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DNA methylation detection using UV nano bowtie antenna enhanced Raman spectroscopy

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ABSTRACT

Methylation in DNA is a controlling factor in gene expression, embryonic development, and has been found to be important in infections and cancer. From a basic biology point of view, great heterogeneity has been found in methylation levels within tissues, so questions arises as to how and why. We show that methylated-DNA (m-DNA) can be distinguished from non-methylated (n-DNA) with nano-bowtie- and resonance- enhanced Raman spectra. By tuning the bowtie antenna to the resonance wavelength, both gains can be realized. Two additional Raman peaks in the 1200 – 1700 cm-1 band appear with methylation: one at 1239 cm^{-1} and the other at 1639 cm^{-1}; a weak peak near 1000 cm-1 also appears with methylation. We also find that the two spectral features, although the latter with slight modification, can be used to distinguish the methylation state even when the DNA is denatured, as we show when we induce crystallization of the salts in the solution with increased excitation power, or allow it to happen naturally via solvent evaporation, and the DNA is trapped within the salt crystals. A comparison between liquid/solution to dried/denatured state m-DNA shows a general broadening of the larger lines and a transfer of spectral weight from the ~1470 cm^{-1} vibration to two higher energy lines. The applicability of the resonance-Raman in these spectra is shown by demonstrating that the Raman spectral characteristics hardly change as the Raman resonance in excitation wavelength is approached. Finally, we comment on real signal gain in this double-resonance system.

Keywords: Resonance Raman, methylation, cytosine, plasmonics, nano-antenna

1. INTRODUCTION

Methylation of DNA is a key regulator in biological growth and gene expression [1,2]. Measuring it in a label-free manner would thus aid these studies and make a practical advance. Raman spectra are often used for label-free studies, since the 'fingerprint' spectra can identify chemical bonding and molecules. In Raman spectroscopy, incident excitation light interacts with a sample by scattering inelastically and creating (in the usual Stokes mode, although it can absorb in the less-strong anti-Stokes mode) a phonon, or quantized vibration, in the material [3,4]. The light leaves with lower energy and thus longer wavelength, which can be detected by a spectrometer. The vibrations are specific to the molecule, thus, the quantized energy loss of the light will also be different in different molecules. Vibrations of higher energy (and easier to detect) are usually from functional groups within a larger molecule, while the whole-molecule vibrations are lower frequency due to the lower restoring forces. These have less energy and are close to the (relatively large) elastically scattered peak, and are therefore harder to detect. The Raman effect is weak, perhaps only one in a million photons will Raman scatter in a non-resonant, typical molecule. Not all vibrations have a Raman interaction, but the functional group vibrations that do Raman scatter contain some information about the molecule, since any charge injected or removed from the group by the rest of the molecule will increase or decrease the inner bond strengths, hence vibration energies. The molecule that the moiety is attached to may also alter relative cross sections of its vibrations. Careful peak position measurements and study of all vibrations present may be required. One of the largest problems with Raman scattering is the weakness of the effect. This is especially stringent when the volume is small so the number

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of molecules is small. We investigate two methods to increase the signal: resonance Raman [5–8], in which the cross section is increased by tuning the excitation to near an absorption of the molecule, and nano-antenna plasmon resonance [9], in which a small, shape and size tuned metal has a resonance in the surface electron density at the excitation wavelength, which dramatically increases the electric field strength in the active region, essentially providing a stronger source field in the small volume where it is needed. Finally, absorption energies are often in the UV spectral range, so we need to do deep-UV plasmonics to make this work.

Whereas neither of these effects is new, using them together for methylation detection is novel. It is not obvious that it will work for several reasons: the metal can interact with the molecule and alter the vibrations [10–12]; the absorption resonance in the Raman signal is not uniform so that some modes may not have increased Raman signal at all [13]; and the resonance Raman probes the excited states [13,14], especially near the absorption peak itself, and these may differ from the ground state vibrations, which can complicate the identification scheme, perhaps making it wavelength-dependent. Resonance Raman tends to increase the Raman signal of the vibrations that involve bonds that interact with the bond that is being broken by the absorption. If this were too far from the methyl group, then the relevant vibrations may not change and the signal of interest may not be detectable. We use a ring absorption in the cytosine [7], which is quite central to the molecule, and thus should interact with the methyl group. We observe that the Raman signal from all the relevant vibrations increase by the same factor. In fact [15], the spectra hardly change as resonance is approached. This also calms the concern about excited state vibrations.

The methylated DNA sample is a double-stranded oligomer of 16 bases with a sequence of 50-AAA AAA AC*/G TTT TTT T-30, where the methylated cytosine group (C*) is located in the center of the oligomer (Invitrogen, Thermo Fisher Scientific corporation). The unmethylated sample is a double-stranded lambda DNA with 48,502 base-pairs (New England Biolabs). The mehtyated and unmethylated DNA are dispersed in 0.1 M phosphate-buffered saline at pH 7.4 and a 10 mM Tris buffer at pH 8 with controlled concentrations, respectively.

2. ABSORPTION RESONANCE

The technique is able to distinguish methylation using a fixed set of metrics that are not affected by absorption or plasmon resonance [15]. A new vibration Raman peak at 1014 cm^{-1} appears with methylation. It resonates and is not changed by proximity to the nano-bowtie antenna. Another notable spectral change is a shift in the relative spectral weight in the 1200-1700 cm^{-1} band. Without methylation, the center of the band is strong, but after methylation, the vibration modes near the edges of the band become comparable in strength. These features can be seen in Fig. 1.

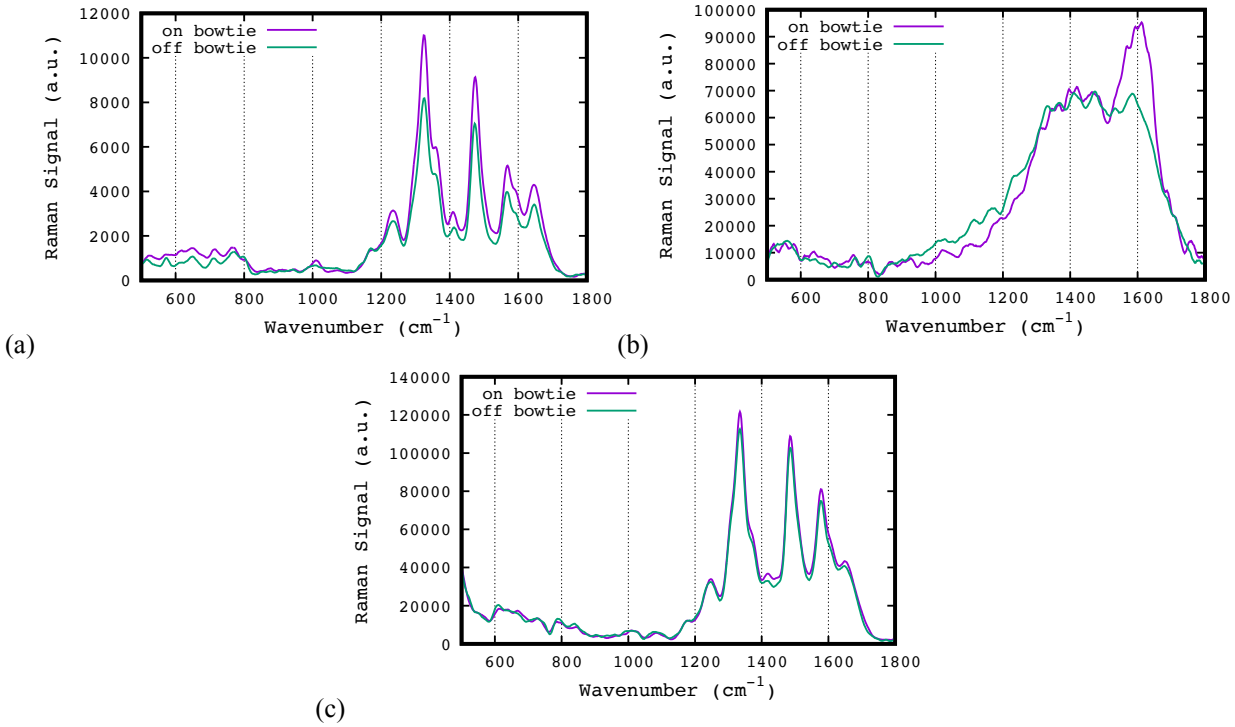

Figure 1. Plasmon resonance gain can be identified by taking spectra on vs. off the bowtie antenna. In this case, the cytosine is uniformly spread on the sample, so relative sampling volumes must also be accounted for. All spectra are from methylated DNA. The relative ratio of areas under the 1200-1700 cm^{-1} peaks are (a) 1.33 for natural DNA dissolved in liquid, (b) 1.07 for denatured DNA in a salt crystal, and (c) 1.08 for natural DNA dissolved in liquid (similar to in (a)).

The strength of the absorption resonance depends upon the wavelength. For symmetry-allowed absorptions, the resonance structure can extend from tens to a hundred nanometers on either side, so the dependence is rather weak. We have found [7] that when a symmetry-forbidden, phonon-allowed vibration is used, the Raman resonates over a much narrower spectral region, typically only a few wavenumbers, and is much stronger, up to a factor of several thousand. In this case, the resonance width is often set by the laser width rather than the intrinsic gain width, but we used a tunable, OPO based DUV laser with ~1 cm-1 resolution, and found [14] a change in the Raman signal level as high as 3600-fold for an excitation energy change of 1 cm^{-1}. The resonance peak was slightly asymmetric, suggesting we had reached the molecular limit. There are clear advantages of such a narrow and strong resonance. It provides a 'double-selectivity' for identifying materials in complex mixtures such as cells or tissue. The narrow resonance (or the on - off comparison) allows separation of the Raman signal from one molecular type -- the absorbing molecule Raman signal can be thousands of times stronger and dominate the measured spectrum. Second, the Raman spectra itself can indicate the particular vibrations present and give information about charge transfer or other bonding changes that may be associated with that molecule in its complex environment.

3. PLASMON RESONANCE

The plasmon resonance is also strong, ~20,000 times [15]. This only applies to the small region with enhanced electric field, between the tips of the two triangles creating the bowtie. This qualification sets limits on practical application of the technique. For example, Figures 1 and 2 show two methods to quantify the gain of the antenna (in the enhanced region). The method in Fig. 1 is based upon placing the excitation light over a region containing a nano-antenna (on), or over a region not containing one (off). The method for Fig. 2 uses the dependence of the plasmon enhancement on the polarization of the incident light.

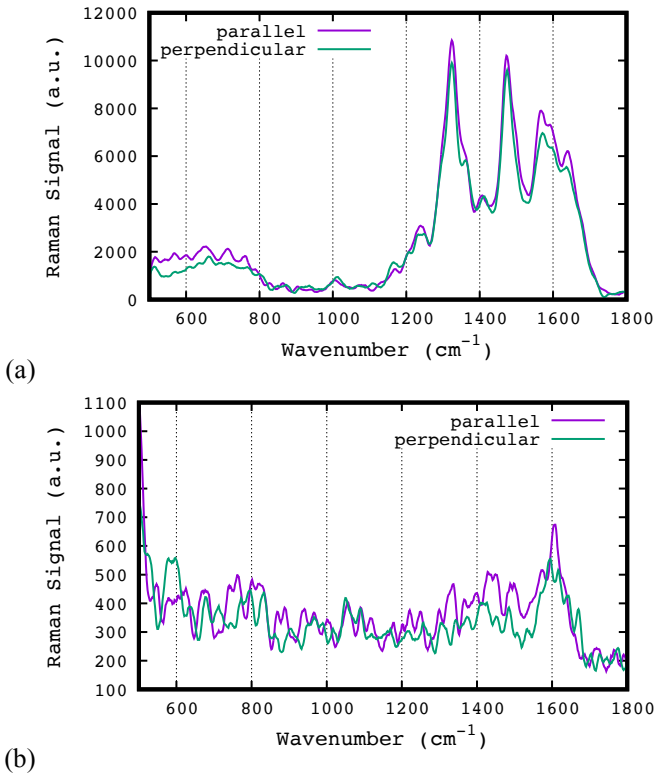

Figure 2. Plasmon resonance gain can be identified by taking spectra with polarization along or perpendicular to the axis of the bowtie antenna. The former results in plasmon gain, the latter only gives minimal increased electric field. In this case, the cytosine is uniformly spread on the sample, so relative sampling volumes must also be accounted for. The relative ratio of areas under the 1200-1700 cm^{-1} peaks are (a) 1.10 for natural, methylated DNA dissolved in liquid, (b) 1.38 for partially denatured non-methylated DNA.

In both figures, the increase in the signal levels is very modest. This does not mean that the gain is small in this case, since the cytosine is present all over the sample, and the ~1 micron spot size of the illumination laser excites (with absorption resonance Raman) all the cytosine it illuminates. To find the gain, one has to consider the relative number of molecules observed in each case and correct for that. For uniform cytosine, the volume ratio is what matters, and the one micron spot gives ~125,000 times the volume of the estimated bowtie enhanced volume, since it has to be a nano-structure to be resonant in the deep-UV. The average resonance gain in all the data from the figures is about 20,000, and the individual gains are within a factor of 2-3 of that. The large variation is due to the strong dependence of the electric field gain on the geometry (actual gain), and of the measurement due to measurement noise (error in measured gain). A 1% error in the ratio determination due to noise in the data, for example, can cause a 10% error in the measured gain for values close to what we measure. The volume ratio is not exact either, so the enhancement values probably contain tens of percent uncertainty in measurement errors alone, which is why we give only 1-2 significant digits in their values.

The other lesson from the figures is that the plasmon enhancement in this case does little to provide increased signal gain if all we want is to measure the methylation of cytosine in a bulk sample. Why is it not practical? Since it only intercepts a small fraction of the incident light and thus can only enhance a tiny volume. If we had a dense array of nano-bowtie antennas, then they could conceivably collect all the incident light and focus it, and we might naively expect most of the ~20k signal gain to appear as an increase in measured signal. There are caveats. We can get an idea of the spacing required from antenna theory. A point antenna should absorb from a region about a wavelength in each lateral dimension. This turns out to be correct, and at closer spacing, the antenna-antenna interactions start to be strong enough that they cannot be neglected. The other caveat is that there is still the volume ratio to be accounted for, as the wavelength-square region does not contribute except for the enhanced nano-region (all the energy has been swept up by

the nanostructure), so the ratio of those two volumes must be applied as a correction to the expected signal. The result is that energy is conserved, and you can't win with a uniformly spread sample (bulk-like sample).

This situation is very analogous to that observed in absorption resonance Raman [16]. In a pure bulk material, the absorption resonance samples only a very thin layer at the surface (due to the very absorption required for it to work), but does so with large cross section. The off-resonance signal comes from a much larger volume, but with lower cross-section. The net result for pure materials is a very modest signal gain. For absorption resonance, trace quantities in a non-absorbing host, or tiny (less than absorption length) volumes in air (such as might be encountered in near-field scanning optical microscopy, NSOM Raman, or tip-enhanced Raman [12,17,18]) are two cases that are strongly aided by the absorption resonance in real signal levels.

When trace quantities are freely diffusing around a sample, the nano-plasmonics real signal level will not be larger than the laser spot signal level, as they are unlikely to be within an antenna's enhanced region at any given time. If, however, a specific binding agent can be applied inside the enhanced region to 'collect' the molecules of interest to the enhanced volume, then real signal gain would be large. Another way to increase signal is to take the NSOM analogue and either move the nano-antenna around to a small volume where the molecules are (assumed immobile), or mobile molecules are constrained to flow through the enhanced region by building a micro-nano fluidics system that pumps the material to where it will be enhanced. This would also bring all the material past the enhanced region and when there, the real signal would be dramatically increased. Thus, there are specific cases in which real signal gain is expected for plasmonic resonance, and for the combination of plasmonic and absorption resonance for Raman spectroscopy.

4. CONCLUSIONS

Raman signal enhancement by a combination of absorption-resonance Raman and plasmon resonance can give huge per-molecule signal gains. The use of deep-UV excitation for the absorption in most materials requires the use of aluminum-based DUV plasmonics. We used electron beam fabricated structures. Considerations about the absorption used and its ability to resonate the vibration signals of interest, and care that the metal does not influence the measurement by forming bonds or absorbing nearby signal are also important. For high-gain, high (double) selective Raman absorption resonance, a symmetry-forbidden, phonon-allowed absorption might be chosen, but it requires a tunable laser to approach the gain region. Several cases in which the real signal level is increased by the plasmon gain are identified, as are cases in which it is less practical. Care must be taken when choosing these gain methods to apply them for maximal real signal gain.

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