

Renato Zenobi
Department of Chemistry and
Applied Biosciences
ETH Zürich - CH-8093 Zürich
Switzerland
zenobi@org.chem.ethz.ch

NEW METHODS FOR MOLECULAR ANALYSIS ON THE NANOMETER SCALE

This article presents novel methods for spectroscopic investigations with nanometer scale spatial resolution. Two basic principles will be discussed, both based on scanning near-field optical microscopy (SNOM) methods: (i) laser ablation through SNOM aperture probes with mass spectrometric detection of the ablation products, and (ii) an apertureless methodology called tip-enhanced Raman spectroscopy (TERS).

The world around us is primarily molecular. Methods for chemical analysis are therefore designed to reveal the molecular nature of matter, and a great variety of ways exists to achieve this goal. Traditionally, these have been wet chemical methods (for example chemical degradation via ozonolysis, precipitation reactions, or specific detection methods based on biomolecular recognition) or instrumental spectroscopy methods (for example nuclear magnetic resonance, infrared spectroscopy, or mass spectrometry) that yield detailed information about the molecular composition of matter. However, most of these methodologies cannot be applied to questions in nanoscience and nanotechnology, because they intrinsically lack spatial resolution.

Established methods for studying matter with nanoscale lateral resolution, such as atomic force microscopy (AFM), scanning tunneling microscopy (STM) and scanning electron microscopy (SEM), typically give very little or no chemical information. Some exceptions exist, for example derivatized AFM tips that can be used for chemical recognition. In general, however, only the shape of nanostructures, the surface topography, and local electric pro-

perties can be measured, albeit in many cases with excellent spatial resolution, down to the atomic scale. However, with the advent of nanotechnology and nanoscience, the need for chemical and in particular molecular analysis has become urgent [1]. Methods of nanotechnology and nanoscience give us judicious control over, and use of properties of matter on the nanoscale (length scale well below 1 μm in at least two dimensions). Nano-objects that are under intense study include subcellular compartments, nanomaterials such as polymer blends, “molecular machines” and small biological objects, nanoparticles with supposed toxic effects, or components of molecular electronics.

There is currently a lack of methods capable of chemical diagnostics and characterization, in particular of the molecular composition, of such nano objects. Fig. 1 illustrates these challenges by two examples, from microbiology and from chemistry. Fig. 1a is a tapping mode AFM (phase) image of a bacteria sample, which clearly shows flagella (indicated by arrow 1) and pili (arrow 2) on one pole of a bacterium [2]. Pili are used by bacteria to attach to surfaces and can be identified in this case as several rigid rods that are spread out in a fan-

like manner. The pili usually have a length of $\approx 1.2 \mu\text{m}$, a thickness of 1-2 nm and a width of 50-100 nm, which are typical dimensions of such structures. Flagella could be distinguished from pili based on rigidity, size, and number. They are used by bacteria for moving in the liquid phase. Typical dimensions of flagella are $>2 \mu\text{m}$ in length, $\approx 4 \text{ nm}$ in thickness and 50-150 nm in width. Besides these linear structures, small spherical-shaped objects can be observed. Shape and dimensions (50-150 nm in diameter and $\sim 3 \text{ nm}$ in thickness) let us assume that these structures are river water colloids consisting of humic substances. Fig. 1b is a SEM of a model catalyst system that was created with the goal of producing uniform "active sites" [3]. A photoresist layer spun on top of a wafer was patterned by laser interference exposure. The grid obtained after removing the exposed parts of the resist is used as an etching mask. Hollows with diameters of 300 nm and depths between 50 and 60 nm were etched into the oxide layer using wet chemical methods. Palladium metal clusters were deposited in a defined way within the hollows. The particles ranged from 10 to 50 nm in height and from 80 to 200 nm in diameter.

In both Figs. 1a and b, the imaging methods employed (tapping mode AFM and SEM, respectively) reveal rich structural features on the nanometer scale, but do not yield any chemical information. It would, for example, be highly desirable to chemically identify the bright and dark "blobs" in the extracellular matrix in Fig. 1a. For studying the effectiveness of the model catalyst in Fig. 1b it would be crucial to obtain the molecular composition of surface adsorbates on the metal grains, on the SiO_2 support, or at a Pd/SiO_2 interface while a chemical reaction is run with the aid of the heterogeneous catalyst. None of this information is available from AFM or SEM imaging. Furthermore, high vacuum conditions are generally used for SEM, rendering this method incompatible with observing a catalyst "in action" under atmospheric or elevated pressure conditions. Standard analytical methods do not possess the necessary spatial resolution to resolve chemical differences on a scale $\ll 1 \mu\text{m}$. A new approach is needed that affords both high spatial resolution and chemical identification capabilities.

Principles of Chemical Analysis by Near-Field Methods

As a platform for nanoscale chemical analysis, we use scanning near-field optical microscopy (SNOM), the "optical cousin" of STM and AFM. Standard "aperture SNOM" works as follows: light from a laser is guided into an optical fiber, whose tip is sharpened and metallized on the outside such that only a very small aperture (diameter down to $<100 \text{ nm}$) passes some laser light. This nanometer light source can be used to illuminate a spot on a sample that is considerably smaller than the optical diffraction limit, $\sim \lambda/2$ (λ =wavelength of incident laser light). Using a method developed in our group based on chemical etching [4], SNOM tips with only 50 nm diameter and a high optical throughput can be fabricated. In order to obtain a high resolution optical image of the sample, the SNOM tip is scanned over the sample surface using a distance feedback circuit, with nanometer accuracy using piezo elements for motion control.

The use of SNOM not only allows one to measure the topographic and optical properties of a sample but also to obtain chemical information, by optically dispersing the emitted or inelastically scattered light to yield fluorescence or Raman spectra (Fig. 2a). This is a nondestructive way of analyzing surfaces. An alternative, albeit destructive way of analyzing sample surfaces is by the use of nano-scale ablation with pulsed laser radiation that is guided onto a sample surface through SNOM tips (Fig. 2b). An obvious way of exploiting the latter scheme for nanoscale chemical analysis would be collection and subsequent analysis, for example by mass spectrometry, of the ablation products. The feasibility of this concept was demonstrated in 1998 by Kossakovski *et al.* [5]. In 2001, our group published a follow-up on this concept that incorporated several significant improvements [6]: (i) operation at atmospheric pressure, i.e., elimination of the need to place the entire SNOM source into the high vacuum of the mass spectrometer; (ii) much better sensitivity (on the order of a few attomoles); and (iii) concomitantly, an excellent spatial resolution of $\approx 170 \text{ nm}$.

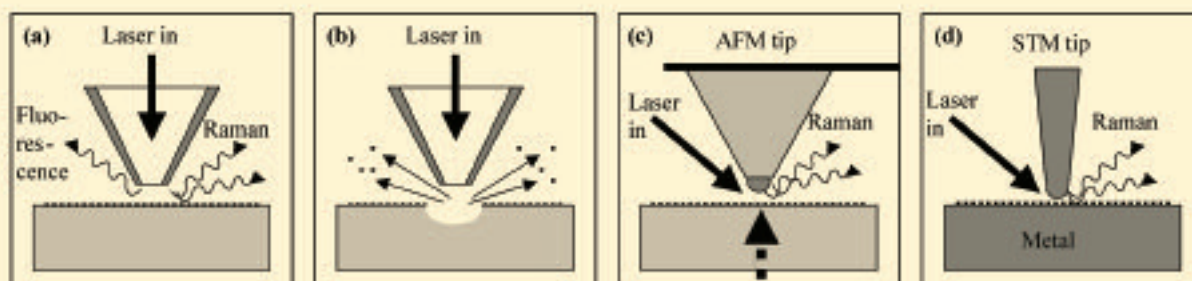


Fig. 2 - Different operating modes of near-field spectroscopic methods: a) aperture SNOM for exciting fluorescence or Raman scattering on a sample surface; b) aperture SNOM for pulsed nanoscale laser ablation with subsequent mass spectrometric detection of the ablation products; c) apertureless SNOM using a modified AFM tip for tip-enhanced Raman scattering (TERS). Note that the excitation laser beam can reach the tip/sample region from above (back illumination) or through a transparent sample (epi-illumination); d) use of a metallic STM tip in very close proximity to the sample surface for gap-mode TERS

The particular challenge in coupling SNOM with Raman spectroscopy is the fact that Raman scattering is an inherently weak effect. In addition, if Raman scattered light originates from a very small (nanometer sized) region, it is normally barely detectable. A way to circumvent this problem is to employ surface-enhanced Raman scattering (SERS). SERS is based on local enhancement of the electromagnetic fields (by up to 6 orders of magnitude), by depositing samples on a nanostructured film of silver. Certain shapes and sizes of silver colloids are even able to enhance the Raman scattering of adsorbed sample molecules by more than 12 orders of magnitude [7]. The special approach we have pioneered makes use of an “apertureless” version of SNOM, where the Raman scattering is amplified locally by the presence of a metallic nanoparticle brought to the sample by means of an atomic force microscope cantilever (Fig. 2c). We call this “tip-enhanced Raman scattering” (TERS) which can be viewed as “SERS upside down”. At the heart of the TERS technique is a ‘hot’ tip, which can highly confine and enhance the electric field at the tip apex, and which can be scanned over a sample surface to image it. The advantage of this approach is that the sample/tip area can be illuminated using conventional optics, avoiding the comparatively lower transmission of an aperture SNOM tip. Such apertureless near-field spectroscopy experiments have been realized using coated AFM tips or sharpened metal tips. It is still not completely clear what the prerequisites are for a highly enhancing site within the metal coating of a TERS tip. Theory suggests that the local electromagnetic field is highest in gaps between metal nanoparticles [8]. A special geometry that promises particularly high enhancement of the Raman scattered light [9] is shown in Fig. 2d (“gap-mode TERS”). In this embodiment, the sample is localized in the gap between a metallic surface and a sharp STM tip. STM feedback is used to bring the tip within a nanometer or less of the surface.

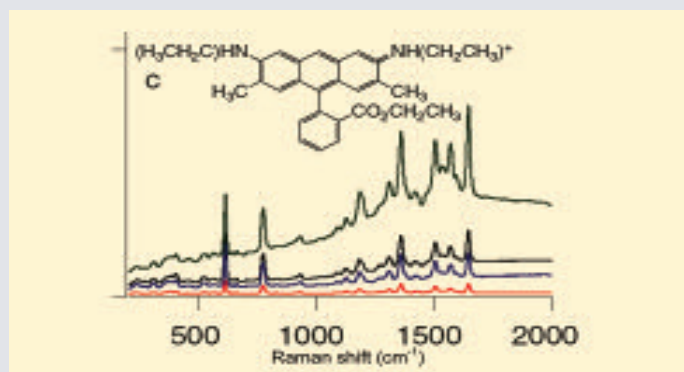


Fig. 3 - SERS spectra of Rhodamine 6G (structure in the inset) spin-coated onto a 6 nm of Ag film deposited on 20 nm thick dielectric layers with different index of refraction: AlF_3 ($n=1.4$, green trace), SiO_2 ($n=1.5$, black), SiO_x ($n=1.5 \dots 2$, blue) or Si ($n=4.4$, red). The intensity of Raman signals is shown to increase as a function of decreasing refractive index of the underlying substrates

Tip-enhanced Raman Spectroscopy in Chemical Analysis

In 2000, our group was the first to experimentally demonstrate TERS [10], closely followed by two other groups who published similar results later the same year [11, 12]. By harvesting the spatial resolution and chemical specificity afforded by scanning probe microscopy (SPM) and Raman spectroscopy, respectively, the surface compositions of brilliant cresyl blue (BCB) and C_{60} thin films have been unambiguously fingerprinted with a 50-nm spatial resolution. A large near-field enhancement of the Raman signals is essential for performing rapid TERS imaging. Unfortunately, the experimental Raman enhancements have been lower than theoretical predictions. This has hampered the development of TERS to become a robust and commercially viable technique. We have been actively involved in the fabrication and design of various TERS probes based on AFM tips, which are intimately connected to the Raman enhancement. A contributing factor is the effect of the AFM tip material on the wavelength of the surface plasmon resonance (λ_{SPR}) of the attached Ag particles. In general, the λ_{SPR} of metal particles is known to increase with higher refractive index (n) of the underlying substrate. This has been studied several times for SERS, and is demonstrated for a SERS experiment in Fig. 3. This finding could then be transferred to tip-enhanced Raman scattering (TERS) with excitation by a 488-nm laser. A huge optical contrast of 70-80 x, corresponding to an enhancement factor of $>10^4$, was achieved for brilliant cresyl blue test analyte using Ag-coated tips made from or pre-coated with low refractive index materials, in particular SiO_2 ($n=1.5$) and AlF_3 ($n=1.4$), which both have a lower refractive index than SiN ($n=2.05$) or Si ($n=4.4$) [13, 14]. Interestingly, the yield of tips giving significant enhancement to the Raman signals was also found to be very high, close to 100%.

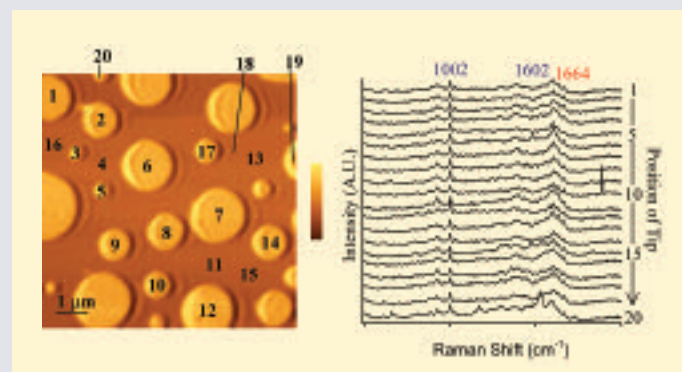


Fig. 4 - a) AFM topography image of a PI/PS film and b) sequence of TERS spectra collected from the positions depicted. Each spectrum was acquired for 60 s. The bands at 1,664 cm^{-1} and at 1,602 and 1,002 cm^{-1} are characteristic vibrational modes for PI and PS, respectively. The spike in Spectrum 10 was caused by cosmic rays

These are crucial steps towards the use of TERS as a robust technique for rapid chemical imaging.

Let us now illustrate the use of TERS to investigate the surface composition of a mixed polyisoprene (PI) and polystyrene (PS) thin film. These two polymers are non-miscible and segregate upon spin casting from a toluene solution. They are not easily studied by Raman spectroscopy, because they are weak scatterers and do not exhibit any resonance enhancement. Nevertheless, we were able to collect high quality TER spectra from spin-cast thin polymer films deposited using 2 μ L of a 1:1 PI:PS toluene solution onto clean glass slides. A wealth of structural information was obtained, some of which cannot be acquired with conventional analytical techniques. Topographic investigation showed depressions (PI) and protrusions (PS) which originate from the segregation of these two materials as the film forms (Fig. 4a). Far-field Raman spectroscopy as well as TERS at many locations confirmed the chemical composition of these depressions and protrusions as PI and PS. However, PI was observed in all the TERS spectra, even on a PS protrusion (Fig. 4b). This can be rationalized by PS being present as a continuous subsurface layer (including in depressions) and the PI forming a thin (20 nm thick) layer at the polymer-air interface. The thickness of the PI film is estimated based on the the depth of view (\approx 20 nm) of TERS. Differences in the PS and PI band intensities, however, suggest that the polymer layers were not uniformly thick, and that nanopores were present under the film surface. The continuous PS subsurface layer and subsurface nanopores have hitherto not been identified. Confocal far-field Raman spectroscopy and X-ray photoelectron spectroscopy were used to corroborate the results and interpretation.

Gap-Mode TERS

As mentioned above, the highest enhancements can be generated in gaps between metallic nanostructures. Based on this insight, we were able to achieve single molecule detection sensitivity using tip-enhanced resonance Raman spectroscopy on brilliant cresyl blue (BCB) sub-monolayers adsorbed on a planar Au surface [15]. A gap of \approx 1 nm between an Ag tip and the Au substrate was employed to create a highly enhanced electric field and to generate Raman scattering from an area of \sim 100 nm².

Three lines of evidence prove the single molecule sensitivity of our experiments:

- 1) extremely dilute samples were used. Estimations show that at most a few molecules were excited by the Ag tip;
- 2) spectroscopic fluctuations, including intensity fluctuations, frequency shifts and lineshape changes were observed. A histogram analysis of the intensity fluctuations of two different BCB covera-

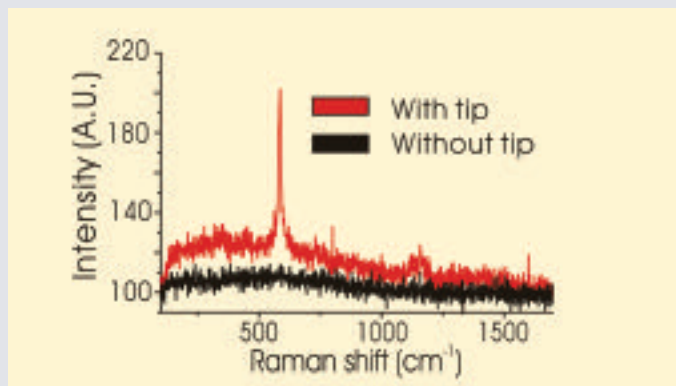


Fig. 5 - Gap-mode TERS: Comparison of a tip-enhanced Raman spectrum and the far-field Raman spectrum of a brilliant cresyl blue (BCB) submonolayer deposited on a Au surface. The estimated Raman enhancement was ca. 10^7

ges was carried out. The results clearly show the features of single molecule behavior;

- 3) discrete signal losses were also observed. This is due to photochemical processes involving single molecules. Besides BCB, which shows a strong resonant absorption at 633 nm, the wavelength of the excitation laser, a self-assembled monolayer of benzenethiol, which does not strongly absorb at 633 nm, was studied. Good quality spectra were recorded with a short exposure time (10 seconds) and time-dependent spectral changes were also observed.

Fig. 5 shows a spectrum of BCB on gold that underscores the very large enhancement factors which can be achieved in this fashion. The single molecule capability of gap-mode TERS has been confirmed by at least two other groups [16, 17].

Chemical Analysis with SNOM-Mass Spectrometry

For interfacing SNOM with mass spectrometry, pulsed laser ablation through a SNOM aperture tip is used. This has been shown to lead to spatially highly localized ablation of matter that can then be ionized and analyzed mass spectrometrically. The SNOM-MS approach is extremely challenging for standard MS instrumentation in terms of detection sensitivity. The following example will illustrate the point: assume an ablation volume of 200x200x100 nm³, and a compound with a density of 2 gcm⁻³ and a molecular weight of 100 gmol⁻¹. This corresponds to an amount of only 80 amol that needs to be introduced into the mass spectrometer, ionized, mass separated, and finally detected.

For the development of SNOM-MS as a nanoanalytical method, we chose an approach where near-field laser ablation takes place at atmospheric pressure, neutrals are sampled into the mass spectrometer with a capillary interface, and ionization takes place inside the

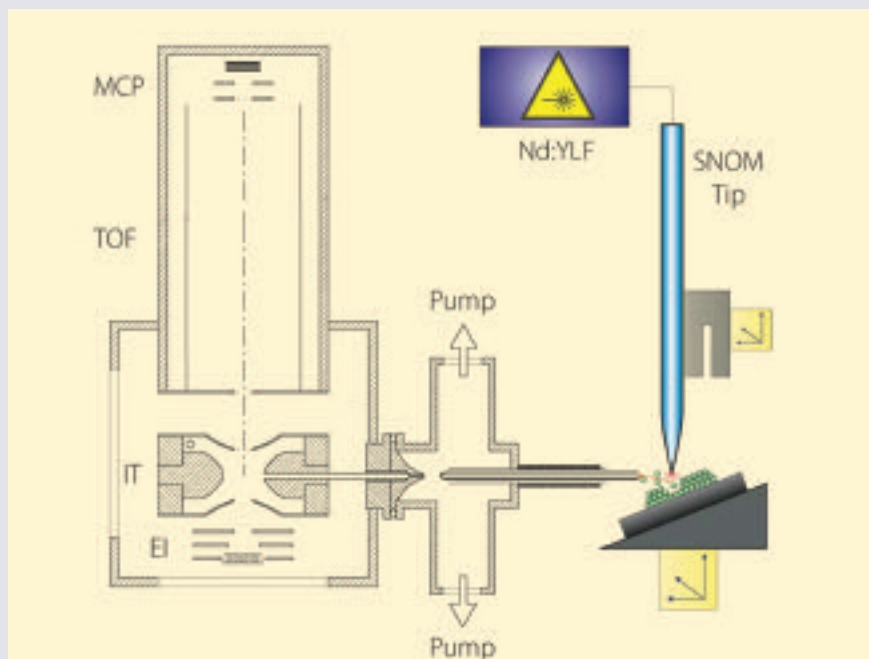


Fig. 6 - Instrumental setup of the SNOM-MS instrument (left part: IT-TOF mass spectrometer; right part: SNOM with UV laser coupled into a SNOM aperture probe). Ablation takes place in the optical near field on the surface of a sample which is kept at atmospheric pressure/ambient conditions. The ablated neutral species are sampled with a capillary and are transported via a nozzle/skimmer arrangement into the mass spectrometer (not drawn to scale)

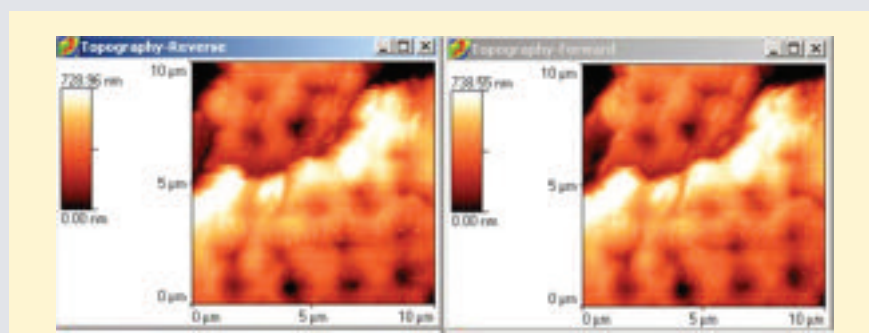


Fig. 7 - Near-field laser ablation pattern on the surface of a tris(8-hydroxyquinoline) aluminum salt crystallite; this material is a common compound in organic light emitting diodes (OLEDs). The image shows a 10x10 μm topographic scan recorded after irradiation of the material by single laser shots of 355 nm wavelength, after "parking" the SNOM tip at an array of predefined locations. The estimated crater size is $<1 \mu\text{m}$ diam, the depth $\approx 300 \text{ nm}$

vacuum of the MS [6, 18]. The most sensitive mass analyzers are time-of-flight (TOF) instruments. However, a direct coupling of atmospheric-pressure near-field laser ablation to a TOF is not feasible due to the temporal spread of the neutral ablation products as they reach the ion source. Therefore, a pre-concentration step between laser ablation and mass separation is necessary. An ion-trap/TOF instrument has been developed in our group for precisely this purpose [18, 19]; a schematic is shown in Fig. 6. After having accumulated the ions of interest within the ion trap, they are extracted in a pulsed fashion into the field-free TOF-region, therefore yielding a better limit

of detection for the overall laser ablation-MS process. Another aspect which is designed to give a too limit of detection is that neutral species from the laser ablation are sampled at atmospheric pressure, transported as neutrals into the vacuum, and ionized only once they enter the ion trap. This is because laser ablation processes (e.g. matrix-assisted laser desorption/ionization, MALDI) are fairly ineffective in producing ions: ion-to-neutrals ratios in the range of 1:1,000 to 1:10,000 are common for UV laser desorption. Much of the ablated material would therefore be "wasted" if one would only sample the ionized species produced during the laser ablation process.

By coupling a UV laser into a fiber tip of a scanning near-field optical microscope, ablation craters much smaller than achievable with conventional laser optics can be obtained. Laser ablation was performed on molecular solids such as dihydroxy benzoic acid (a MALDI matrix compound), anthracene and pyrene. After storing the generated ions in the trap for 10 ms, they were extracted into a sensitive time-of-flight spectrometer. Spatially resolved molecular analysis at atmospheric pressure with a lateral resolution of $5 \mu\text{m}$ (FWHM) and a sensitivity of $\approx 60 \text{ fmol}$ of solid anthracene was so far possible, along with topographical analysis of the sample with the same instrument. The efficiency of transporting the ablation products into the mass spectrometer, the ionization efficiency, and the detection sensitivity of the MS instrumentation is directly related to the achievable spatial resolution: an improvement of a factor of 1,000 would immediately result in a spatial resolution well below $1 \mu\text{m}$. Current limitations are the fact that a

major fraction of the ablated material appears to leave the sample in the form of large clusters/small chunks, which are virtually impossible to sample by the "choked flow" of the transfer capillary, i.e., they may never even enter the MS instrument. Furthermore, electron impact is currently used for ionization, which is not a particularly efficient method. By improving the sampling efficiency for the neutral ablation products and the ionization yield, significant gains in sensitivity and better spatial resolution for SNOM-MS are expected.

Fig. 7 demonstrates a first step towards SNOM-MS imaging. Regular sampling patterns can be "drawn" by the ablation laser onto the sur-

face of a sample, and in principle, a mass spectrum can be recorded for every such pixel. While this is obviously a destructive type of chemical analysis, this drawback is compensated by the very high chemical information content available from mass spectrometric data.

Applications

At the moment, SNOM-MS is still under development, but TERS has matured into a fairly generally applicable spectroscopic method for analyzing a wide variety of materials and samples. One application in the area of polymer science was shown above, and we certainly expect more results from similarly complex polymer nano-composites. For example, the chemical content of the polymers, fillers, and additives in rubber tyres - which are in fact a modern nanomaterial - could be derived from TERS data. We also see a large potential of this method in the area of nanobioscience. The feasibility to spectroscopically investigate calcium alginate fibers, a model for extracellular biofilms has been shown [2, 20]. Cell walls have been studied by TERS [21]. TERS is being promoted as a nondestructive way for DNA sequencing [22], and thanks to its high spatial resolution, it has been used to specifically probe the protein vibrations of cytochrome

C (a heme protein) [23] which are normally completely swamped by resonance Raman scattering of heme bands.

Conclusions

Near-field Raman spectroscopy and near-field laser ablation mass spectrometry are developing into powerful analytical methods for the nano world. Raman spectroscopy is a very attractive, nondestructive and label-free method for identification and analysis. In the near field, it can in principle be performed both by aperture and apertureless SNOM methods. The latter has the potential for excellent sensitivity - reaching single molecule detectability - and very high spatial resolution. Mass spectrometric analysis of SNOM-laser ablation products is predicted to become a powerful method of chemical analysis and imaging as well. Although it is destructive, this disadvantage is easily outweighed by the very high chemical information content available from mass spectrometry.

Acknowledgments: The author would like to thank his co-workers (Gerado Gamez, Grace Leong, Thomas Schmid, Thomas Schmitz, Johannes Stadler, Boon-Siang Yeo and Weihua Zhang) for their valuable contributions to this research.

References

- [1] R. Zenobi, *Anal. Bioanal. Chem.*, 2008, **390**, 215.
- [2] T. Schmid *et al.*, *Anal. Bioanal. Chem.*, 2008, **391**, 1899.
- [3] M. Schildenberger *et al.*, *Catal. Lett.*, 1998, **56**, 1.
- [4] R.M. Stöckle *et al.*, *Appl. Phys. Lett.*, 1999, **75**, 160.
- [5] D.A. Kossakovski *et al.*, *Ultramicroscopy*, 1998, **71**, 111.
- [6] R. Stöckle *et al.*, *Anal. Chem.*, 2001, **73**, 1399.
- [7] K. Kneipp *et al.*, *Phys. Rev. Lett.*, 1997, **78**, 1667.
- [8] J.P. Kottmann *et al.*, *Opt. Express*, 2001, **8**, 655.
- [9] I. Nottingher, A. Elfick, *J. Phys. Chem.*, 2005, **109**, 15699.
- [10] R.M. Stöckle *et al.*, *Chem. Phys. Lett.*, 2000, **318**, 131.
- [11] M.S. Anderson, *Appl. Phys. Lett.*, 2000, **76**, 3130.
- [12] Hayazawa *et al.*, *Opt. Commun.*, 2000, **183**, 333.
- [13] B.S. Yeo *et al.*, *Appl. Spectrosc.*, 2006, **60**, 1142.
- [14] B. S. Yeo *et al.*, *Anal. Bioanal. Chem.*, 2007, **387**, 2655.
- [15] W. Zhang *et al.*, *J. Phys. Chem. C*, 2007, **111**, 1733.
- [16] C.C. Neacsu *et al.*, *Phys. Rev. B.*, 2007, **75**, 236402; *ibid.* 2006, **73**, 193406.
- [17] K.F. Domke *et al.*, *J. Am. Chem. Soc.*, 2006, **128**, 14721; J. Steidtner, B. Pettinger, *Phys. Rev. Lett.*, 2008, **100**, 236101.
- [18] T. Schmitz *et al.*, *Anal. Chem.*, 2008, **80**, 6537.
- [19] P.D. Setz *et al.*, *Rev. Sci. Instrum.*, 2006, **77**, 024101.
- [20] T. Schmid *et al.*, *Anal. Bioanal. Chem.*, 2008, **391**, 1907.
- [21] U. Neugebauer *et al.*, *ChemPhysChem.*, 2006, **7**, 1428.
- [22] E. Bailo, V. Deckert, *Angew. Chem.*, 2008, **47**, 1658.
- [23] B.S. Yeo *et al.*, *J. Phys. Chem. C*, 2008, **112**, 4867.

ABSTRACT

Nuovi metodi di analisi molecolare su scala nanometrica

Questo articolo presenta nuovi metodi di indagine spettroscopica a risoluzione spaziale in scala nanometrica. Vengono discusse le due principali metodologie basate sui metodi di microscopia a scansione ottica a campo vicino (SNOM = scanning near-field optical microscopy): (i) ablazione laser mediante sonde aperte SNOM che permette la determinazione delle specie di materiale asportato mediante spettrometria di massa e (ii) la metodologia, detta SNOM senza apertura, combinata con la spettroscopia Raman e chiamata Tip-Enhanced Raman Spectroscopy (TERS).