Chapter 5

Excitation in optical resonance: Malachite green isothiocyanate

The combination of spectroscopy and microscopy at nanometer scale renders TE(R)RS an interesting analysis tool that could provide also promising advantages for the ultra-sensitive characterization of biophysically relevant species down to the single-molecule (SM) level. The sensitivity of surface-enhanced Raman spectroscopy, however, has not quite been reached yet with TERS. Enhancement factors of up to 10^{14} have been reported to be necessary for single-molecule detection with SERS. For TERS, maximum enhancement factors of 10^6 to 10^7 have been achieved, which seems to be many orders of magnitude away from the SM detection limit.[53]

In this chapter, the possible application of tip-enhanced resonance Raman spectroscopy as a single-molecule analytical tool is demonstrated. TERR spectra of the triaryl dye malachite green isothiocyanate (sketched in Fig. 5.1) on Au(111) are investigated. We chose MGITC, because its absorption maximum coincides with our excitation wavelength at 632.8 nm (Fig. 5.2). Thus, we make use of additional resonance Raman enhancement. MGITC is adsorbed at a Au(111) single crystal from six differently concentrated solutions. Even at the lowest adsorbate concentration of approximately 0.7 pmol/cm^2 (around 5 molecules present in the enhanced-field region), the unambiguous identification of the target molecule is possible. When taking into account theoretical calculations of the near-field extension along the surface,

as presented in Chapter 2.5.1, we indeed reach the SM detection level. From the recorded STM images, it is apparent that, at maximum coverage, MGITC does not form a densely packed monolayer on Au(111) upon the given experimental conditions. Despite the suboptimal resolution, single MGITC molecules are discernible at low adsorbate concentrations in the images in form of ~ 1 nm sized dots. In correlation with the corresponding STM images, the sensitivity of TERRS as a probe for the detection and semi-quantitative determination of the dye coverage is highlighted.

5.1 Experimental part

MGITC is purchased from Invitrogen and dissolved in ethanol to give 4.9 x 10^{-5} M, 1.2 x 10^{-6} M, 2.3 x 10^{-7} M, 1.2 x 10^{-7} M, 2.3 x 10^{-8} M and 1.2 x 10^{-8} M solutions which are used to prepare samples with different adsorbate concentrations as described in Chapter 3.

Essential for obtaining a good TERR spectrum – and the main difficulty of the experiment – is the precise focussing of the laser beam onto the tip apex. At the same time, the focussing time has to be reduced to a minimum in order to avoid (photo)bleaching of the dye. For each concentration, several TERR spectra are recorded at different tip positions along the substrate surface to always guarantee a fresh, unbleached sample, searching for maximum spectral intensities. At low dye concentrations, very few sample locations tested for TERR scattering lead to a reasonably intense and well-defined spectrum, because the statistical chance of "finding" an adsorbate molecule adds to the problems of degradation of the species and bad focussing. Only fingerprint spectra that exhibit the typical MGITC Raman features (and no carbon signals) are used for further analysis.

5.2 TERR fingerprint of MGITC

Another advantage of MGITC is that it is known to bind strongly to gold with the sulphur atom of its isothiocyanate group. Therefore, it is quite immobilized and stable

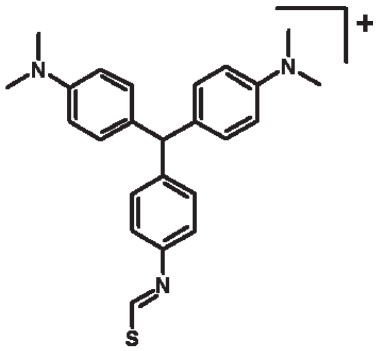

Figure 5.1: Malachite green isothiocyanate (MGITC) is a triaryl dye with two amino (-NR_2) and one isothiocyanate (-NCS) functional groups. With the latter, it can form a strong sulphur-Au bond with the substrate.

on the substrate surface and we assume a stable, well-defined adsorption geometry during the duration of the STM and Raman experiments, even when working in air.

In Fig. 5.3, a typical TERR spectrum of a monolayer MGITC adsorbed at a Au(111) substrate is shown. Its characteristic spectral features such as band positions and relative intensities can be compared to spectra reported in the literature for MGITC in solution [110] and allow the unambiguous identification of the triaryl dye. In the absence of the tip, no Raman bands are detectable (at short integration times). One of the advantages of carrying out Raman spectroscopy at metal substrates is that the fluorescence of the resonant adsorbate is sufficiently quenched for Raman bands to be easily detected. The three most prominent bands appear at 1618 cm^{-1}, 1370 cm^{-1} (with a shoulder at 1393 cm^{-1}) and 1180 cm^{-1}, in addition to several other characteristic features, like the strong bands at 1586 cm^{-1}, 807 cm^{-1} and 448 cm^{-1} (with a shoulder at 428 cm^{-1}). Although no thorough assignment of the Raman bands of MGITC has been published in the literature, these spectral features represent a fingerprint of the target molecule which allows its clear identification.

5.3 MGITC single molecule analysis

The investigation of single molecules has several advantages over the study of molecular ensembles: In order to get a more complete picture of the molecular states and environment, multiparameter studies that provide information on spectral intensity,

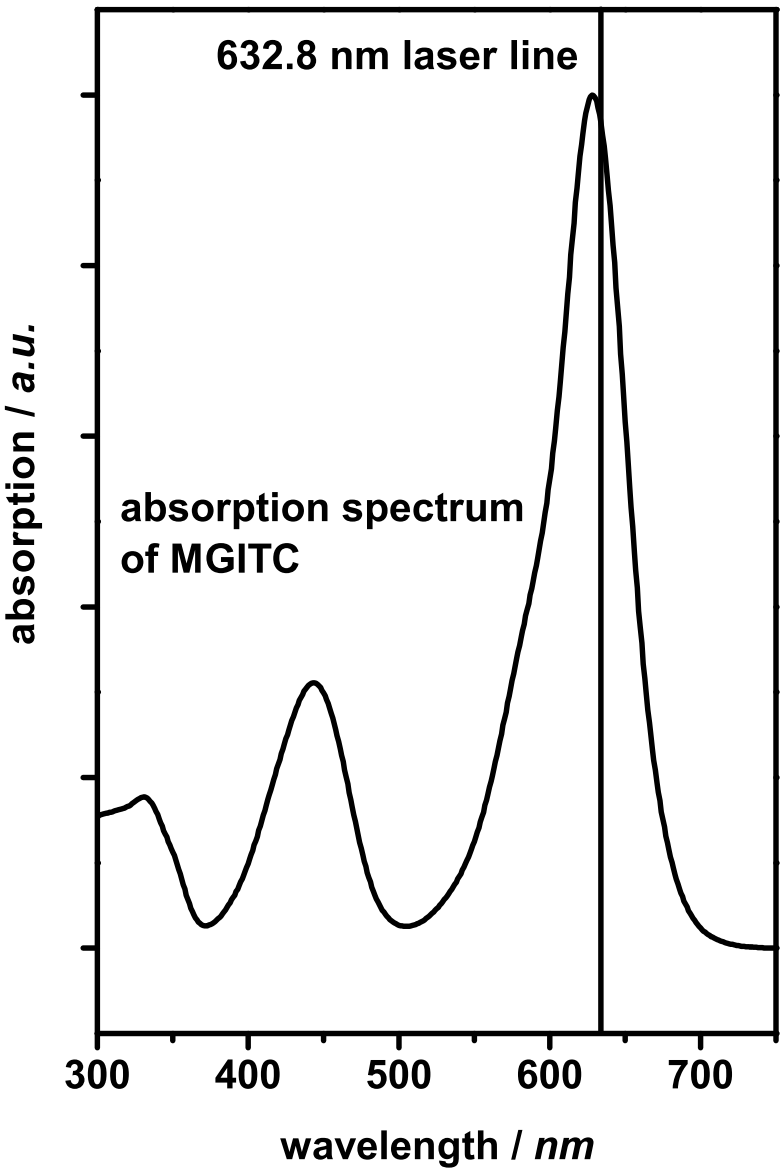

Figure 5.2: The absorption maximum of malachite green isothiocyanate coincides with the 632.8 nm laser line, i.e. MGITC has vibrational transitions which are in resonance with the excitation frequency. Absorption spectrum reproduced from Ref. [109].

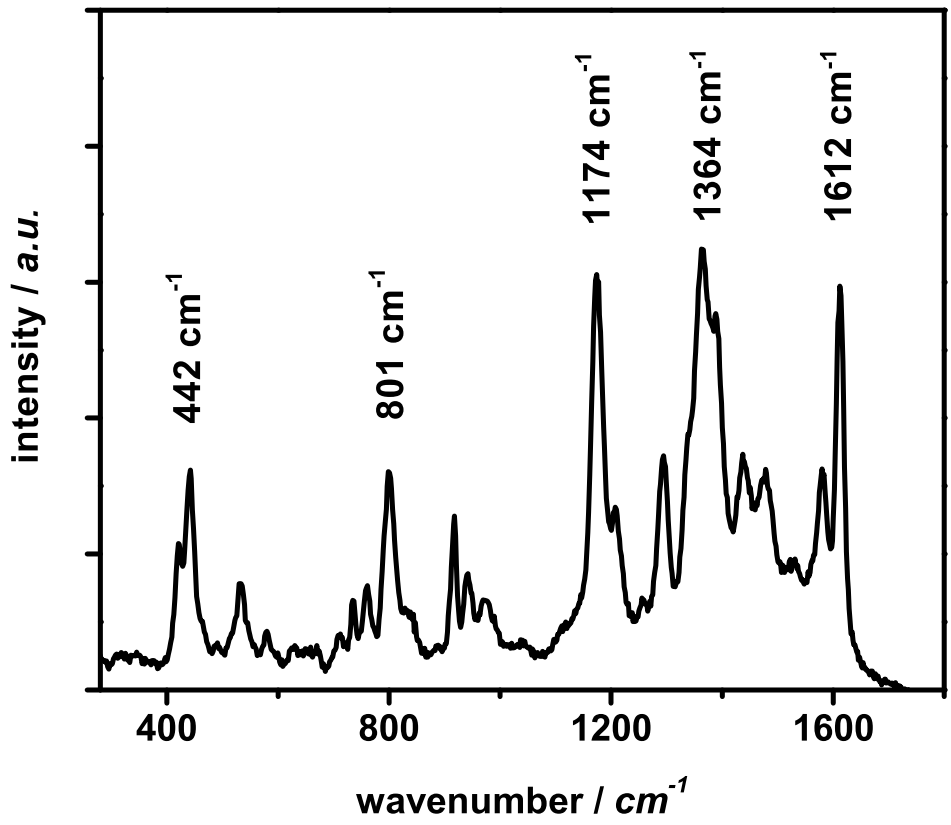

Figure 5.3: The TERR fingerprint spectrum (background-corrected) of a monolayer malachite green isothiocyanate adsorbed at Au(111) exhibits extremely intense Raman bands, because in addition to the strong field enhancement, the resonance of the molecule itself enhances the scattering intensities. Integration time: 1 s, laser power at sample: 2 mW.

lifetime, polarization anisotropy and band position at the same time are carried out. In ensemble studies, an average picture of the properties of individual molecules that might not be identical is created.

Chemical differences, like protonation states or tautomerisms, geometrical variation, such as cis/trans- and R/S-conformers or folding behaviour, or diverse adsorption at terrace, step or kink sites as well as adsorbate interactions are fundamentally stochastic features which cannot be observed in bulk measurements, because the molecules in an ensemble are difficult or impossible to synchronize. SM studies help to gain a deeper understanding of molecules and their chemical and physical behaviour.

Up to date, optical detection of single molecules is mainly carried out by fluorescence spectroscopy because of the relatively high photon count rates that can be reached.[111–114] However, the number of molecules with intrinsic fluorescence is limited,[115] and often the investigated species have to be modified by a fluorescent moiety that acts as a "reporter".[116, 117] The study of single molecules by enhanced Raman spectroscopy is developing into an interesting alternative as it gives comparable photon count rates, allows the analysis of unmodified species (no labelling) and provides more detailed information about molecular structure than fluorescence spectroscopy.[32, 33, 65, 71, 118–120]

We have carried out a concentration study on MGITC/Au(111) in order to test the detection limit of TERRS. Figure 5.4 shows STM images of the Au(111) surface after adsorption of MGITC from differently concentrated solutions. Figure 5.4A displays the substrate densely covered with adsorbate after 15 hours adsorption from a 1.2 x 10^{-6} M solution. It is clearly seen that the MGITC molecules do not form a uniform monolayer (ML) even after an extended adsorption time, but give a rather irregular distribution of dot-like structures. Figures 5.4B to 5.4F display a series of images recorded after one hour adsorption time from increasingly diluted solutions (4.9 x 10^{-5} M, 2.3 x 10^{-7} M, 1.2 x 10^{-7} M, 2.3 x 10^{-8} M and 1.2 x 10^{-8} M, respectively). The images have been plane-flattened, but not modified any further.

For Figs 5.4B to 5.4F, the number of adsorbates discernible in the images diminishes with decreasing concentration. Size and geometry of the small, dot-like structures do not vary. At the highest investigated concentration, the Au surface is densely covered after one hour adsorption time; however, the adsorbate does not seem as

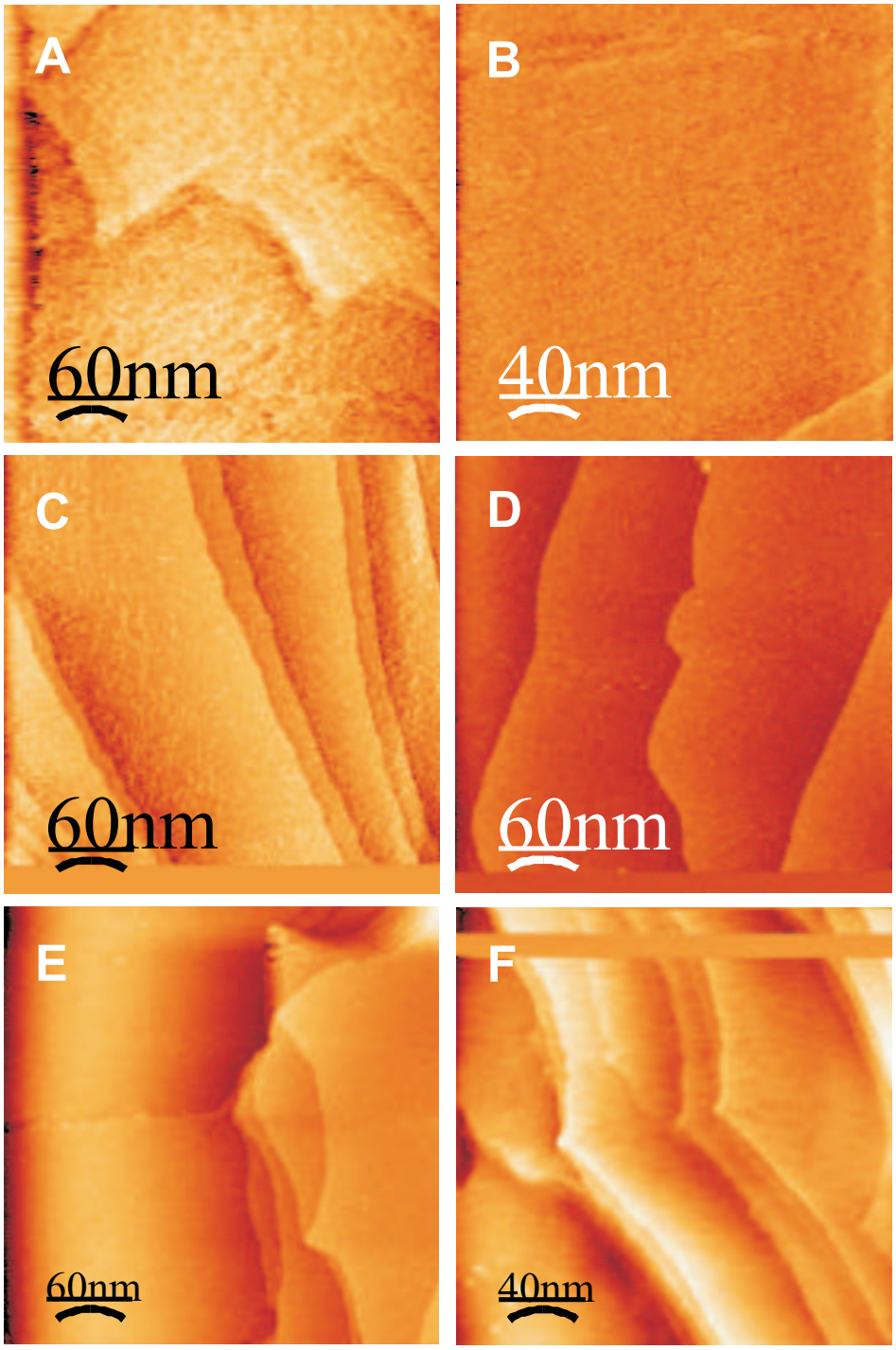

Figure 5.4: STM images of MGITC/Au(111) at different estimated adsorbate concentrations: A) 170 pmol/cm^2, B) 73 pmol/cm^2, C) 33 pmol/cm^2, D) 24 pmol/cm^2, E) 1 pmol/cm^2, and F) 0.7 pmol/cm^2 (refer to text for detailed description of the determination of the surface coverages). U_{bias} = -100 mV, I_t = 1 nA, scan area as indicated.

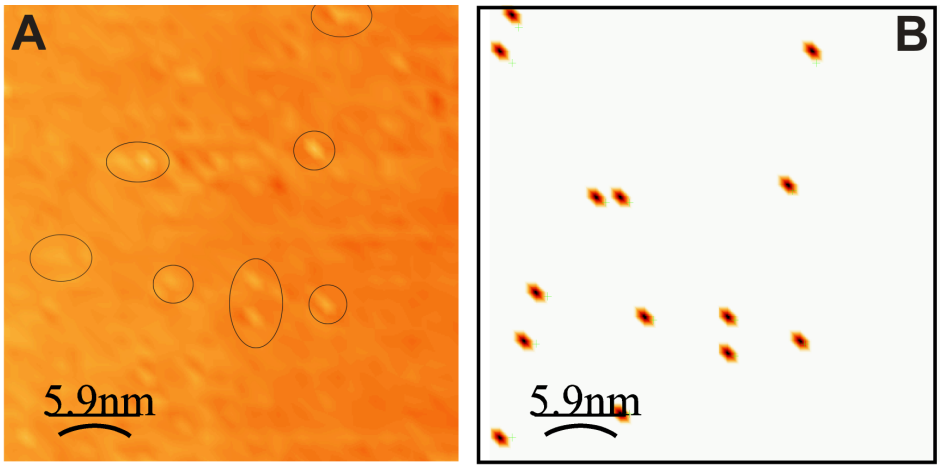

Figure 5.5: Visualization of nanometer-sized dots in STM images. A) Zoom into an STM image recorded at medium high surface coverage of MGITC at Au(111) (approx. 1 pmol/cm^2). Circled areas mark the nanodots of around 0.15 nm height that have been detected by the flood scan (parameters: size minimum = 0 nm, height = 0.15 nm) of the zoom image depicted in B).

tightly packed as in Fig. 5.4A (15 hours adsorption). At the lowest investigated concentration, the Au substrate seems smooth aside from monoatomic steps in the substrate. No adsorbate molecules can be discerned in the image, and the few dots that should appear cannot be separated from noise.

A flood scan of a zoom image visualizes the nanometer-sized structures that appear in the image recorded of around 1 pmol/cm^2 coverage. Fig. 5.5A shows the zoom image; some of the detected nanostructures are circled as a guide to the eye. The flood scan parameters were set to size minimum = 0 nm and height = 0.15 $\pm$ 0.01 nm. The resulting scan is depicted in Fig. 5.5B. There are clearly more nanodots visible in the zoom, but they are not detected by the flood scan as they do not match the height parameter setting.

In order to get a size estimate of the adsorbate structures seen in the images, we compare them to the monoatomic steps of the substrate present in every image. Figure 5.6 shows two typical surface profiles for a clean, atomically smooth Au(111) surface (Fig. 5.6A) and for Au(111) covered with MGITC (Fig. 5.6B), respectively. The height of a Au step is found to be around 2.4 Å. The small structures are of approximately 1.5 nm diameter and appear visibly lower than the Au steps (around

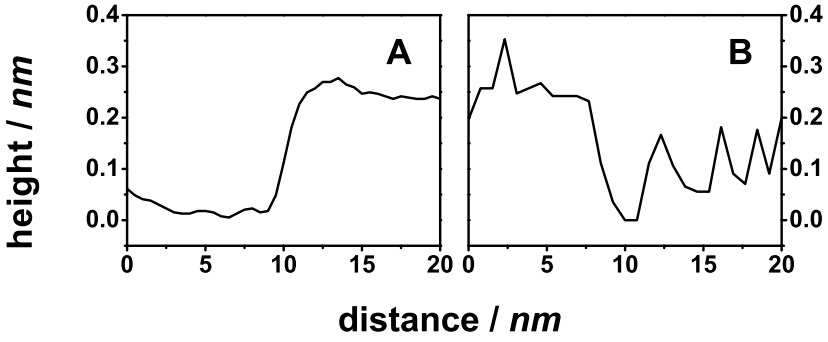

Figure 5.6: Typical STM line profiles for a Au(111) surface without/with MGITC adsorbate. A) Atomically smooth (clean) Au(111) surface, Au step height approximately 0.24 nm. B) The observed adsorbate structures appear of approximately 0.13 to 0.18 nm height and 1.5 nm diameter. A monoatomic Au step of 0.24 nm height is clearly distinguishable from the nanodots.

1.3 to 1.8 Å). They do not form a densely packed monolayer, but are separated by approximately 1 to 1.5 nm. Unfortunately, the experimental set-up does not allow atomic or molecular resolution because of missing vibration and temperature isolation and thus aggravates an accurate size analysis of the structures.

In the recorded STM images, an apparent dependence of the surface coverage on the concentration of the adsorption solution is seen: Even for a high concentration of 4.9 x 10^{-5} M (which corresponds to an adsorbate concentration of 2 x 10^{16} molecules/cm^2 if all molecules from a 5 mL volume are adsorbed), completion of a ML cannot be observed after one hour adsorption time. Kikteva *et al.* determined the maximum coverage of malachite green (MG), a similar dye lacking the SCN-group, to be 1.6 x 10^{14} molecules/cm^2 (one molecule occupying an area of approximately 60 Å^2) on silica by optical second harmonic generation (SHG) experiments.[121]

At a first approximation, taking into account typical bond lengths, one MGITC molecule occupies a volume of $1.2\,\mathrm{x}\,1\,\mathrm{x}\,0.2\,\mathrm{nm}^3$. It is known from literature that MG adapts an inclined orientation ($\sim 55°$) at silica or mica surfaces.[121, 122] Hence, a similarly tilted adsorption geometry is assumed for MGITC on gold, which is supported by the intense TERR spectra that are recorded. According to the surface

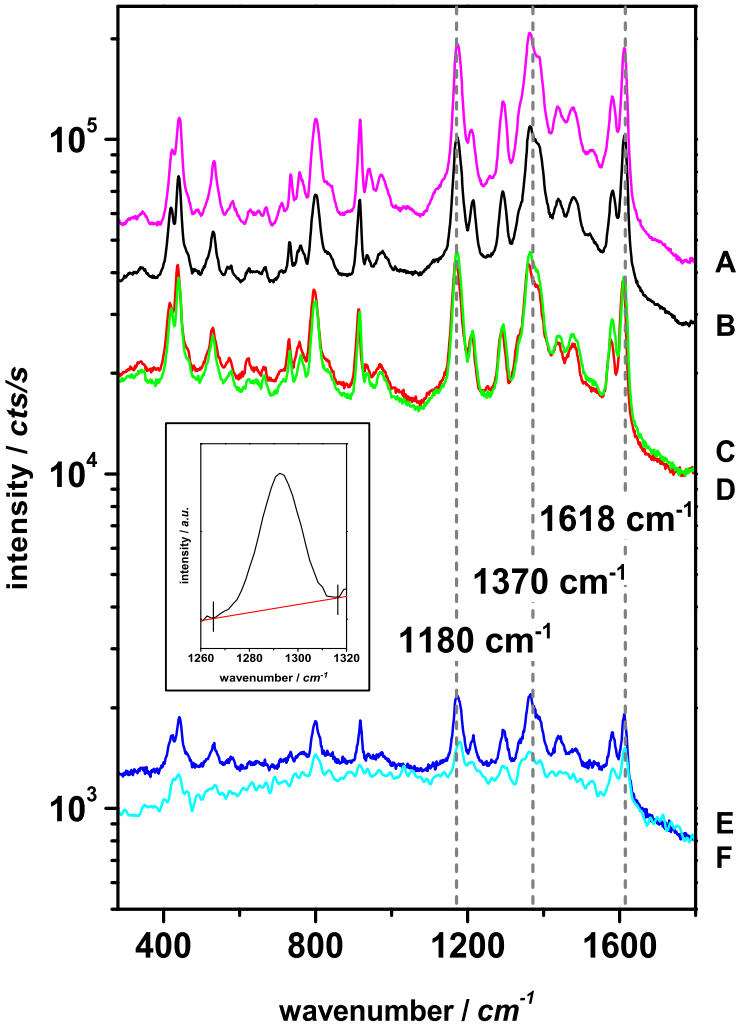

Figure 5.7: Average TERR spectra of MGITC/Au(111) at different adsorbate concentrations A (highest coverage) to F (lowest coverage), normalized to full laser power (2 mW at sample) and 1 s integration time. The inset shows an example of the straight baseline employed to obtain the Raman band intensity.

selection rules, only vibrational modes with changes of polarizability in z-direction perpendicular to the surface will be strongly enhanced.[123] For a 55°-inclined MGITC molecule, the surface area occupied amounts to approx. $0.6\,\mathrm{x}\,1\,\mathrm{nm}^2$ according to the above-mentioned dimensions of the molecule. The structures found in the STM images are somewhat larger than expected for single MGITC molecules. This is attributed to the suboptimal lateral resolution of our instrument.

In spite of the chemical S-Au bond, surface diffusion of the molecules at room temperature cannot be ruled out. Supplementary experiments performed upon UHV conditions with a high-resolution STM did not produce better images[1]. Cooling down the system to 200 K might help to improve the resolution and enable to monitor single MGITC molecules in detail. This work is in progress.

Note that it was very difficult to obtain STM images at high MGITC coverage due to the high resistivity of the adsorbate. A similar attempt by Möltgen and Kleinermanns to obtain well-resolved STM images of MG on graphite also failed because of the small electrical conductivity of the adsorbate.[124] As the tunneling current perceives the high resistivity of the molecule, the tip approaches further. Thus, we measure a small effective molecular height of 0.2 nm, although an inclined MGITC molecule sticks out approximately 1 nm from the surface.

Nevertheless, we conclude that the observed structures represent single MGITC molecules. The mean distance between the clusters is in the range of the cluster size which results in a mean area of around 100 $\mathrm{Å}^2$ per MGITC molecule or 1 x 10^{14} molecules/cm^2, a slightly lower ML coverage than estimated by Kikteva *et al.* for MG at silica.[121] The actual surface concentration could not be directly determined up to now, for instance, from fluorescence spectroscopy of re-desorbed species, because it was below the detection limit.

Figure 5.3 shows a typical fingerprint TERR spectrum of MGITC. The three prominent bands at 1618 cm^{-1}, 1586 cm^{-1} and 1370 cm^{-1} (with a shoulder at 1393 cm^{-1}) are assigned to phenyl-N stretch, and to the ring breathing and stretching of the aromatic ring according to Lueck *et al.*[110] In Fig. 5.7, an overview of the TERR spectra recorded at different adsorbate coverages shows that a fingerprint of MGITC

[1]UHV experiments were performed in the laboratory of Dr. Thorsten Kampen, Fritz Haber Institute

is obtained even for a very low number of scatterers.

The corresponding six TERR spectra from MGITC/Au(111) at different surface coverages are plotted in a semi-logarithmic plot in Fig. 5.7. The spectra are averaged over five spectra recorded at different sample locations, and spectrum 5.7F has been smoothened to improve the signal-to-noise ratio. Interestingly, band and background intensities decrease co-linearly. This is evident from Fig. 5.7 by comparing the geometrical heights of the various peaks in curves A-D and the corresponding backgrounds. Even at very low surface concentrations of MGITC, the spectral characteristics allow an unequivocal identification of the molecule. All experimental spectra display a large background due to fluorescence scattering (compare Chapter 4.3).

As no band shifts or changes in relative band intensities are observed with variation of the adsorbate concentration, it is concluded that neither the orientation of the molecules with respect to the surface nor the interaction between the adsorbed molecules vary substantially with the surface coverage and thus are negligible for the resulting Raman spectra. Even at the lowest investigated surface concentration, contamination of the sample does not cause problems, although the experiments are performed in air.

Note that the concentration of the solution cannot be related directly to the adsorbate concentration at the surface, although roughly a factor of ten less TERR spectral intensity is found when decreasing the adsorbate solution concentration from 10^{-7} M to 10^{-8} M (compare Fig. 5.7). In general, this relation will, among other factors, depend on the substrate geometry, the surface diffusion of the molecules and the concentration itself. It has been shown in concentration-dependence SERS studies that linearity between Raman intensity and solution concentration is given only for very low concentrations in the range of 10^{-9} M to 10^{-7} M.[125–127] As can be seen from our experiment, the adsorption time also plays a crucial role.

In fact, the only linear indicator for the surface coverage and, thus, the number of Raman scatterers is the measured Raman band intensity. As the saturated photon absorption regime is not approached in these experiments, it is reasonable to expect a linear relationship between the number of Raman scatterers and the Raman band intensity.

To calculate the number of scatterers at each surface concentration, we take into

account the maximum coverage and the corresponding spectral intensity, following the linear dependence of the TERR band intensity on the surface concentration of MGITC. To obtain the Raman band intensities, a straight baseline is set at the bottom of the Raman band and integrated between the intersects of baseline and band feet, as shown in the inset of Fig. 5.7. The area of the enhanced field is estimated to $A_{ef} = \pi r_{ef}^2 = 1257$ nm^2 with $r_{ef} = 20$ nm at Heaviside approximation. Comparing the size of a MGITC molecule with the structures found in our STM images, we estimate that one molecule occupies approximately 1 nm^2. Thus, we presume a highest surface coverage N_A of 1 x 10^{14} molecules/cm^2, which corresponds to around 1260 molecules present in the enhanced-field region at maximum coverage.

We analyze the intensities of three different MGITC bands (deliberately chosen) at 1298 cm^{-1}, 1586 cm^{-1} and 1618 cm^{-1} as well as the total spectral intensity between 100 and 1800 cm^{-1} to determine the number of resonant Raman scatterers present in the enhanced field. According to $(I_A/N_A) = (I_X/N_X)$, we calculate the number of scatterers N_X present for different concentrations X from the integrated intensities I_X. For example, the 1298 cm^{-1} band gives a total intensity I_A of 9.84 x 10^5 cts/s for the highest adsorbate concentration N_A, or 783 cts/s per molecule - and consequently, we calculate that only 5 molecules are present in the enhanced field at the lowest concentration. Table 5.1 contains an overview for the series of experiments with different concentrations of MGITC.

For 2.3 x 10^{-7} M (2.3 x 10^{-8} M) solutions, an average number of scatterers of 248, 261, 261 and 338 (8, 9, 9 and 22) is calculated for the different MGITC bands and the background, respectively. For the lowest adsorbate concentration investigated in this study, this calculation leads to remarkably low numbers of 5, 6, 6 and 18 MGITC molecules detected with TERRS. Relating to the band intensities, which are unquestionably due to inelastic scattering of the adsorbate, the sensitivity reached with TERRS has approached the SM detection level.

The measured band intensities of the three bands give similar number of scatterers for all measured solution concentrations, whereas the integration of the total spectral intensity leads to a slightly higher number. This may be due to the fact that there are indeed some small contributions from contamination or from weak luminescence of the metal substrate itself. The discrepancies are quite small, however, and the

comparison underlines the potential of TERRS as a SM analytical tool.

In addition, this calculation has the tendency to yield too large a number of scatterers. The real number of scatterers is likely to be smaller for two reasons:
1) According to theory, the effective TER(R) radius (i.e. the radius of the surface area from which 63% of the signal originates) is somewhat smaller than the radius of the tip and the enhanced-field radius.[53, 56] Approximating the distribution of the enhanced field by a Gaussian profile, the TER(R)S radius is half the field radius ($r_{TER(R)S} \approx (1/2)r_{ef}$), reaching a lateral resolution of 10 nm.[53] According to Rendell *et al.*,[56] the localization length of the field is $\sqrt{2\,r_{tip}\,d} = 7$ nm for a tip radius of 20 nm and a tunneling distance of $d = 1$ nm, while Eqn A15 from Ref. [53] yields 9 nm. The lateral resolution is then 14 nm and 18 nm, respectively. Recalculating the number of scatterers present in the enhanced field with $r_{ef} \leq 1/2r_{tip}$, i.e. with the theoretically calculated area from which the Raman scattering stems, we clearly arrive at 1 or even <1 molecule. However, as these relations have not been proven experimentally, the approximation $r_{TER(R)S} = r_{ef} = r_{tip}$ is very generous, but safer.

Table 5.1: An overview on the integrated spectral intensities (I) and the corresponding calculated numbers of scatterers in the enhanced-field region (sc/ef) for the differently concentrated adsorption solutions A-F, respectively.

analyzed band		**A** $\mathbf{1.2 \times 10^{-6}}$ **M**[a]	**B** $\mathbf{4.9 \times 10^{-5}}$ **M**	**C** $\mathbf{2.3 \times 10^{-7}}$ **M**	**D** $\mathbf{1.2 \times 10^{-7}}$ **M**	**E** $\mathbf{2.3 \times 10^{-8}}$ **M**	**F** $\mathbf{1.2 \times 10^{-8}}$ **M**
100-1800 cm^{-1}	I $[cts/s]$	1.32×10^{8}	8.02×10^{7}	3.55×10^{7}	3.46×10^{7}	2.28×10^{6}	1.89×10^{6}
	sc/ef	1257	764	338	330	22	18
1298 cm^{-1}	I $[cts/s]$	9.84×10^{5}	4.90×10^{5}	1.94×10^{5}	1.66×10^{5}	6.33×10^{3}	3.80×10^{3}
	sc/ef	1257	626	248	212	8	5
1586 cm^{-1}	I $[cts/s]$	1.32×10^{6}	5.80×10^{5}	2.74×10^{5}	1.91×10^{5}	9.80×10^{3}	5.40×10^{3}
	sc/ef	1257	552	261	182	9	5
1618 cm^{-1}	I $[cts/s]$	2.34×10^{6}	1.26×10^{6}	4.58×10^{5}	4.33×10^{5}	1.46×10^{4}	1.04×10^{4}
	sc/ef	1257	677	261	233	9	6

[a] 15 hrs adsorption time

2) Besides, it has been shown that the surface-enhanced scattering intensity per molecule is smaller for a densely packed dye layer due to resonant absorption than for lower adsorbate concentrations.[128–130] Pettinger *et al.* found that it varies inversely with the square root of the number of scatterers: $I_{ERS}/n \approx n^{-1/2}$.[131, 132] This means that decreasing the surface coverage by two orders of magnitude from 1000 to 10 molecules present in the enhanced field leads to roughly a factor 10 more scattering intensity per molecule. Therefore, the alleged scattering rates per molecule have to be corrected for lower adsorbate concentrations. As a result, the real number of scatterers is expected to be clearly smaller than stated above, in fact reaching the SM level, where only one molecule is present in the enhanced-field region.

It is often stated in literature that an enhancement of 10^{14} or 10^{15} is needed in order to explain SM SERS.[32, 71] However, the degree of electromagnetic and resonance enhancement should be discussed. Typical cross sections of normal Raman scattering for small, nonresonant molecules like water lie in the range of 10^{-30} cm^2. For the usually employed, much larger dye molecules, we can expect an increase of a few orders of magnitude to $\sim 10^{-28}$ cm^2. In addition, resonance enhancement of the adsorbate plays a significant role and further increases the cross section.[32, 133, 134] A reasonable effective cross section for a resonant dye would then lie around 10^{-23} cm^2. In order to reach the SM detection level of around 10^{-16} cm^2, an additional electromagnetic enhancement of 7 orders of magnitude thus is sufficient. This has been reached with TERRS, as shown in Ref. [53] and indirectly with the high sensitivity reported in this chapter. Hence, single molecules can be detected with TERRS.

Often, SM SER(R)S studies report of a "blinking" phenomenon.[32, 119, 135] Nie *et al.* state that Raman signals suddenly disappear or change after a few minutes of continuous illumination, which suggests that each molecule is adsorbed at a different site (on a rough surface).[32] Michaels *et al.* find an "on/off behaviour" of the investigated silver colloids, as well as variations in the integrated intensities and slight band shifts of rhodamine 6G.[119] The "blinking" is attributed to thermally driven desorption and readsorption of the molecule(s).

In our case, we do not find any blinking of the TERR spectra, even for the lowest adsorbate concentrations. The only changes observed between individual spectra could always be attributed to carbonaceous contamination, i.e. degradation of the

adsorbate. This is in agreement with results from Hartschuh *et al.*, who did not observe blinking behaviour for TER spectra of carbon nanotubes on glass.[136] The main difference between SERRS and TERRS lies in the fact that the latter is carried out on smooth, single crystal surfaces. This means that, in principle, all adsorption sites for MGITC are spectroscopically equal, and molecular diffusion will not lead to changes in the spectra as long as the molecules stay in the enhanced-field region.

A similar SM study was performed by Neacsu *et al.* for MG on evaporated Au films.[65] However, they do not obtain fingerprint spectra of the adsorbate at SM concentration, which would allow clear identification of the molecule. Exposing the sample to long-time illumination (100 s with full laser power of 2 mW), it is likely that the dye is (photo)bleached and that the spectra then show carbonaceous contamination (see Chapter 5.4 for discussion on the contamination problem in enhanced Raman spectroscopy).

As we observe a linear decrease of the background with the Raman scattering intensity (and thus with the surface coverage), we confirm that the background stems from the adsorbate (or a metal-adsorbate complex) and represents enhanced fluorescence, as discussed in Chapter 4.3. For the recorded TERR spectra from Au covered with adsorbate, contamination bands are of negligible significance even at lowest surface coverages, because the resonantly enhanced bands of MGITC (as well as the enhanced fluorescence background) always appear much stronger. The new set-up described in Chapter 7 will allow the working in controlled environment, such as inert gas atmosphere or vacuum.

5.4 The carbon problem

Sometimes it appears to be difficult to distinguish between the target molecule and contamination or decomposition of the sample, and, unfortunately, it is not always easy to ensure that the observed Raman features actually stem from the investigated species. It is likely that a significant number of publications on enhanced Raman spectroscopy present data that exhibit spectral features of carbonaceous species that may stem either from decomposition or from contamination of the sample. The vital

question that arises in enhanced Raman studies is: How can we distinguish between the target molecule and carbonaceous species?

Far-field (normal) Raman spectra, in general, do not vary substantially from the corresponding near-field spectra. Only small changes in the band positions between near-field and far-field spectra have been observed, which are due to the interaction of the adsorbed molecule with the metal substrate. Examples can be found in the literature for resonant (rhodamine 6G [15, 137] and malachite green [110, 138]) as well as for non-resonant adsorbates (DNA bases [139–141]). Large band shifts are not expected, especially not for physisorbed large organic molecules like the often employed dyes, because the large molecular electronic structure is hardly affected by a small orbital overlap.

Often, changes in the surface selection rules are called upon in order to explain a drastic change in a near-field versus a far-field Raman spectrum.[65] This has never been proven neither theoretically nor experimentally. Modeling of the field enhancement shows that vibrational modes with changes of the polarizability in z-direction (perpendicular to the surface, parallel to the enhanced field) are more strongly enhanced than those with strong x- and/or y-components. This means that a near-field surface- or tip-enhanced spectrum may display vibrational modes which cannot be observed under normal Raman conditions.[123, 140] However, it does not mean that Raman bands appear which do not belong to the inherent vibrational modes of the molecule under investigation.

Unfortunately, the possible degradation of the molecule under investigation during SE(R)R experiments is often not ruled out or considered when interpreting the spectra, although many SE(R)R studies on the photodynamics of adsorbate species on metal surfaces have been published.[53, 142–150] Especially when exposed to strong EM fields or laser light in resonance with their absorption, the molecules are likely to decompose quickly. In this case, it is impossible to identify the original target molecule, because the resulting Raman spectra have changed, as one would expect, significantly and usually show typical bands of carbonaceous species, resulting from the photobleaching or decomposition of the adsorbate. Fig. 5.8 compares TERR spectra of intact and decomposed MGITC/Au(111) at monolayer and single-molecule coverage. It is evident that the photobleached dye does not give the typical Raman

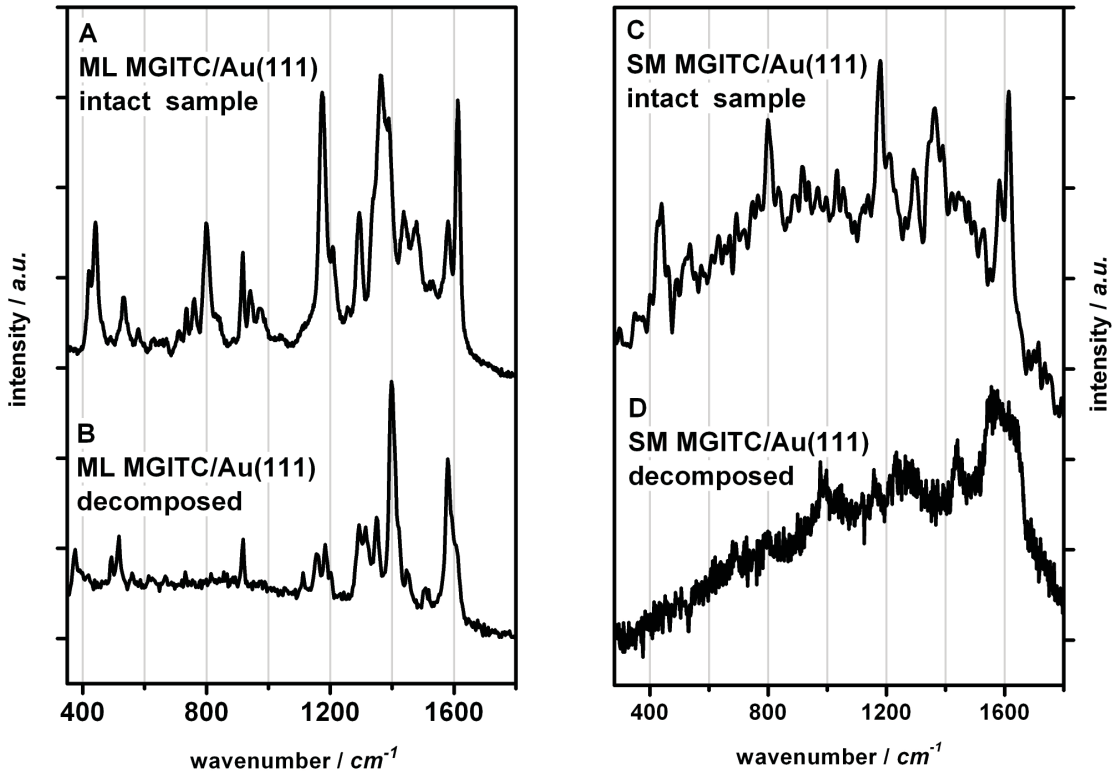

Figure 5.8: A comparison of ensemble (A, B) and SM TERRS (C, D) of MGITC/Au(111) is presented. Even at lowest coverage, intact MGITC can be easily identified by its characteristic Raman spectrum (C). Contamination/degradation of the sample is easily noticed, when the characteristic fingerprint of MGITC disappears (B, D).

fingerprint anymore, and that such spectra cannot be employed for the analysis of the target species, neither at full nor at low coverage.

In order to obtain satisfactory TERR spectra, it is indispensable to regularly move to fresh surface areas, where no photobleaching has taken place. Spectral integration time as well as the incident laser power have to be adjusted respectively. One has to find a proper compromise between too little field enhancement, which will only give a weak Raman signal, and too strong laser power, which is likely to quickly destroy the sample, in order to get a spectrum free of artefacts.[151–153]

Another carbon source besides degradation of the investigated species is contamination of the sample. Undoubtly, metal surfaces handled under ambient laboratory working conditions will be exposed to impurities.[154] Therefore, extreme cleanliness is essential to obtain valuable spectral information.[155, 156]

Under usual SE(R)R and TE(R)R conditions, where oxygen is omnipresent in the air or solution environment, the electronic (π-)system of the adsorbate is easily attacked and oxidation is likely to occur.[157] Also, the often employed Ag substrates that readily incorporate oxygen, suffer from chemical attack and support the oxidation of the adsorbate.[156, 158] Experimental conditions that exclude oxygen (vacuum or inert gas atmosphere) would help to decrease the rate of oxidative decomposition of the molecule when exposed to extremely strong fields. The use of chemically inert Au substrates, which also have strong localized surface plasmon resonance in the visible or near IR, helps to prevent from unwanted chemical reactions.[159]

An important aspect in TE(R)R spectroscopy is the cleanliness of the near-field probe. As the metal tip always exhibits a certain surface roughness, adsorbate or contamination molecules that stick to it give large, but unwanted SE(R)R spectra. The laser focus has roughly a diameter of 1 μm, and the tip surface illuminated is much larger than the enhanced field region at the sample surface. SE(R)R scattering from such a large area conceals any signal that stems from the TE(R)R near-field region of $\sim$ 10 nm diameter, rendering the detection of the desired (single) molecules on the atomically smooth substrate impossible.[160] Two ways to discriminate SE(R)R spectra from the tip are i) to exchange the substrate for a clean one and record TE(R)R spectra with the same tip or ii) to simply retract the tip by several tens of nanometers, so that the tip-enhanced field does not reach the surface anymore. As

the tip is still illuminated in the laser focus, SE(R)R scattering still shows.

In general, measuring and interpreting SM SE(R)R spectra is challenging and the results are not without controversy. Some SM enhanced Raman spectra presented in the literature do not resemble the fingerprint (far-field) spectra of the investigated adsorbate. In a critical survey, Otto showed that SE(R)R spectra of adsorbed tyrosine and photodecomposed pyridine closely resemble Raman spectra of evaporated carbon.[161] However, in order for the SM Raman studies to contain valuable scientific information, the identification of the molecule according to its characteristic Raman spectrum has to be sufficiently convincing.

A typical characteristic observed in SM enhanced Raman spectra are spectral fluctuations with time, often compared to the blinking observed in SM fluorescence experiments.[162] If the time scale of the molecular changes is smaller than the measurement time, orientational changes, molecular interactions, diffusion to different adsorption sites or photodesorption can be visualized in the spectra.[163]

Time series of SM SE(R)R spectra often reflect narrowing or splitting of bands, small band displacements of $\pm$ 5 cm^{-1} or fluctuations of the relative band intensities.[32, 37, 81] These are ascribed to lateral motion/diffusion of the molecule on the substrate and changes in the adsorption geometry.[32, 81] In similar SM TE(R)R spectra (Chapter 5.3, Ref. [75]), no band displacements are observed, and are not expected, because all adsorption sites are equal at an atomically smooth surface. The same holds for more strongly adsorbed molecules which do not change their orientation with respect to the surface and the enhanced field. In spite of spectral fluctuations, SM SE(R)R and TE(R)R spectra resemble very much the corresponding ensemble spectra of the target molecule.[15, 71, 75, 141, 156, 164]

As we will emphasize in the following, the observation of spectral fluctuations in enhanced Raman spectra alone, however, is no sufficient prove that single molecules are being monitored.

Statistical analysis of SER time series is often employed as a "proof" for single-molecule detection. One has to be careful, though, when performing such an analysis, because of several reasons: All long-tail distributions exhibit discrete peaks when a low number of samples is analyzed. In a recently submitted article by Le Ru *et al.*, it is explained that, in order to apply a reliable statistical Poisson analysis of SER

spectra (as often performed), at least 10 000 sample events have to be recorded.[165] This number exceeds by two orders of magnitude the number of spectra generally presented in a SERS time series in the literature. Poisson-like distributions from such small sample series alone are no proof of SM SERS.

The transfer of Poisson statistics from the SERS to the TERS case is also not trivial. In the TERS case, one hot spot is purposely generated, and the spectrum is due to the molecules present in the enhanced field. This means that, statistically, identical TER spectra should be recorded at any tip position above the sample surface at full coverage. As described in Chapter 3, this is not the case, because the quality of the laser focus on the tip apex highly influences the spectral intensity. Experimental experience with ML MGITC/Au(111) reveals that it is meaningless to compare signal intensities obtained from different samples on different days.

If we consider low adsorbate coverages, assuming that tip and focus are held constant and spectral fluctuations solely occur due to the diffusion of species into and out of the focus, as suggested by Neacsu *et al.*,[65] the problem is hardly less complicated. Now, the lateral intensity distribution of the near-field (see Chapter 2.5.1) has to be taken into account: A single molecule located right underneath the center of the tip experiences a much stronger field than one located slightly off-center. The latter is expected to give much less scattering intensity, because of the weaker field. One molecule drifting across the enhanced-field region is to give a vast variety of signal intensities, depending on its exact position with respect to the tip, thus rendering a simple statistical analysis as proposed by Neacsu *et al.* impossible.

Another difficulty arises from the fact that irregular carbon networks on a surface produce similar time-dependent spectral fluctuations as expected for single target molecules, because of rapid C-C or C-O bond creation and breaking.[166–169] The enhanced Raman cross-section of carbonaceous molecular structures is exceptionally large, and even slight traces of carbonaceous contamination are easily detected with SE(R)R or TE(R)R spectroscopy. As a result, it is very easy to confuse these two sources of fluctuations. Also at the single-molecule level, photobleaching of the sample is known to be a problem, despite the efficient energy transfer from the adsorbate to the metal substrate,[147] and sometimes carbon spectra are presented as SM spectra in the literature.

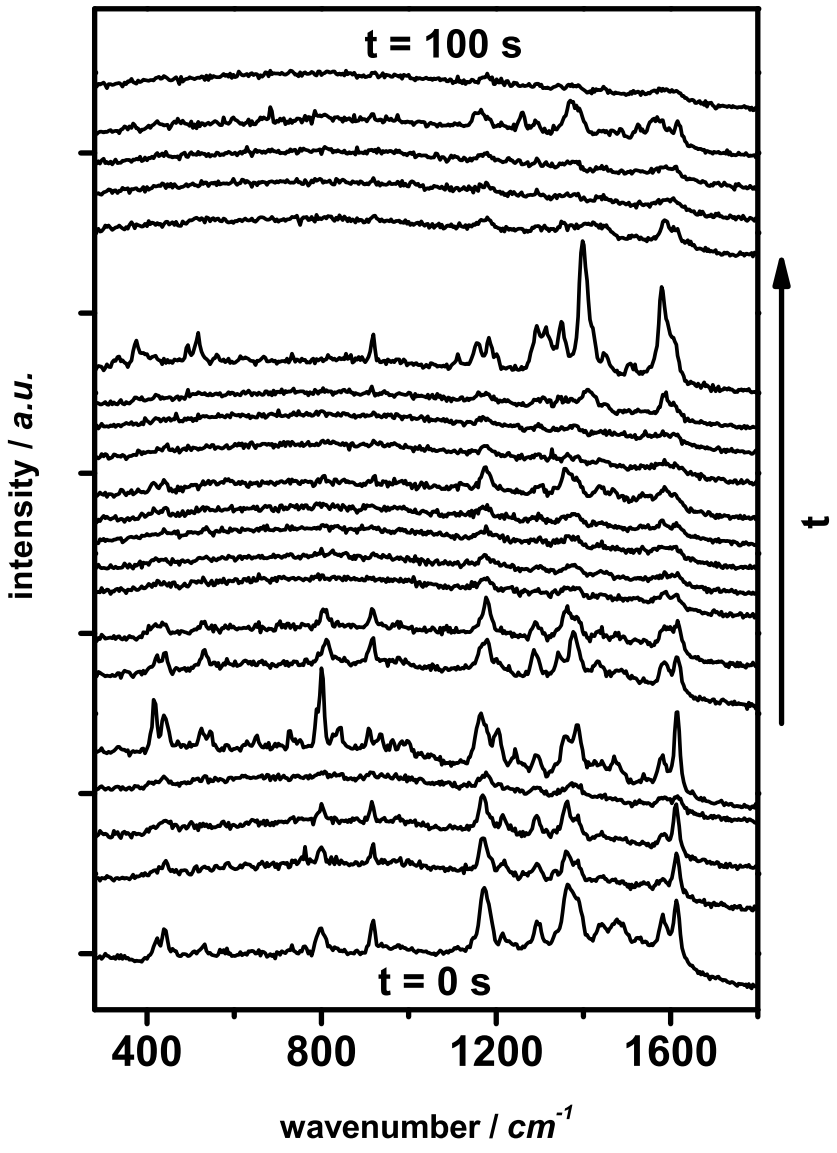

Figure 5.9: The time evolution of TERR spectra of MGITC/Au(111) monitors the (photo)decomposition of the sample Laser intensity at sample: 0.2 mW, integration time per spectrum: 5 s.

To illustrate the problem, a TERR spectral time series recorded from a monolayer of malachite green isothiocyanate (MGITC) adsorbed at Au(111) is presented, where roughly 1260 molecules are present in the enhanced-field region underneath the STM tip (Fig. 5.9). In the beginning, the characteristic MGITC fingerprint spectrum is recorded. With continuing illumination of the sample, the band intensities decrease. At 15 s, the spectrum can hardly be distinguished anymore, and at 20 s, a new Raman spectrum with clearly different band positions in comparison to the first spectrum appears. From then on, spectral fluctuations occur that cannot be assigned to MGITC anymore. A study on the bleaching behaviour of MGITC under the same experimental conditions showed that the bleaching time constant has a value of $\tau = 0.7$ s (for 2 mW incident laser power), and that the molecules readily decompose under the influence of the strong field, with the result that only carbonaceous bleaching products are observable in spectra recorded with full laser power.[53]

Similar spectral fluctuations at high adsorbate concentrations were presented by Bjerneld *et al.*[170] Intriguingly, typical "SM spectral blinking" was also observed by several groups even without a specific target molecule [135, 156, 167, 171] Obviously, spectral blinking alone does not guarantee a SM spectrum, and determining the source of the fluctuations is a significant challenge.

First, spectral changes over time that are transient and nondestructive, where the signal quality and unambiguousness are maintained, have to be clearly distinguished from spectra that deteriorate in intensity and simplicity and reflect a permanent (chemical) alteration of the target molecule.[156]

Second, another important difference between SM and carbon fluctuating Raman spectra is easily detected when summing up the individual spectra: The sum over carbon fluctuations gives a typical carbon broad band structure with peaks at around 1350 cm^{-1} and 1580 cm^{-1},[167, 168] whereas the sum over a series of SM spectra leads to the typical ensemble (far-field) spectrum of the molecule.[172]

To carry out and present a thorough, unambiguous (SM) enhanced Raman study, it is highly advisable to

- prevent sample contamination at any stage of preparation and during the experiment,

- take into consideration the experimental environment (oxidative atmosphere) and possible photodecomposition of the sample,
- find a decent compromise between reduced laser power and signal intensity,
- carry out a careful interpretation of the spectra,
- present (SM) enhanced Raman spectra in comparison to the corresponding far-field Raman spectra,
- point out spectral features which cannot be ascribed to the species under investigation.

To take these factors into account when carrying out SM Raman studies is of great importance for the development of SE(R)R and TE(R)R spectroscopy into an attractive and reliable SM analytical tool.

5.5 Conclusions & outlook

In this Chapter, we have proven that the enhancement reached with TERRS is sufficient to perform single-molecule Raman spectroscopy on resonant species adsorbed at atomically smooth Au(111). No change in band positions or relative band intensities were observed with varying adsorbate concentration. Even at the SM level, the characteristic Raman fingerprint allows the unambiguous identification of MGITC. Despite the suboptimal resolution of the STM, we can distinguish single nanodots of approximately the size of one MGITC molecule in the images.

However, satisfying TERRS signals are not easy to obtain. Irreproducible focussing leads to large intensity variations (roughly a factor 10) at full coverage. In addition, it is unclear how different tips employed at difference times during the experimental series influence the signal strength. At low coverages, it is impossible to distinguish between low signal intensity due to bad focussing or the presence of only one or a few molecules. Therefore, we chose to average over the five spectra that gave maximum scattering intensity at the respective adsorbate coverage during the whole

experimental series. The second difficulty to occur, especially when working with resonant species, is the (photo)decomposition of the adsorbate.

In order to substantiate the TERS single-molecule detection, we have started a collaboration with Thorsten Kampen (Fritz Haber Institute) who provides a temperature-controlled UHV STM. Working at low temperature with a high-resolution STM will hopefully enable us to record STM images that allow us to actually "count" the number of molecules present in the enhanced field. Alternatively, we are preparing to implement a photodetector to our home-built TERS set-up that would allow to record Raman images of the surface. A correlation of such Raman images and the STM images would contribute to the identification of the nanodots. In addition, it would provide insight into the lateral optical resolution of our instrument. Of vast interest for the general applicability of TERS in the single-molecule regime is the use of nonresonant species. We have tested the sensitivity of TERS for adenine/Au(111), and arrived (unoptimized) at around 500 molecules present in the enhanced field, about two orders of magnitude above the single-molecule detection limit. However, we hope to improve the detection limit with the newly designed set-up introduced in Chapter 7.