-thiothymidine, the sulfur substituted analogue (middle), and 4-thiouridine, the model compound used in this study (right). The presence of the sulfur atom in the base shifts the absorbance maximum of the nucleoside from around 280 nm to 350 nm providing a selective probe for Resonance Raman spectroscopy.

However, despite the potential of the 4S-dT as a spectral probe there have been no further reports on its use in studies of DNA binding. This can be ascribed to a number of potential problems, stability of 4S-dT containing DNA to oxidation, the challenges of Resonance Raman in general. The main issue however is the cost of obtaining sufficient DNA to undertake the study of sequence context that will be necessary to provide a firm basis for interpretation of spectral changes on protein-DNA binding. It is therefore valuable to develop systems that will allow these studies and other investigations of valuable biological material with very small samples.

In previous reports we discussed the development of a sample environment for Resonance Raman Spectroscopy that allows the use of sample volumes as small as 5 µL in the form of a frozen droplet.2)3) Here we discuss the adaptation of this system for use on samples in the liquid state and demonstrate the collection of spectra with a 15 second acquisition from a 10 µL sample of 50 µM 4-thiouridine, a model compound with similar spectroscopic characteristics to 4-thiothymidine.

The experimental setup

The setup for RR spectroscopy comprised an argon-ion laser (363.8 nm), collection optics, Raman spectrograph, CCD detector and a sample holder. The sample holder, a modified nickel top hat, similar to those used in protein crystallography, was magnetically held on a goniometer head for positioning. The sample sits in a photo-etched brass loop of 1.5 mm internal diameter attached to an integral stem of 40 mm length. The brass stem loops were glued to the top hat. A stream of argon was flowed continuously over the sample (Figure 2).

Figure 2. Sample setup for Raman Spectroscopy of small liquid samples. The sample loop holds 5 – 10 µL.

Stability of the sample

A significant problem with Raman spectroscopy of thionucleosides is the photodegradation of the chromophore.4) As the photoproducts are Raman silent this does not produce spectral artifacts but leads to a continuous degradation of signal to noise in the spectra. Our first experiments with liquid samples free in the loop and without any inert gas stream showed rapid degradation of the spectra over 10 – 20 seconds exposure to the laser beam.

The introduction of a stream of argon reduced the rate of degradation significantly. Peaks in the Raman spectrum could still be discerned after minutes of laser exposure. The optimum laser power to provide a balance of signal intensity and sample stability was between 20 – 30 mW at the sample. Under these conditions it took five to ten minutes for aqueous samples to evaporate. Samples of volatile liquids, including the toluene used for calibration, evaporate over the course of a few minutes.

Detection limits in the sample system

Having established the optimal conditions for spectra collection we investigated the detection limits of the system. As the ultimate aim of these studies is to examine DNA-DNA and protein-DNA binding processes it would ideally be possible to work at concentrations above and below the dissociation constants for the processes of interest. The binding constants

for protein-DNA binding processes lie in the micromolar to millimolar range. We therefore investigated the detection limit for solutions of 4-thiouridine at concentrations ranging from 1 mM downwards.

With the optimized setup useful spectra of 4-thiouridine could easily be obtained from 10 µL samples with concentrations of 1 mM to 100 µM. With concentrations below 100 µM spectral features were still discernible at concentrations from 50 µM to 12.5 µM with a 15 second acquisition (Figure 3). Sample degradation took place over a period of 30 seconds to one minute under these conditions making it possible to collect multiple spectra. Thus it should be possible to work at concentrations ranging down to the low micromolar. This opens up the possibility of studying a wide range of biological processes.

Figure 3. Limits of detection of 4-thiouridine by Resonance Raman spectroscopy with a 10 µL sample in an argon stream. Spectra were acquired for 15 seconds with a laser power of approximately 25 mW at the sample. A polynomial baseline was fitted to the data and subtracted but there was no other manipulation of the data. The spectra are offset on the Y axis for clarity.

Conclusions

Useful Resonance Raman spectra can be readily obtained from 10 µL samples of dilute solutions using the sample environment described here. In the case of thionucleosides photo-oxidation of the chromophore is a potential problem. This is significantly reduced by placing the sample loop in a stream of argon. This does increase the rate of evaporation of the sample but allows longer acquisition times.

Spectra of solutions of 4-thiouridine with concentrations as low as 50 µM could be analysed with acquisition times of 15 seconds. More dilute solutions can be analysed with longer acquisitions times. Under our conditions the spectrum degrades over about a minute.

Using this sample environment and spectroscopy setup it is possible to record spectra of valuable biological samples in a short time from extremely small samples. In addition the sample setup is easily accessible and sample changes are straightforward. This approach should therefore open up opportunities for the application of Raman spectroscopy to a wider range of samples where previously the cost of producing sufficient quantities of sample was prohibitive. In particular this makes it possible to investigate the structural and energetic details of protein-DNA binding processes using thionucleosides as positions specific spectral probes.

References

