

Monitoring the Electrochemistry of Single Molecules by Surface-Enhanced Raman Spectroscopy

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Abstract: Coherent control of chemical species in complex systems is always subject to intrinsic inhomogeneities from the environment. For example, slight chemical modifications can decisively affect transport properties of molecules on surfaces. Hence, *single-molecule* (SM) studies are the best solution to avoid these problems and to study diverse phenomena in biology, physics, and chemistry. Along these lines, monitoring SM redox processes has always been a “holy grail” in electrochemistry. To date, claims of SM electrochemistry by spectroscopy have come only from *fluorescence quenching* of polymers and redox-fluorescent molecules. In unconnected developments, the potential of the bianalyte surface-enhanced Raman scattering (SERS) method as a technique with SM sensitivity has been demonstrated. Raman spectroscopy has the potential to explore SM detection of *any* molecule, independent of its chemical nature. We provide definitive proof of SM events following redox cycles using SERS. The superior sensitivity and spectral richness of SERS makes it general enough to study, in principle, SM electron transfer of any (label-free) molecule.

Chemical species are always subject to the effect of the environment,¹ resulting (in spectroscopic terms) in the presence of an *inhomogeneous broadening* in the response of an ensemble. Slight changes in bonding geometries and/or electronic interactions on surfaces, for example, are known to have measurable effects on the transport properties of molecules.^{2–5} Therefore, *single-molecule* (SM) studies are deemed to be the only way to avoid the averaging process of the ensemble (i.e., the origin of the inhomogeneous broadening) and have indeed been pursued for the understanding of different phenomena and mechanisms in biology,^{6–8} physics,⁹ and chemistry.¹⁰

Redox processes at the SM level¹¹ have the potential to unravel the details of the coupling of individual molecules to the underlying surfaces (acting as electrodes). From the optical spectroscopy point of view, *fluorescence quenching* in polymers^{12,13} and redox-fluorescent molecules¹⁴ have been used in monitoring SM redox processes. However, Raman spectroscopy offers further additional advantages through its potential to explore SM detection of *any* molecule, independent of its chemical nature¹⁵ (i.e., not necessarily a fluorophore). In this respect, the potential of the bianalyte surface-

enhanced Raman scattering (SERS) method as a technique with SM sensitivity has been extensively studied^{15–18} (including sophisticated versions using isotopically edited probes). In this communication, we provide an experimental proof of SM events using SERS performed in the background of an underlying redox cycle. Specifically, we followed the spatial and temporal evolution of SM electrochemical events, thus revealing their individual contributions to the overall redox response.

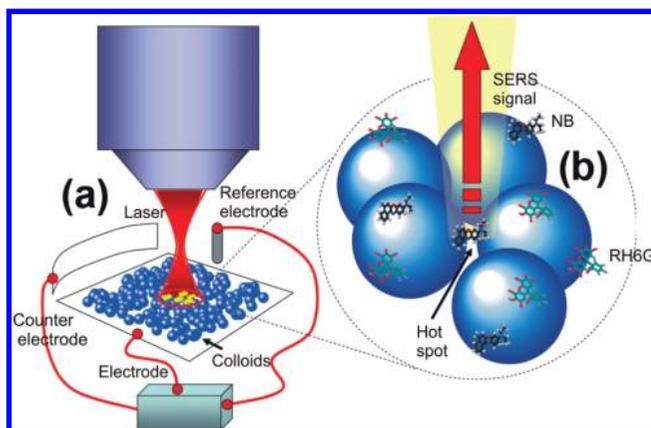


Figure 1. Experimental setup: (a) Ag colloids premixed with a suitable combination of bianalyte SERS partners (RH6G and NB at 2–5 nM concentration) are deposited on a working electrode (Ag) in an open-frame electrochemical cell (with a wide-area Pt counter electrode and a Ag/AgCl reference electrode) where we can perform microscopy through a water/air interface. (b) Occasionally, one molecule is at a SERS hot spot¹⁹ in a gap and dominates the SERS signal. SM electrochemistry can then be probed indirectly through the time evolution of the SERS spectrum.

In contrast to SM electron transport experiments, in which molecular junctions are built, we create in our experiments the conditions under which many different SMs at different places can experience redox cycles, and we select one of them to be analyzed through its SERS response. Several aspects of this problem are related to the exact conditions under which single molecules can be detected (and reliably identified as such). Most of these have already been solved in the field of SM-SERS,¹⁹ which has now reached a mature state to address issues beyond the mere demonstration of SM sensitivity.^{17,20} Therefore, the initial question of how we know that we are actually measuring single molecules with SERS has been resolved with the bianalyte SERS technique,¹⁶ which has now been extensively developed to different levels of sophistication^{15,17,18,21} and to which we add the possibility of

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performing electrochemical measurements. Another important question is how we assess that the detected SM cases are electrochemically active. In fact, electrochemistry provides an external “switch” to modulate the intensity of the Raman signal. Moreover, it is possible to differentiate typical random SM-SERS fluctuations from the effect of the electrochemical modulation. This point is further discussed later. In what follows, we focus only on electrochemically modulated signals.

Figure 1 shows a simplified schematic of our experimental setup. To the conditions normally used for bianalyte SM-SERS, we have added the ingredient of simultaneously performing electrochemistry. We chose two bianalyte partners, rhodamine 6G (RH6G) and Nile blue (NB), for very specific reasons. From the Raman point of view, NB changes from an oxidized state with a resonant SERS spectrum at 633 nm excitation to a barely visible (nonresonant) reduced state. The electrochemical cycle has a much less dramatic effect on RH6G.^{22,23} For all practical purposes here, we can consider RH6G as a “constant” (i.e., unchanged by the electrochemistry) bianalyte SERS partner of NB; this is desirable for an “electrochemical bianalyte” experiment. Further details of this choice are provided in the Supporting Information (SI). From the bianalyte SERS method point of view,^{15,20,21} the addition of observing the statistics with a “time domain” for individual events (to follow the effect of the electrochemical cycle) opens up a completely different optimization problem with tight constraints. Among these are photobleaching of the dyes, the readout time of the charge-coupled device (CCD), the period of the electrochemical cycle, the stability of colloids on the working electrode, the presence of distinguishable fingerprint modes at nearby Raman shifts, comparable SERS cross sections at the excitation laser, and so on. These conditions are discussed in more detail in the SI. Ultimately, we succeeded in following signals that we can attribute to SM-SERS in the background of a running electrochemical cycle, and the main results are discussed in what follows.

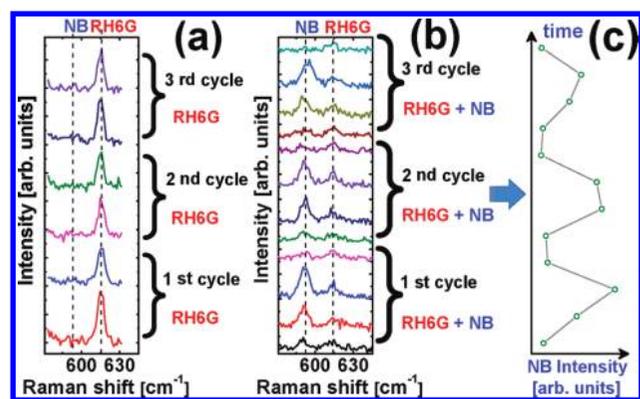


Figure 2. Electrochemically modulated bianalyte SERS spectra. (a) Single RH6G event, where the 610 cm^{-1} mode of RH6G was observed over three electrochemical cycles at a scan rate of 1 V s^{-1} . (b) “Mixed” event (NB + RH6G) with fingerprint modes at 590 (NB) and 610 cm^{-1} (RH6G). (c) Time evolution of the NB SERS intensity from (b). Over several full electrochemical cycles (scan rate = 0.5 V s^{-1}), the NB signal follows the modulation while the RH6G signal remains basically constant.

We observed all of the cases expected in the bianalyte SERS method.^{15,16,21} Figure 2a shows an example of a single RH6G signal (no NB) that remained unperturbed for several electrochemical cycles. In contrast, Figure 2b shows spectra of a mixture of RH6G and NB, where the latter (former) follows (does not follow) the successive electrochemical cycles, as shown in Figure 2c. Such mixed signals are expected in the bianalyte SERS method,²¹ but they are discarded for the analysis of the SM statistics. Still, Figure

2b,c shows how one molecule is affected by the underlying electrochemical cycle while the other one is not. The experimental conditions under which the data of Figure 2 were obtained were adjusted to allow the SM-SERS spectra to be followed over several electrochemical cycles. However, there is a trade-off here, namely, the longer we monitor the signal (for several cycles), the smaller the power density we can use (photobleaching), and the smaller the signal itself. We can compensate for this by using a longer integration time, which in turn results in a poorer time resolution of the cycle. The opposite obviously holds. In Figure 2c, for example, the electrochemical cycle is not perfectly resolved in time, but several cycles can be observed.

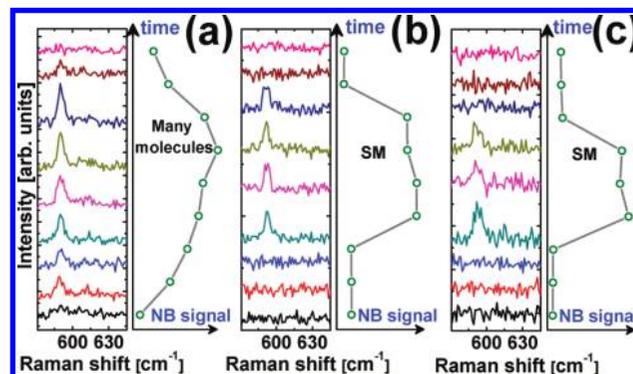


Figure 3. Many-molecule and single-molecule SERS electrochemistry. (a) Time evolution of SERS spectra over a full electrochemical cycle (scan rate = 0.3 V s^{-1}) for many molecules. (b, c) Two examples of SM events. A smoother time evolution of the signal was obtained for many molecules (25 nM NB with no RH6G in the cell). This is unlike the SM cases (at 2 nM), where a sudden appearance/disappearance of the NB SERS signals was observed.

The opposite limit of a shorter time resolution displays the most novel results, which are the main contribution of this work. In Figure 3, we aimed to resolve the electrochemical cycle with the smallest time resolution, 0.35 s (limited by the CCD readout time of 0.25 s and the signal integration time of 0.1 s); this is comparable to the charge-transfer dynamics times for redox couples involving two electrons and two protons²⁴ (see the SI). In order to do so, we used the largest power density to achieve enough signal per spectrum, but as a result of photobleaching, molecules did not last for more than a few electrochemical cycles. To avoid artifacts associated with photobleaching, only cases where the SM signal was present before and after the cycle were considered. A new aspect emerged in this limit. All of the NB SM-SERS signals showed a characteristic “rectangular” pattern under the electrochemical modulation. This is seen in Figure 3b,c and summarized for several NB SM-SERS cases in Figure 4. This behavior is drastically different from the smoother curves expected and observed for a large number of molecules (Figures 3a and 4a). The SM-SERS cases in Figures 3 and 4a therefore confirm the ubiquitous signature of SM electrochemistry and provide the possibility of simultaneously monitoring the Raman spectra and the electrochemical features of a SM event with no statistical averaging.

Figure 4a illustrates how various SM-SERS events last for different times in the “on” (oxidized) state before reduction. Hence, the NB molecules are oxidized at quite different potential values. This behavior reflects the random characteristics of the local redox properties at the different surface hot spots. A measurable surface heterogeneity is, indeed, expected in colloids.^{25,26} Further analysis of the redox behavior of SMs is depicted in Figure 4b. While some NB molecules exhibit reversible oxidation–reduction events (1 and 3 in Figure 4b), others show a more irreversible process (2 and 4

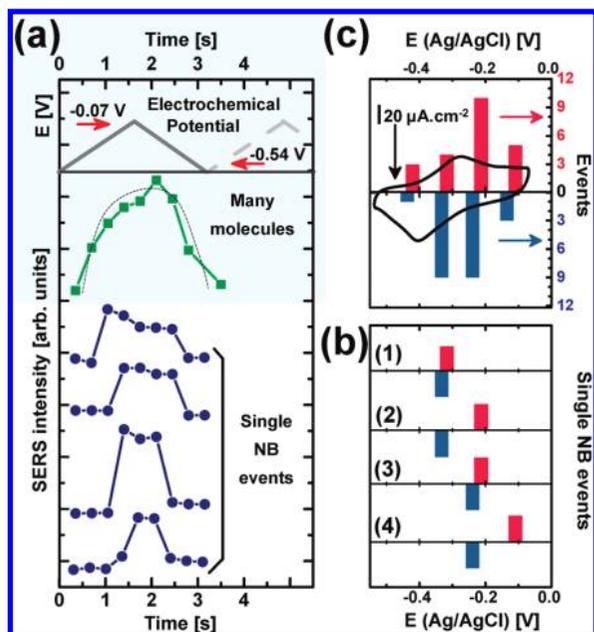


Figure 4. Variations in redox properties of different SM events. (a) Many-molecule and SM-SERS intensity cases for NB. The varied durations of the signals in the “on” (oxidized) state reveal the different redox potentials for that particular molecule. (b) Electrochemical potential for oxidation (red)—reduction (blue) on—off events for the single NB SM-SERS processes shown in (a). (c) Histogram showing the distribution of SM oxidation (red) and reduction (blue) on—off events with the applied potential. The voltammogram recorded for NB at nearly full coverage (black) is also shown.

in Figure 4b). Such a variation for other surface-confined redox molecules has been attributed to many causes ranging from lateral molecular interaction to variation in redox-site/electrode electronic coupling to microenvironmental variance in properties such as surface charge or molecular orientation.²⁷ As a consequence, both the redox potential and the charge-transfer rate constant evidenced a random behavior at the SM level (Figure 4b).

Let us focus on the behavior of a single molecule along two successive voltammetric cycles (Figure 5a,c). Inspection of the data indicates that the same molecule experiences oxidation and reduction at different potentials. This instability is lost when a statistical average is performed in the many-molecule limit (Figure 5b). For the example shown, the molecule is oxidized at -0.21 ± 0.05 V and reduced at -0.42 ± 0.05 V in the first cycle, while in the second cycle it is oxidized at -0.32 ± 0.05 V and reduced at -0.24 ± 0.05 V. All of these features are signatures of the stochastic nature of SM events and reveal temporal fluctuations in the environment, as predicted theoretically.²⁸

An interesting question is whether one can recover the “average” behavior of the system from “single” SM events. The connection between the behavior of our SM measurements and the voltammogram recorded for full coverage is depicted in Figure 4c. The correlation of the histogram built from the SM events with the voltammetric cycle at large concentration (in particular, the broadening of the oxidation peak in relation to the cathodic one) is evident. There is, however, a subtle difference: the histogram is shifted to more positive values. In fact, the formal potential $E^{\circ'}$ derived from the histogram is $E^{\circ'} \approx -0.22$ V, which is ~ 0.10 V more anodic than that estimated from the voltammogram for adsorbed NB at full coverage ($E^{\circ'} = -0.32$ V). This shift could reflect the change in intermolecular interactions as the system moves from nearly full coverage of NB to only few molecules.²⁶ However, it could also be due to the lack of enough single events to recover

the “average” voltammogram. In fact, it has been shown that even in the case of hundreds of molecules, corresponding to a low coverage of immobilized proteins, the redox behavior differs from that observed in conventional electrochemical measurements, i.e., it involves a larger contribution from events with low electron-transfer rates.²⁷ These conclusions can also be expected to hold for the temporal convolution regarding SM and many-molecule events in Figure 5.

From Figures 4 and 5, one can conclude that the standard redox potential is the spatial and temporal convolution of the potentials of a large number of molecules with similar but not identical local conditions and that it can be extracted only from SM measurements where these properties are not averaged.

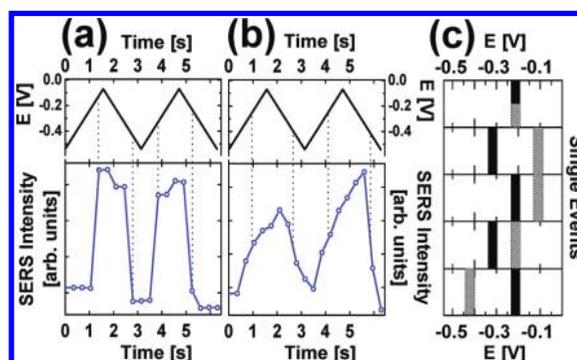


Figure 5. (a, b) Variations in redox properties for two consecutive cycles, as determined from the SERS intensity of the 590 cm^{-1} peak of NB along two voltammetric cycles: (a) single molecule; (b) many molecules. The electrochemical cycle shown at the top. Dashed lines are guides to the eye for the oxidation/reduction potentials of each event. (c) Temporal variation for single molecules. Each of the five rows illustrates the temporal variation of the oxidation potential in two successive redox events for a single NB molecule (black, first event; dashed, second event). The top curve corresponds to a molecule whose oxidation potential did not change in the two observed periods, while those of the other four did. All of the potentials are referred to the Ag/AgCl reference electrode.

Our results were obtained at the limit of current experimental capabilities. Their importance relies mainly on their potential. The combination of SM-SERS with an underlying electrochemical modulation provides clean examples of SM electrochemistry. Measuring SM electron-transfer processes is out of reach for state-of-the-art electrochemistry.^{11,29} Hence, SERS provides an indirect “amplification” method for monitoring SM electrochemistry with high specificity (the Raman spectrum). It is not difficult to envision further developments from here, such as real-time experiments mapping single redox centers in living cells or studying spatial and temporal fluctuations of individual components with biological redox activity.

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Supporting Information Available: Methods, selection of the bialyte SERS probes, sample preparation, electrochemical cycles,

optimization of SM-SERS EC experiments, discrimination of electrochemically modulated SM events versus random SM events, principal component analysis (PCA) method, and further details and discussions of the experimental conditions and the analysis of the data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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SUPPORTING INFORMATION

Monitoring the electrochemistry of single molecules by surface-enhanced Raman spectroscopy

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S1 – Methods

Experiments were performed with a three-electrode cell constructed with a Ag/AgCl (1M KCl) and a high-area platinum foil as reference and counter electrodes, respectively (Fig. 1). All the potentials reported here are referenced to the Ag/AgCl (1M KCl) electrode and the electrolyte solution used is 0.25 M phosphate buffer (pH=6). For the experiments presented in the main text (Figs. 3, 4 and 5) a scan rate of $v=0.30$ V/s was used. Potentials were scanned from -0.54 V to -0.7 mV. The whole arrangement is placed inside an open electrochemical cell that allows focusing on the working electrode with a long working distance objective (x20, when we want to average over large areas with many molecules; or x100, when we search for SM-SERS signals) through a water/air interface. Cyclic voltammetry with digital data acquisition is performed with a computer controlled potentiostat. In order to allow exploration of different clusters on the working electrode, the cell is placed on top of a motorised x-y stage for microscopy. The Raman signal is collected by a BX41 Olympus microscope attached to a Jobin-Yvon LabRam spectrometer with a notch filter. The minimum acquisition time to follow

the dynamics of the electrochemical cycle is limited by the readout time of our CCD (which is 0.25 sec), and the integration time of the SERS signal, 0.1 sec in this case. All the experiments were performed with the 633nm line of a HeNe laser with varying input powers in the range 0.3-3 mW depending on the signal level. Data analysis was performed by Principal Component Analysis method (PCA).

S2- Selection of the bianalyte SERS probes.

NB has a well-understood electrochemistry and undergoes reduction/oxidation (redox) transformations at $\sim -0.32\text{V}$ on Ag (vs Ag/AgCl reference electrode) in a phosphate buffer solution pH=6, involving a two-electron, two-proton (2e2H) process.¹ The electron transfer rate constant of NB at pH 7 is $\sim 1.4 \pm 0.1 \text{ s}^{-1}$. Similar transfer rate constant values (6-17 s^{-1}) are obtained for adsorbed phenoxazines under different conditions.^{3,4} Brolo et. al.⁵ reported on the onset of electrochemical reduction of RH6G at $\sim -0.4\text{V}$ under similar experimental conditions as used here. This results in a slight increase of the intensity in the average SERS spectrum of RH6G at 633 nm laser excitation. Compared to the dramatic change in NB, this effect is mostly secondary.

S3- Sample preparation.

Rhodamine 6G (RH6G) and Nile Blue (NB) were obtained from Sigma-Aldrich, and mixed at the appropriate concentrations with borohydride-reduced Ag colloids.⁶ This is done to avoid a citrate capping layer (present in citrate-reduced colloids). The colloids+dyes ($\sim 2-5\text{nM}$ dye concentration) are mixed with 20 mM KCl to induce the pre-formation of small clusters⁶⁻⁸ before drying. RH6G and NB attach to the colloids in solution through electrostatic interactions. The colloidal solution is subsequently drop-casted and dried on a clean Ag working electrode; previously cleaned with a piranha solution (to avoid any residual organic contamination of the electrode). Hence, the dyes were adsorbed beforehand on the colloids, and not dissolved in the electrolyte solution itself. The colloids themselves stick to the working electrode by Van der Waals forces, and remain attached to it upon re-immersion in the

phosphate buffer solution. Accordingly, the working electrode provides all the necessary conditions where SERS enhancements produced by localized hot-spots (gap-plasmon resonances)⁹ can coexist with the ability to perform electrochemistry on the attached molecules. We tested that at much higher concentrations (~ 1mM) the electrochemistry of the dyes on the colloids is indistinguishable from the one where they are directly attached to the Ag foil; i.e. the presence of the adsorbed colloids does not introduce any electrochemical artefact. Similar experimental conditions to observe single molecules in SERS using the bianalyte technique have been reported previously.^{7,8,10}

S4- Electrochemical cycles

Under our experimental conditions each voltammetric run lasts ~3.13 sec (scan rate $v=0.30 \text{ V}\cdot\text{s}^{-1}$). SM-SERS spectra are acquired with a total time of 0.35 sec (integration time + readout time of the CCD); so a complete electrochemical cycle corresponds to 9 SM-SERS spectra (3.15 sec). We have verified that under these conditions the dephasing time (between potentiostat and spectrometer) is less than 0.1%. The read-out time of the CCD and the SM-SERS signal integration time are the main limits in the resolution of the SM-SERS electrochemical events. With our present resolution each SM-SERS spectrum corresponds to ~0.10 V in the voltammetric run.

S5 - Optimization of SM-SERS EC experiments.

As mentioned in the main text, our results are the product of a complex optimization procedure amongst the multiple variables of the problem, which impose stringent and sometimes contradicting requirements. As complementary information here, we expand a bit further on the explanation of the different requirements of the experiments. These include:

- Photobleaching: dye molecules last for a finite time under SM-SERS conditions. While this can be somewhat improved with the laser power density being used, there is an obvious trade-off with the signal-to-noise level for the Raman signal

on the CCD for a fixed integration time. Molecules under the present experimental conditions (typically) do not last for more than ~ 9-11 sec, in the best of cases.

- Readout time of the CCD: There is a minimum read-out time imposed by the CCD in our system of 0.25 sec. This limits the minimum time resolution with which we can follow the electrochemistry of a single molecule (provided there is enough signal on the CCD, *vide supra*). Charge transfer rate constants for this kind of redox processes (2e2H) are within this time window.

- Electrochemical cycle period: the potentiostat limits the maximum scanning rate with which we can complete a redox cycle. Ideally, we want to have as many cycles as possible during the (photobleaching) lifetime of the molecule. But the fastest scan rates have to be in accordance with the minimum integration times possible on the CCD, which is decided by the readout time in combination with the signal-to-noise ratio of the actual signal.

- Stability of the colloids on the working electrode: This effect is convoluted in fact with the stability due to photobleaching (and it is indistinguishable from it in practical terms). The configuration producing hot-spots in the colloid clusters has to be stable enough to produce a steady SERS signal from single molecules during the total observation time. This turns out to be the case in our experimental conditions.

- Presence of distinguishable fingerprint modes at close-by Raman shifts: This is desirable -but not essential- to simplify the bianalyte SERS statical analysis, which otherwise might have to deal with issues of plasmon-dispersion corrections to make a fair comparison of peaks that are too far apart. This is a well known and documented phenomenon in SERS¹¹ that can be basically avoided if two analytes with close-by Raman peaks are selected. This has been one of the reasons to choose RH6G and NB in our case, in addition to the electrochemical constraints.¹²

- Comparable SERS cross sections at the excitation laser: This is also desirable to have the simplest type of analysis in the bianalyte SERS technique. If the probes have cross sections that are too different, there are issues with small signals that could be below the signal-to-noise level for one analyte but not the other. This produces a skewed statistics, which is a reflection of the inappropriate choice of molecules, rather than an intrinsic limitation.

S6- Discrimination of electrochemically modulated SM-events vs random SM-events.

An important point regarding electrochemically modulated SM-SERS events is how to differentiate them from random SM-events. Under our experimental conditions 25% of the NB SM-events can be assigned to electrochemically modulated events and only those cases were considered for this work. Basically, the main problem is to ensure that the molecule is present at the hot-spot along the complete voltammetric cycle. We therefore considered only events where the NB SM-signal is present both before and after a complete oxidation/reduction cycle. On the other hand, the stability of both (reduced and oxidized) NB species at the working electrode has been already shown.^{1,12}

S7 - PCA method.

The SERS intensities of both RH6G and NB as a function of time are obtained by analyzing the spectra with Principal Component Analysis (PCA). While this is not necessary (any data analysis capable of extracting the intensities of the peak as a function of time should render exactly the same results), it is done for convenience. PCA allows us to identify the *principal components* through which the spectra can be decomposed (as a linear combination). In the case of two independent dyes (NB and RH6G), the principal components (or eigenvectors) are the average intensities of the corresponding fingerprint modes for both compounds. An example of the two principal components used in the decomposition of the spectra is shown in Fig. S1. The PCA analysis method for the study of multiple-analytes in SM-SERS has been described in detail in Ref.[8] (see also the supplementary information of Ref. [8]). In order to facilitate the PCA analysis, spectra have also been background-subtracted with the algorithm developed by C. M. Galloway *et. al.*¹³ As with the use of PCA, this is not actually necessary, but we found it convenient for a fast and reliable analysis of the intensities. None of the conclusions depend on the particular choice of these methods.

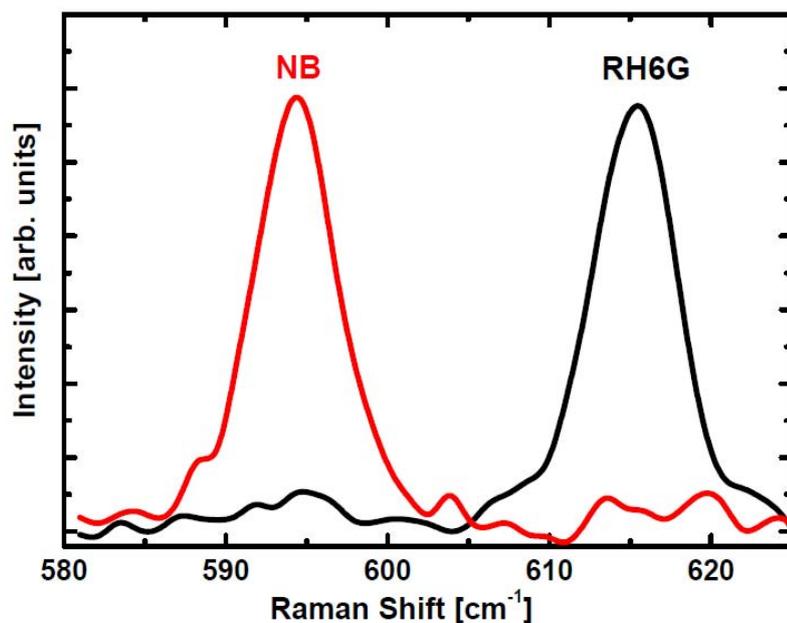


Figure S1: The 1st and 2nd PCA eigenvectors contain the two main “fingerprints” spectra for RH6G and NB in this spectral region of interest ($\sim 580\text{-}625\text{ cm}^{-1}$). A linear decomposition of the spectra in the eigenvectors results in the coefficients (proportional to the respective intensities) of NB and RH6G, respectively.

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