Pushing the limits

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In 1997, Kneipp et al. and Nie and Emory independently reported the first examples of single-molecule detection using surface-enhanced Raman scattering (SERS). These seminal works sparked a surge of interest in SERS, while introducing a new question: how can it be conclusively proven that just one molecule is being probed?

Refers to Kneipp, K. et al. Single molecule detection using surface-enhanced Raman scattering (SERS). *Phys. Rev. Lett.* **78**, 1667-1670 (1997), and Nie, S. and Emory, S.R. Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. *Science* **275**, 1102-1106 (1997).

Vibrational spectroscopies are ensemble measurements, yielding a spectrum that is the average result for all molecules within the illuminated area. In 1997, two papers were published that described the first examples of single-molecule detection using surface-enhanced Raman

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scattering (SERS)[1,2]. Although these articles had a pivotal role in expanding interest in SERS, the observations and conclusions sparked much debate within the community.

The approach of Kneipp and colleagues[1] involved the use of aggregating silver nanoparticles (AgNPs) in the presence of NaCl to form silver clusters. Aggregation of the AgNPs led to the formation of 'hot-spots' between adjacent AgNPs capable of dramatically increasing the SERS signal. The authors estimated that a final concentration of 3.3×10^{-14} M crystal violet corresponded to 0.6 molecules of crystal violet per the estimated 100 silver clusters within the probed 30 pl volume. The key evidence supporting the claim of single-molecule detection was the presence of a Poisson distribution in the peak intensity at 1174 cm^{-1} for the 100 SERS spectra. Poisson distributions describe the probability of an event occurring when the frequency of that event is low. In this case, the event described is the number of molecules present within the probed volume. With a Poisson distribution of 0.5 best fitting the results, the authors ascribed the four local SERS intensity maxima to the probability of finding 0, 1, 2, or 3 molecules in the probed volume (Fig. 1a, b). Given the low distributions at 2 and 3 molecules, and the previously estimated number of molecules present in 30 pl, the authors concluded that single-molecule detection had been achieved.

Nie and Emory[2] also used AgNPs, which were first incubated in 2×10^{-11} M rhodamine 6G (R6G), to obtain an estimated 0.1 molecules per AgNP. The treated AgNPs were subsequently immobilized onto a glass surface, yielding isolated particles. This work argued that 'hot particles' (those with a strong intrinsic capacity for SERS) were the source of the single-molecule detection capabilities. The evidence supporting single-molecule detection came from two observations. First, unlike in bulk SERS measurements, when orthogonal polarizations of the incident light were used, different SERS intensities were found. The secondpiece of evidence was the presence of

fluctuations in the SERS signal under repeated irradiation of a single AgNP. Often referred to as 'blinking', the fluctuations include the appearance, disappearance and shifting of the Raman bands (Fig. 1c).

With such different observations, debate arose not only about the mechanism underlying single-molecule SERS, but also the evidence used to justify the conclusions. Simply put, should there be a Poisson distribution, peak fluctuations, or are there other experimental methodologies or spectral features that could be used to better confirm single-molecule detection?

In 2007, Etchegoin and co-workers[3] addressed the observation of a Poisson distribution. For the frequency distribution of the normalized peak heights to be described by a Poisson distribution, the signal intensity must only be proportional to the number of molecules. However, this is not the case in SERS, as the hot-spots generated within an aggregate vary between clusters. Even a small change in the enhancement can cause an intense variation in the SERS signal. Furthermore, the Poisson distribution was no longer observed when the number of measurements increased from 100 to 3,000 spectra[3]. Instead, a long tail was found in the histogram comparing the SERS intensity with the number of spectra.

Fluctuations in SERS signals have been shown to arise from conditions other than single-molecule detection. Kudelski and Pettinger[4] demonstrated that when carbon films are deposited onto rough silver or gold surfaces, the resulting SERS spectra show temporal changes when the measurements are performed in the presence of O_2. Given the strong electric field enhancement within hot-spots and considering the laser power densities used during experiments, the possibility of carbon contamination at the nanoparticle surface must be considered if signal fluctuations are to be used as evidence for single-molecule detection.

An alternative approach to verify single-molecule SERS was introduced based on a thought experiment. If two analytes are present at equally low concentrations, it is likely that a given nanoparticle would have a molecule of one analyte or the other adsorbed on its surface, with only a few having both[5]. The term bi-analyte SERS (BiASERS) was used to describe this effect, with R6G and benzotriazole dyes acting as the initial analytes. However, given that different molecules have different scattering cross sections and affinities for metal surfaces, an alternative approach was subsequently introduced relying on the use of isotopologues[6]. A comparison of two isotopologues of R6G showed that the main difference was a shift of the 622 cm^{-1} vibrational mode of hydrogenated R6G (R6G-d_0) to 610 cm^{-1} in the deuterated sample (R6G-d_4). The distribution of the isotopologues was investigated by interrogating 50 nanoparticle aggregates, with one spectrum acquired from each aggregate. Of these spectra, 24 had the peak of R6G-d_4, 22 had the peak of R6G-d_0, and only 4 had both peaks, which indicates that the experiment agrees with theory in terms of single-molecule measurements. It is important to recognize here that, once again, a small sample size was used. Relying on just 50 spectra may not be sufficient. Transitioning towards quantifying analyte concentrations at ultra-low concentrations can also benefit from the use of the isotopologue approach and is applicable to molecules other than dyes[7]. Raman mapping provides a straightforward means of acquiring large numbers of spectra, which is important because at ultra-low concentrations, only a few pixels within the map will have an active spectrum (Fig. 1d, e).

In the past decade, there have been more studies looking at ways to expand the types of configurations for single-molecule SERS beyond aggregates of AgNPs. The introduction of a DNA framework between two adjacent gold nanoparticles makes it possible to trap analytes of interest within the hot-spot, with the size of the framework being tuned to the analyte[8]. Likewise, nanoparticle-on-mirror[9] and nanostar–nanohole[10] geometries have also shown success in single-

molecule and near-single molecule measurements. Ultimately, with debate about data being a necessary part of science in fostering new ideas, applications and improving our fundamental understanding, the sub-field of single-molecule SERS remains dynamic thanks to these two papers from 1997 (ref.[1,2]).

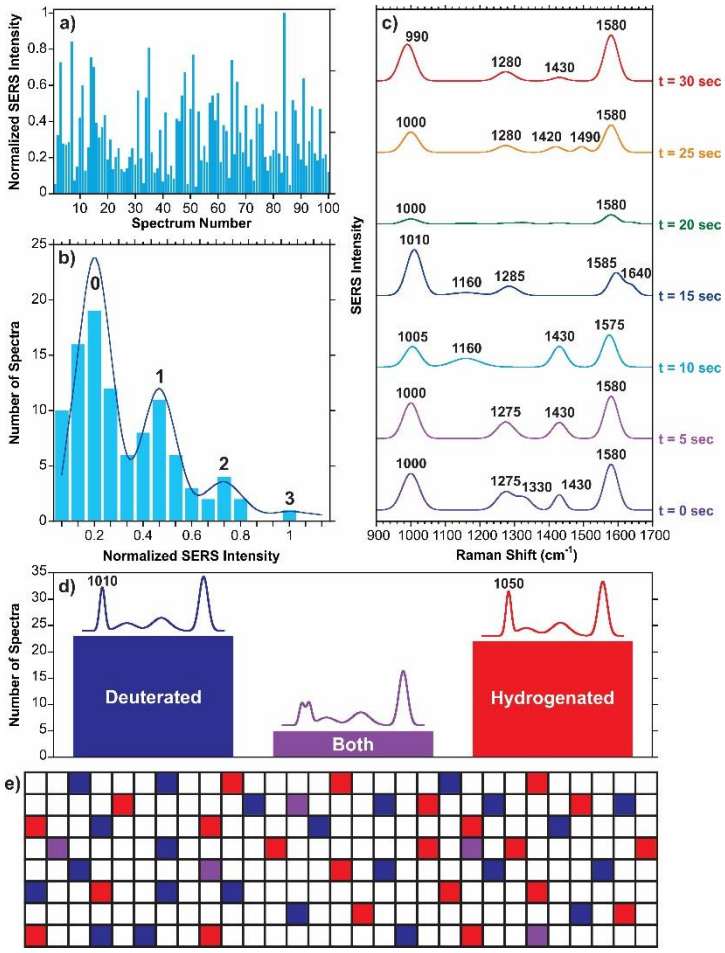

Figure 1. **Evidence for single-molecule detection a**| 100 normalized SERS intensities of a vibrational mode of a theoretical molecule. **b**| The corresponding Poisson distribution assuming an average of 0.5 molecules within the probed volume. The solid curve corresponds to the idealized Gaussian distributions and with the numbers indicating the number of molecules within the probed volume. **c**| The variation of the peak intensities, peak positions and number of peaks of theoretical

SERS spectra with time. The spectra are vertically offset for clarity. **d**| Examples of spectra obtained with BiASERS using deuterated and hydrogenated analytes, including the number of each type of spectra observed from a sample size of 50 theoretical spectra. The location of a peak is determined by whether the analyte is hydrogenated or deuterated, and when both modes are present, both peaks are observed. The numbers above the peaks indicate the shift in the SERS peak in the hydrogenated to deuterated analytes **e**| The corresponding hypothetical distribution map of analyte positions in a SERS map with blue, red and purple squares indicating deuterated analytes, hydrogenated analytes and those with both vibrational modes, respectively.

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COMPETING INTERESTS

The authors declare no competing interests.

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