# Origins of Blinking in Single-Molecule Raman Spectroscopy

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# Origins of Blinking in Single-Molecule Raman Spectroscopy

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We observe spectral and intensity fluctuations in Raman scattering from single molecules of 4-mercaptopyridine (4-Mpy) adsorbed on textured silver surfaces. We present evidence that the root cause of these fluctuations is thermal. Analysis of the spectra shows that in some cases the spectral changes are associated with molecular reorientation and in others with chemical reactions of the 4-Mpy. Analogous surfaces fully covered with 4-Mpy exhibit similar behavior, indicating that monolayer Raman spectra are dominated by a few molecules at most.

## Introduction

Due to the structural specificity of vibrational spectroscopy, early observations of Raman scattering from single molecules adsorbed to silver or gold surfaces[1−8] have given a boost to the prospects for realizing the dream of "watching" single molecules undergo chemical reactions. In practice, single-molecule Raman intensities and spectra are hypersensitive to the position and orientation of the molecule relative to electromagnetic hot spots that make giant enhancement possible. This leads to complex spectral variations in time reminiscent of "blinking" in single-molecule fluorescence studies.[9−14] Fluctuations in single-molecule fluorescence experiments have been studied extensively and are widely believed to occur when molecules are promoted via resonant excitation to metastable nonabsorbing[9−11] or nonemissive states[12−14] such as triplets or ions. Blinking in single-molecule Raman scattering is not as easy to rationalize as in fluorescence, particularly for molecules apparently far from optical resonance. Suggested explanations of single-molecule Raman blinking include thermal diffusion of the molecules in and out of hot spots,[2−5] photoionization via charge-transfer states,[6,7] and rearrangement of the silver substrate morphology.[8] In the present paper, we address the mechanism for intensity and spectral fluctuations in a study of Raman scattering from single molecules of 4-mercaptopyridine (4-Mpy) on fractal silver surfaces. We find that the spectral and intensity fluctuations are not simply described by any of the above ideas and arise mostly from thermally stimulated molecular reorientation. In addition to rearrangements of single 4-Mpy molecules, we also observe thermally assisted chemical processes such as protonation and deprotonation of the nitrogen atom. We compare blinking for several different molecules and compare single molecules to saturated monolayers of 4-Mpy.

## Experimental Section

Silver films were fabricated on glass substrates using the Tollens reaction,[15] which produces fractal morphologies that can enhance cross-sections enough to observe single-molecule Raman scattering.[16,17] We spin-cast 4-Mpy molecules onto these silver substrates from $10^{-9}$ M deionized water solutions to produce very low coverage surfaces for single-molecule studies.

For comparison, we also applied saturated solutions of 4-Mpy to form dense self-assembled monolayers. Excitation and collection of Raman scattering were done in a confocal geometry using an inverted microscope with a 1.4 numerical aperture oil-immersion objective. The excitation source was a He−Ne laser operating at 632.8 nm. After removal of the scattered excitation light with a holographic notch filter, the remaining light was observed using either a spectrometer with a liquid-nitrogen-cooled charge-coupled device (CCD) detector to measure spectra or a single-photon-counting photomultiplier with a 100 $\mu$m pinhole to image samples and to measure total Raman scattering intensity transients. Raman images were acquired by scanning the sample with a computer-controlled 2D piezoelectrically driven scanning stage. Spectroscopy and dynamics of molecules were studied at "hot spots" exhibiting high Raman intensity that are located using the spatial scans.

## Results and Discussion

The spectroscopy of 4-Mpy has been studied extensively both in the solid form and adsorbed to various types of silver surfaces.[18−22] The spectroscopy of 4-Mpy is greatly complicated by its ability to assume several tautomeric forms.[21] Nevertheless, pH-dependent spectroscopic studies of 4-Mpy complexes on silver[18,19] have permitted definitive assignments of protonated and deprotonated forms of the molecule as well as cationic and anionic states. 4-Mpy has $C_{2v}$ symmetry so that all of the modes are Raman allowed although $a_1$ and $b_2$ modes tend to be stronger. Surface selection rules for molecules adsorbed on metals can also have important effects on relative intensities[23] and greatly simplify the assignment of SERS spectra.

**Time Dependence of Single 4-Mpy Raman Spectra.** Figure 1 illustrates a typical time series of Raman spectra from a single molecule of 4-Mpy found by scanning over a silver-treated substrate where 4-Mpy has been applied by spin casting from extremely dilute solution. The spectra typically remain similar for relatively long periods so that, even though we average over several seconds to obtain good signal-to-noise ratios, large spectroscopic variations with time are not washed out. Substantial blinking of the overall Raman intensity and dramatic variations of the spectra with time are observed. There are general spectroscopic patterns that tend to recur, and it is unlikely that the molecules are degrading with time or being converted to amorphous carbon as has been suggested in the

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**TABLE 1: Assignments for Selected Raman Modes Observed in These Experiments**

| frequency (cm$^{-1}$) | assignment[a] | frequency (cm$^{-1}$) | assignment[a] |
|---|---|---|---|
| 700 | 11b$_1$ out-of-plane C–H def | 1150 | 15 b$_2$ C–H def |
| 720 | 6a$_1$ in-plane ring deformation with C=S | 1206 | 9a$_1$ in-plane C–H def with protonated nitrogen |
| 760 | 4b$_1$ out-of-plane ring def | 1220 | 9a$_1$ in-plane C–H def |
| 815 | 10b$_1$ out-of-plane C–H def | 1250 | in-plane N–H def |
| 870–940 | out-of-plane N–H def | 1287–1318 | 3b$_2$ in-plane C–H def |
| 892 | 10a$_2$ out-of-plane C–H def | 1395 | 14b$_2$ ring stretch |
| 920 | 5b$_1$ out-of-plane C–H def | 1440 | 19b$_2$ ring stretch |
| 962 | out-of-plane def for N–H$^+$ | 1470 | 19a$_1$ ring stretch |
| 1013 | 1a$_1$ ring breathing | 1580 | 8b$_2$ ring stretch with deprotonated nitrogen |
| 1030–1050 | 18a$_1$ in-plane C–H def | 1604 | 8b$_2$ ring stretch with protonated nitrogen |
| 1085 | 18b$_2$ in-plane C–H def | 1620 | 8a$_2$ ring stretch |
| 1099 | 12a$_1$ trigonal ring breathing with C=S | | |

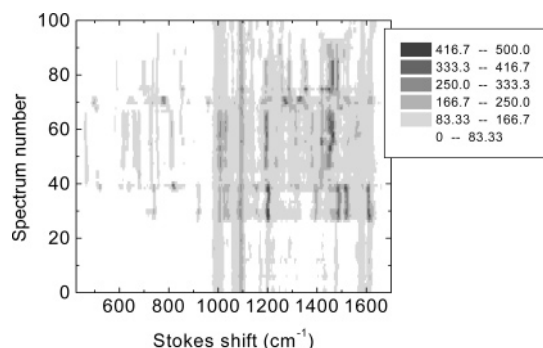[a] Ring modes are denoted in terms of Wilson notation.



**Figure 1.** Time evolution of the Raman spectrum of a single 4-Mpy molecule on silver. The data include 100 consecutive spectra with 2 s integration times at an excitation intensity of 40 $\mu$W. The gray scale for the signal amplitude is shown in the inset in units of total counts.

literature.[24] It is relatively straightforward to assign the Raman modes to those typical of 4-Mpy spectra as is done in Table 1, where the assignments are mostly derived from previous work.[18–22] The spectra are most often more complex than literature spectra of 4-Mpy, and this can be attributed in part to the fact that we are averaging over 5 s worth of configurations and in part to the extreme local field enhancements and their spatial and polarization profiles relative to the adsorbed molecule. Previous work[16] has calibrated the Raman enhancement for 4-Mpy on similar silver surfaces due to local hot spots to be ∼10$^{14}$ times, and the electromagnetic field strengths must therefore be textured even on the size scale of the 4-Mpy molecule. This can undoubtedly distort the vibrational spectra in ways that are difficult to predict or analyze.

**Vibrational Spectroscopy of 4-Mpy.** Previous SERS studies enable us to address a variety of questions about the state of the 4-Mpy molecules under study. For example, it is possible to assign relatively unambiguous spectroscopic signatures for sulfur covalently binding to the silver, for ring orientation with respect to the local metal surface, for protonation versus deprotonation of the pyridine nitrogen atom, and for thiol versus thione tautomeric forms. Many of these properties are, of course, coupled. For example, sulfur binding to the silver typically enforces a ring orientation with the plane perpendicular to the metal surface, and protonation of the nitrogen tends to be correlated with the thione tautomer. We review the spectroscopic rules of thumb briefly so that we can analyze what is happening to a number of molecules for which we report time series of Raman spectra below. In the spectral region we have covered (∼600–1700 cm$^{-1}$), covalent binding of sulfur to the silver is marked by strong enhancement of the 12a$_1$ mode due to effects of the altered C–S bond on the ring vibrations.[21] The frequency

of the 6a$_1$ mode around 710 cm$^{-1}$ is also shifted to lower frequency than in the bulk spectrum due to Ag–S bond formation.[21] Ring orientation perpendicular to the metal is associated with strength in the in-plane modes[19] and with preferential strength in 12a$_1$, 9a$_1$, and 8b$_2$ relative to those in the solid spectrum.[21] When the pyridine ring is parallel to the surface, a$_2$ modes that are otherwise very weak can often be observed.[21] Protonation is clearly indicated by a shift of the 8b$_2$ mode to higher frequency (from ∼1580 to ∼1605 cm$^{-1}$) as well as the appearance of in-plane N–H modes around 1250 cm$^{-1}$ and out-of-plane N–H modes in the region between 870 and 940 cm$^{-1}$.[22] In addition, the 1100 cm$^{-1}$ 12a$_1$ ring breathing mode weakens substantially,[18] and the 19b$_2$ ring stretch upshifts in frequency by as much as 100 cm$^{-1}$ upon protonation.[22]

**Interpretation of Single-Molecule Spectral Dynamics.** Figure 2 illustrates sequences of events for two different molecules. The first molecule (Figure 2A) initially has spectra
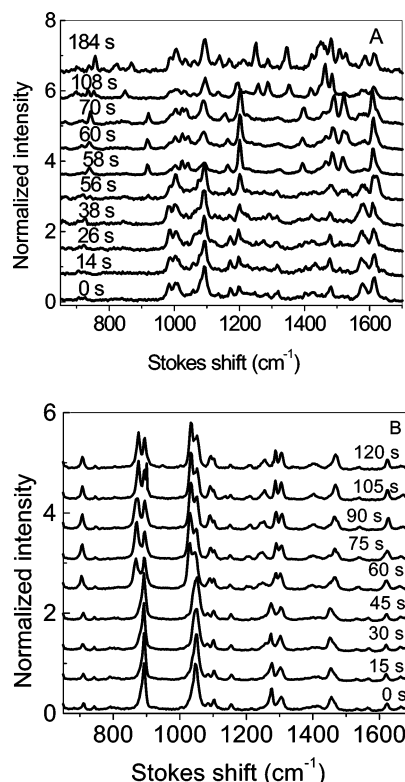


**Figure 2.** Time evolution of the Raman spectrum of two single molecules of 4-Mpy on silver. The laser excitation power is around 10 $\mu$W. The integration time for each spectrum is 2 s for the molecule in (A) and 15 s for the molecule in (B). The spectra are normalized to the amplitude at the scattered wavelength with the maximum number of counts.

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reminiscent of solid 4-Mpy.[21] The most dramatic difference is the strength of the 12a$_1$ mode ($\sim$1100 cm$^{-1}$), which reflects the formation of a Ag$-$S bond to the substrate. The spectrum is stable over a long period of time but changes dramatically after 56 s of observation. The fairly complete spectral switch confirms that we are looking at only a single molecule. The spectrum is characteristic of 4-Mpy with the ring oriented perpendicular to the substrate primarily in its deprotonated form. The abrupt change in spectroscopy observed in the 58 s spectrum and those following clearly reflects protonation of the 4-Mpy nitrogen atom. The 8b$_2$ mode no longer has intensity at 1580 cm$^{-1}$, and the N$-$H stretch appears around 900 cm$^{-1}$. These are accompanied by the characteristic changes in the ring stretching modes, with 14b$_2$ gaining intensity and 19b$_2$ being upshifted by nearly 100 cm$^{-1}$. These changes can be understood in terms of protonation favoring a more quinoidal ring structure and commensurately higher ring stretching vibration frequencies. In addition, the 1100 cm$^{-1}$ mode intensity drops. Concomitant with the protonation, we also observed b$_1$ modes at low frequency, indicating that the 4-Mpy molecule remains strongly bound to the substrate through the thiol and perpendicular to the surface. A new spectrum is observed at 108 s that more closely resembles the original spectrum and may be associated with return to the deprotonated form. This spectrum and that at 184 s, however, also exhibit additional structure and may reflect photodegradation of the molecule.

The 4-Mpy molecule of Figure 2B exhibits much different spectroscopy. Surprisingly, in the spectra from 0 to 45 s, there is no evidence of many of the modes such as ring breathing that typically dominate the spectrum. Instead, the strong modes are associated with C$-$H bending motions (10a$_2$ at $\sim$890 cm$^{-1}$, 18a$_1$ at $\sim$1050 cm$^{-1}$, and 3b$_2$ at $\sim$1300 cm$^{-1}$). The out-of-plane C$-$H bend at 890 cm$^{-1}$ is an a$_2$ symmetry mode that is usually extremely weak unless the ring is lying flat on the silver. The absence of a strong 12a$_1$ band and silence of the 1a$_1$ mode confirm the lack of binding through the sulfur and add support to the hypothesis that interactions between the ring's $\pi$ electrons and the silver are the surface attachment mechanism. In addition, we note the presence (albeit weak in this geometry) of ring stretch (8b$_2$) intensity at 1580 cm$^{-1}$, a marker indicating that the 4-Mpy nitrogen is not protonated. This deprotonation marker disappears in the spectra from 60 to 120 s coincident with the appearance of two bands characteristic of N$-$H attachment, the 1250 cm$^{-1}$ in-plane N$-$H deformation, and a band at 870 cm$^{-1}$ that we assign as out-of-plane N$-$H deformation. Thus, we associate the change in spectroscopy in this case with protonation of the 4-Mpy molecules during the period between 45 and 60 s.

The ability to do single-molecule spectroscopy is essential to being able to observe 4-Mpy molecules with their rings lying flat on the surface since these are sufficiently rare that they would not contribute to an ensemble spectrum. It is also conceivable that they are only present on very sparsely covered surfaces where traditional Raman spectroscopy would not be adequately sensitive to see them. The observation of strong out-of-plane modes (700, 870, and 890 cm$^{-1}$) is consistent with the surface selection rules one might expect since the field would be preferentially perpendicular to the local silver surface and therefore also orthogonal to the plane of the ring. By the same token, we do not see these modes in molecules covalently bound and oriented perpendicular to the silver so that the field would be preferentially in the plane of the ring. The surface selection rules cannot explain, however, why these modes are weak in bulk 4-Mpy spectra. We speculate that out-of-plane modes for
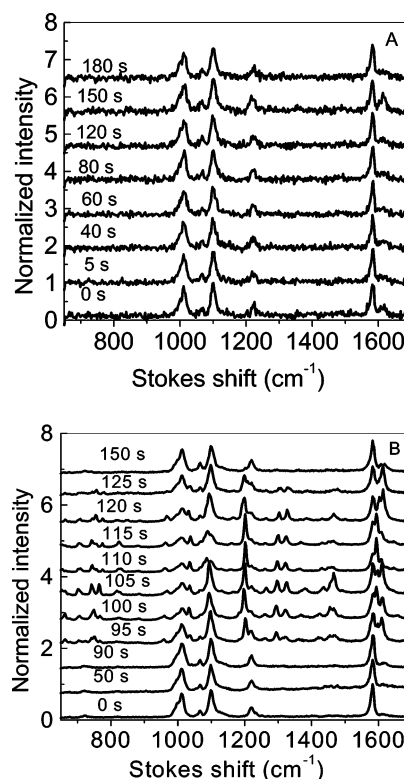


**Figure 3.** Representative Raman spectra versus time at the same location on a saturated 4-Mpy monolayer at low excitation intensity (A, 1.3 $\mu$W) and high excitation intensity (B, 5 $\mu$W). Integration times for all scans are 5 s. The spectra are normalized to the amplitude at the scattered wavelength with the maximum number of counts.

the 4-Mpy molecule lying flat on silver are enhanced because of the importance of resonant charge-transfer states between 4-Mpy and silver to the overall Raman enhancement. There is strong reason to believe that resonance enhancement by charge-transfer bands plays a role in the Raman scattering intensity based on calculations of the magnitude of electromagnetic field enhancement that can be achieved in silver aggregates[17] and on the observation of charge-transfer resonance in similar systems such as pyridine on silver.[25] The contribution of these states would be expected to be strongest for molecules lying flat, a geometry that facilitates strong interactions between the $\pi$ electrons and the metal. Charge-transfer state resonance enhancement would be largest for field polarizations perpendicular to the metal surface, a geometry that can only be achieved for molecules lying flat on the surface. Thus, any electromagnetic fields in the correct orientation to excite out-of-plane Raman-active modes in molecules oriented perpendicular to the silver would not be oriented to take advantage of strong charge-transfer resonance enhancement of the Raman scattering. It is plausible that out-of-plane vibrational modes would enhance the charge-transfer resonance contribution when the geometry is as we have postulated for the molecule in Figure 2B. We do not understand why in-plane bending modes such as 18a$_1$ and 3b$_2$ also appear to be associated with resonance enhancement of the charge-transfer band since we do not observe these when the ring is perpendicular to the plane of the silver.

**Raman Spectroscopy of Densely Adsorbed 4-Mpy.** For comparison, we have studied saturated monolayers of 4-Mpy on similar silver surfaces. The temporal evolution of the Raman spectra for a saturated 4-Mpy monolayer at two different powers on the same hot spot is illustrated in parts A (low power) and B (high power) of Figure 3. The spectrum at low power is very stable and is dominated by only a few modes. It is characteristic
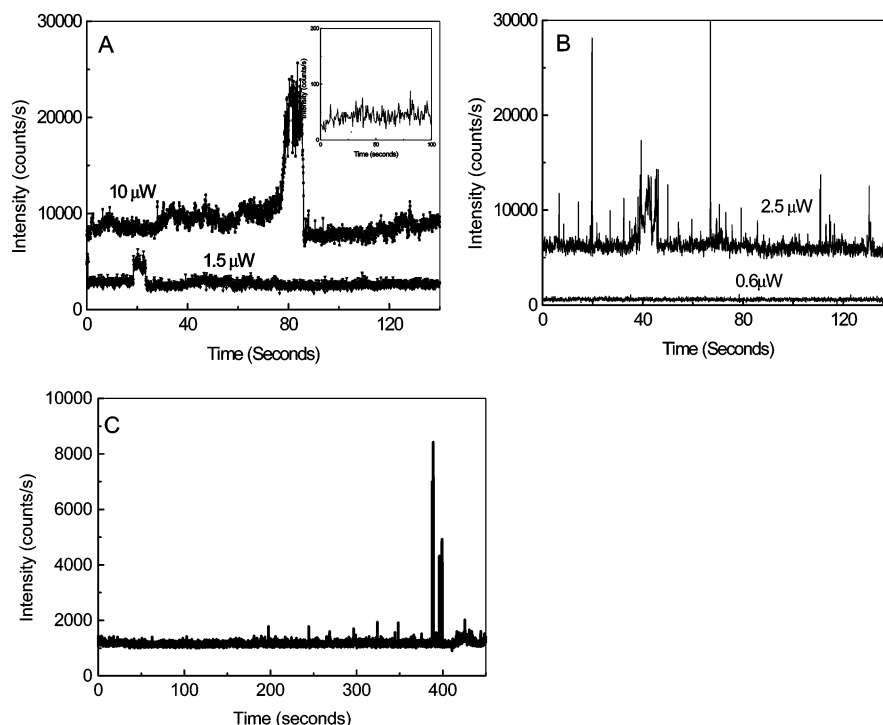
**Figure 4.** Spectrally integrated surface-enhanced Raman scattering versus time for single 4-Mpy molecules (A) and saturated 4-Mpy monolayers (B) at low excitation intensity (lower traces) and high excitation intensity (upper traces) as labeled in the Figure. The inset in (A) is a measurement of the dark counts. The case for saturated monolayers at high excitation intensity as a function of temperature (i.e., during evaporation of liquid nitrogen) is shown in panel C.

of deprotonated 4-Mpy molecules covalently bound to the silver through a Ag−S bond with the ring perpendicular to the metal surface. Increasing the power leads to the onset of blinking, although generally not as dramatic as for the case of single molecules under similarly high power. One remarkable feature of the data is that the magnitude of Raman scattering from single molecules is comparable to that from the saturated monolayer. A rough estimate says that the illuminated region in our confocal microscope is on the order of 0.3 $\mu$m in diameter or $\sim 10^{-9}$ cm$^2$ so that our signal should derive from a dense monolayer containing $\sim 10^5$ adsorbed molecules. Because electromagnetic enhancements of the Raman scattering can be as large as 14 orders of magnitude when molecules reside in hot spots,[8,16] it is still possible for one or a few molecules to dominate the spectrum of the monolayer. In fact, since hot spots only concentrate the incident field to small dimensions but do not increase the intensity averaged over the beam, this result is not surprising. The dominance of only a few molecules also explains why it is possible to see large fluctuations in Raman intensity even when the Raman scattering is collected from such a large ensemble. The diminution in the amount of spectral fluctuations relative to that of the single-molecule case is consistent with the reduction of orientational freedom in a densely packed layer.

**Evidence for Thermal Origin of Raman Blinking.** Figure 4 depicts the Raman intensity integrated over all modes from 650 to 1700 cm$^{-1}$ for the case of single 4-Mpy molecules (Figure 4A) or saturated monolayer coverages of 4-Mpy (Figure 4B). In this case, the data are recorded with much higher time resolution using a photomultiplier with counting electronics. At low excitation intensities, the signal is quite stable but exhibits strong fluctuations when the intensity is increased. The data of Figure 4C illustrate that the fluctuations observed at high laser intensity can be suppressed when the surface is cooled with a drop of liquid nitrogen but resume when the nitrogen evaporates. This implicates laser-induced heating of the silver surface[26] as

the trigger for the blinking and apparently contrasts with the conclusions of Weiss and Haran, who ascribed the blinking to charge-transfer excitations in their elegant Raman studies of rhodamine on silver.[7] We do not think that photoionization of 4-Mpy via charge-transfer excitations would be intrinsically temperature dependent, but it is certainly possible that thermal reorientation of the molecules can greatly affect the resonance enhancement of Raman scattering by charge-transfer states as we have discussed above in connection with the spectroscopy observed in Figure 2. The fact that we can understand the spectral changes in terms of microscopic processes such as reorientation or protonation also mitigates against photoinduced charge transfer and subsequent recovery as the reason for blinking. Finally, we have seen little evidence for Raman spectra characteristic of ionic forms of 4-Mpy whose spectroscopy is well-known.[18] Our conclusion is that thermally induced orientation and chemistry are the root cause of Raman blinking though some of their effect may be through modulating the contribution of charge-transfer resonance to the Raman enhancement.

We also believe that changes in the silver configuration are unlikely to be responsible for the blinking in our experiments. We have never observed the disappearance of a hot spot, and we often see nearly reversible spectroscopy such as in the case of the molecule studied in Figure 2A.

## Conclusions and Perspectives

We have showed that molecular rearrangements and chemistry that are stimulated by thermal effects are important root causes of spectral and intensity fluctuations in single-molecule Raman spectroscopy. Assignment of time series of spectra provides clear evidence that protonation and deprotonation of single 4-Mpy molecules can be observed. These chemical transformations and other molecular reorientation can be suppressed in 4-Mpy by using very low laser power or by working at low temperatures. The ability to track structural and chemical

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transformations of single molecules is remarkable and may someday provide useful insights into reaction mechanisms. Our data also show that single molecules can dominate the Raman spectra of even large ensembles when the laser focus is on a hot spot. This may complicate interpretation of surface-enhanced Raman spectroscopy in potential sensor applications. More work needs to be done to control the location of hot spots and to interpret observed Raman spectra in the presence of complex silver morphologies and local electromagnetic field distributions.

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