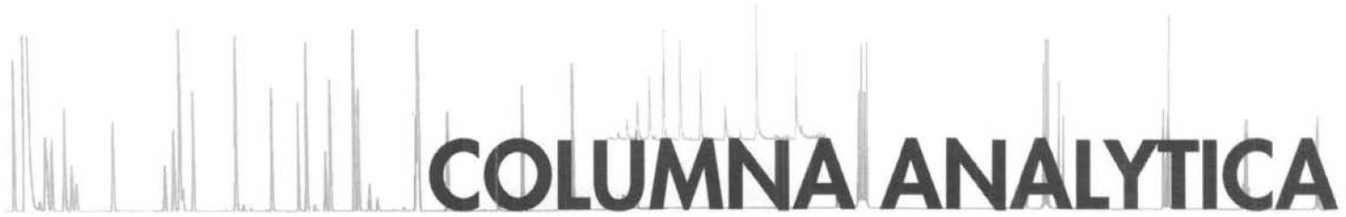
CHIMIA 1999, 53, No. 1/2



Column Editor: Prof. Renato Zenobi, ETH-Zürich

Chimia 53 (1999) 35–37

ISSN 0009–4293

Single-Molecule *Raman* Spectroscopy – Fact or Fiction?

Single-molecule detection represents the ultimate sensitivity limit in chemical analysis. Spectroscopic studies may even allow identifying the chemical structure of a single molecule, offering far-reaching opportunities in basic and applied research. Recent advances have allowed detection and dynamic studies of single molecules under both cryogenic and ambient conditions [1]. Most of these studies are based on laser-induced fluorescence, a method that provides ultra-high sensitivity but is limited in the amount of molecular information. Vibrational spectroscopy, for example *Raman* spectroscopy, would be a preferred method for single-molecule studies because of the very high chemical information content. *Raman* scattering, however, is a very weak effect, with cross sections between 10^{-30} cm^2 and 10^{-25} cm^2 per molecule, the larger values occuring only under favorable resonance *Raman* conditions. Such small *Raman* cross sections require a large number of molecules to achieve adequate conversion rates from excitation laser photons to *Raman* photons, thereby making single-molecule *Raman* spectroscopy 'science fiction'.

This situation is dramatically improved if surface-enhanced *Raman* scattering (SERS) is used. The exciting phenomenon of a strongly increased *Raman* signal from molecules attached to metallic nanostructures was discovered in 1977 by *Van Duyne*, *Jeanmaire*, *Albrecht* and *Creighton* [2]. Very recently, and almost simultaneously, two groups, the one of *Kathrin Kneipp* and the other of *Shuming Nie*, unexpectedly observed enhancement factors much larger than the ensemble-averaged values derived from conventional measurements, on the order of 10^{14} to 10^{15} for compounds adsorbed on metal nanoparticles or colloids [3–5]. Such large enhancement factors were inferred by a straightforward method based on steady-state population redistribution due to the pumping of molecules to the first excited vibrational state *via* the strongly enhanced *Raman* process [3]. Following these initial studies, *Käll*'s group [6] in Sweden has confirmed the presence of optically 'hot' metal nanoparticles and has reported ultrasensitive SERS spectra of biological molecules.

The proof whether these SERS signals truly result from single molecules is not straightforward. In this column, *Kathrin Kneipp* (Technische Universität Berlin and MIT) as well as *Shuming Nie* and *Steve Emory* (Indiana University) present arguments and data in support of true single-molecule SERS; they also share some thoughts on the nature of the SERS enhancement and on the use of single-molecule *Raman* spectroscopy as an analytical tool.

***Kathrin Kneipp* writes**: In single-molecule SERS, the effective *Raman* cross sections are on the order of 10^{-16} cm^2/molecule, which is comparable to effective fluorescence cross sections of common laser dyes. As we know from fluorescence experiments, such cross sections are sufficient for single-molecule detection. *Fig. 1a* shows typical single-molecule *Raman* spectra measured at about 150 mW non-resonant near-infrared excitation. To achieve the required large SERS enhancement, single target molecules are attached to colloidal silver clusters in aqueous solution. The spectra provide a clear spectral 'fingerprint' of single molecules by showing the characteristic *Raman* lines. Strong fluctuations in the SERS signals appear due to *Brownian* motion of the colloidal silver particles which carry single target molecules into and out of the probed volume. The change in the statistical distribution of the *Raman* signal from *Gaussian* to *Poisson* (*Fig. 1b*) when the average number of analyte molecules in the scattering volume is one or fewer shows that single-molecule *Raman* scattering is in fact the observed effect [4].

Very large field enhancement, as predicted for fractal colloidal clusters by *Shalaev*, *Moskovits* and co-workers, provides a rationale for single-molecule SERS on colloidal clusters [7], but there might also be a small 'chemical' contribution to the total SERS enhancement [8]. In general, for full understanding of single-molecule SERS further investigations are crucial.

Another approach to single-molecule *Raman* spectroscopy exploits a favorable superposition of surface-enhancement and molecular resonance *Raman* enhancement [9]. For instance, *Nie* and *Emory* measured SERS spectra of single rhodamine 6G molecules on isolated 'hot' silver particles at visible resonant excitation [5].

Due to the mainly electromagnetic origin of the extremely large SERS enhancement, at least in the experiments performed on colloidal clusters, it should be possible to achieve cross sections sufficient for single-molecule *Raman* spectroscopy for a broad range of molecules [10].

***Shuming Nie* and *Steve Emory* write:** Single-molecule *Raman* is a true vibrational technique. It is capable of providing rich molecular information and does not suffer from rapid photobleaching (*Raman* scattering has very short excited-state lifetimes). Furthermore, nonfluorescent molecules such as nucleotides and amino acids might be detected and identified at the single-molecule level. At present, there is compelling evidence to support single-molecule *Raman*, but the accumulated experimental data do not constitute a 'proof' in the absolute sense. In the following, we briefly discuss the current evidence for single-molecule *Raman* as well as important issues that need to be resolved in the future.

The most obvious evidence for single-molecule *Raman* comes from low-concentration studies, in which the number of colloidal particles far exceeds the number of analyte molecules such as rhodamine 6G or crystal violet [4–6]. Assuming random *Poisson* distribution, the probability of finding more than one molecule on a single particle is extremely small. Thus, the surface-enhanced *Raman* signals observed from a single nanoparticle should correspond to a single analyte molecule. Other lines of evidence come from the strongly polarized nature of the emitted *Raman* signals (which is never observed in population-averaged SERS) and the observation of sudden spectral fluctuations [5] (*Fig. 2*). The latter behavior is similar to intermittent light emission that has been reported for single dye molecules [11][12], single fluorescent proteins [13], and single CdSe quantum dots [14]. It should be noted, however, that the observed SERS fluctuations contain both a frequency (*Raman* shift) and an intensity component. These results are consistent with the expected behavior of a single molecule, but do not rule out the possibility that molecular aggregates are formed on the particle surface. If all the adsorbed molecules interact with each other and have the same orientation, a molecular aggregate could behave as a single molecule. Recent studies in our group indicate that single colloidal nanoparticles often show weak but detectable SERS signals from the absorbed citrate molecules [15]. The citrate spectrum is replaced by the R6G signals in one step, and a mixture of citrate and R6G signals is rarely observed. This result indicates that a single citrate ion is replaced suddenly by a single R6G molecule. A molecular aggregate is unlikely to form or to be replaced in a single step.

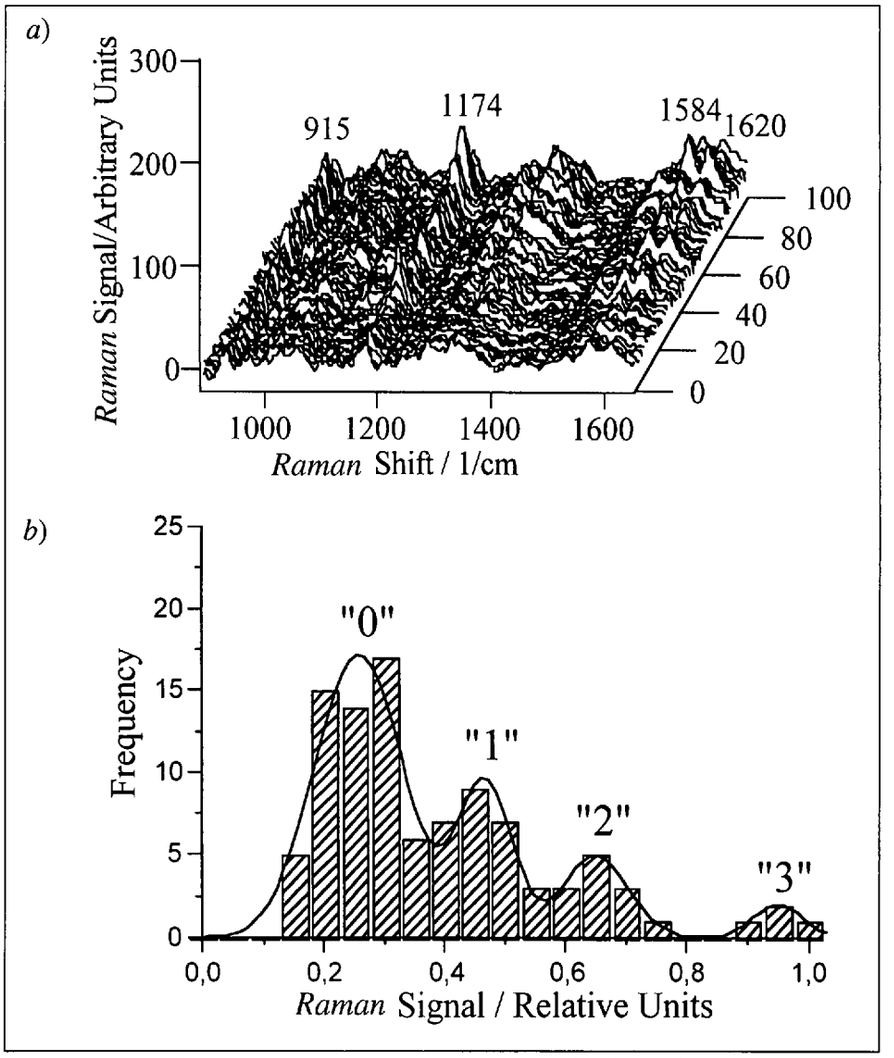


Fig. 1. *a) 100 SERS spectra collected in time sequence from a 30pl scattering volume containing an average of 0.6 crystal violet molecules.* Each spectrum is acquired in 1 s. *b) Statistical distribution of the SERS signal, which can be reasonably fit by the superposition of four* Gaussian *curves whose areas are roughly consistent with a* Poisson *distribution for an average number of 0.5 molecules, in relatively good agreement with the experimental situation.* The four peaks reflect the probability to find just 0, 1, 2 or 3 molecules in the scattering volume.

A remaining key issue is the nature of optically active and inactive sites on the particle surface. Early work by *Hildebrandt* and *Stockburger* [16] suggests that the SERS-active sites are high-affinity binding sites (65 kJ per mole) that are associated with adsorbed anions such as Cl^- or Br^-. The number of active sites per colloidal silver particle is very small, *ca.* 3.3 per particle. By using this active-site model, one can estimate the probabilities that a single molecule occupies an active site, an inactive site, or remains in free solution. For the optically active Ag nanoparticles, we estimate that a R6G molecule at adsorption equilibrium has a 90% probability of residing at an active site, 3% probability at an inactive site, and 7% probability of remaining in free solution. This partitioning effect could account for the 'missing molecules' – those molecules that are not detected in SERS [5].

A further issue is the origin of SERS-signal fluctuations in single metallic nanoparticles. Direct heating and cooling studies suggest that this behavior arises from thermally activated diffusion (site-to-site hopping) of single adsorbed molecules on the particle surface [17]. The fluctuating SERS signals also show a clear clustering effect, which is consistent with the neighborhood effect in random walking. In other words, a single molecule is likely to move across a single site several times when it diffuses to that area or neighborhood. A similar observation has been reported for single-molecule diffusion in free solution [18]. The activation energies of surface diffusion and site-to-site hopping are on the order of 5 kcal per mole, which is well within the reach of low-

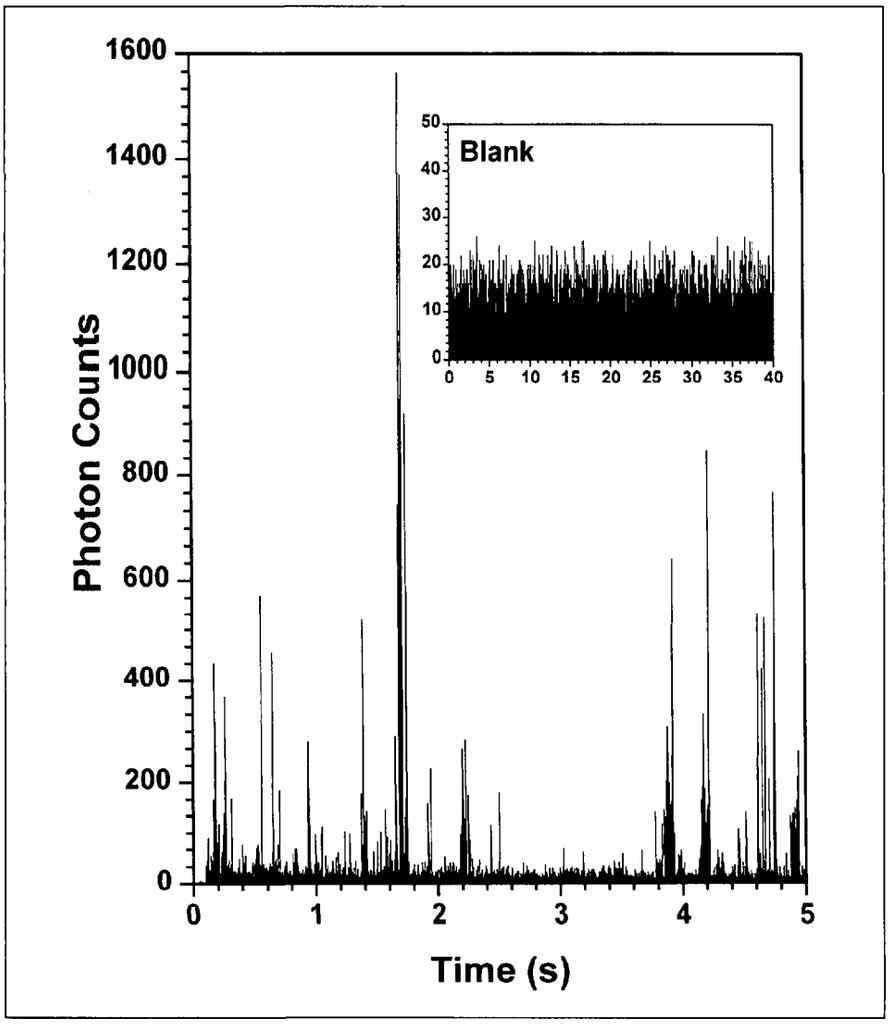


Fig. 2. *Intermittent surface-enhanced* Raman *scattering of rhodamine 6G observed from a single colloidal Ag nanoparticle.* The time-resolved data were recorded with a photon-counting avalanche photodiode and a multichannel scalar at 200 data points per second (5-ms integration time). To reduce background contributions, a narrow bandpass filter (560 DF 6, *Chroma Tech*, Brattleboro, VT) was used to select the *Raman* lines at 1647, 1566, and 1502 cm^{-1}. Continuous-wave laser excitation at 514.5 nm was provided by an argon ion laser (*Lexel Laser*, Fremont, CA). The laser power at the sample was about 0.1 mW.

energy thermal processes. Because the number of active sites on a single particle is small, surface diffusion of the adsorbed molecules will lead to large fluctuations in single-particle SERS. This low-energy thermal process is distinctly different from the light-driven mechanisms of intermittent light emission in single molecules and single quantum dots [11–14]. If this surface-diffusion model can be confirmed in the future, it will provide the strongest evidence for single-molecule *Raman*.

In addition to potential applications in ultrasensitive chemical analysis, an important outcome of single-molecule *Raman* might be the final elucidation of SERS. After 20 years and 2500 publications [19][20], we still do not have a complete understanding of the enhancement mechanisms. At present, it is believed that both a long-range electromagnetic (EM) effect and a short-range chemical effect are simultaneously operative in surface enhancement. Unlike homogeneous chemical systems, the SERS system (*i.e.*, molecules adsorbed on the surface of small metal particles) is highly complex and heterogeneous. As such, the SERS effect might be best studied and understood at the level of single molecules and single nanoparticles.

Received: January 7, 1999

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Addresses of the Authors:

Katrin Kneipp
Department of Physics
Technical University Berlin
D-10623 Berlin
and
G.R. Harrison Spectroscopy Lab.
Massachusetts Institute of Technology
Cambridge, MA 02139, USA
E-Mail: Kneipp@usa.net
Fax: 0049 30 427 17 92

Steven R. Emory and Shuming Nie
Department of Chemistry
Bloomington, IN 47405, USA
E-Mail: nie@indiana.edu / semory@indiana.edu
Fax: 812 855 83 00