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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, s*ingle-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 level[11] 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 molecules[14] 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 nature[15] (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.

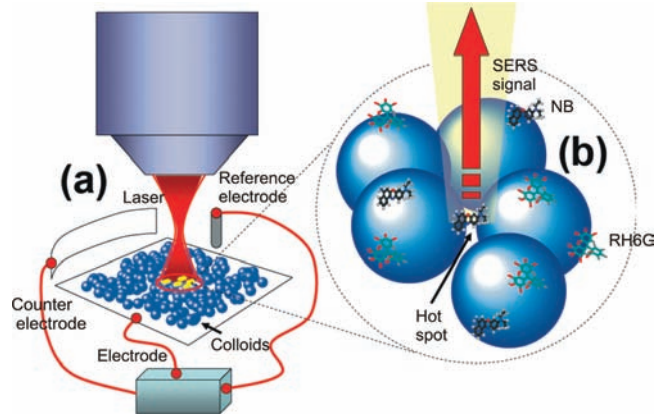

Figure 1. Experimental setup: (a) Ag colloids premixed with a suitable combination of bianalyte SERS partners (RH6G and NB at 2−5 nM concentration) are deposited on a working electrode (Ag) in an open-frame electrochemical cell (with a wide-area Pt counter electrode and a Ag/AgCl reference electrode) where we can perform microscopy through a water/air interface. (b) Occasionally, one molecule is at a SERS hot spot[19] 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.

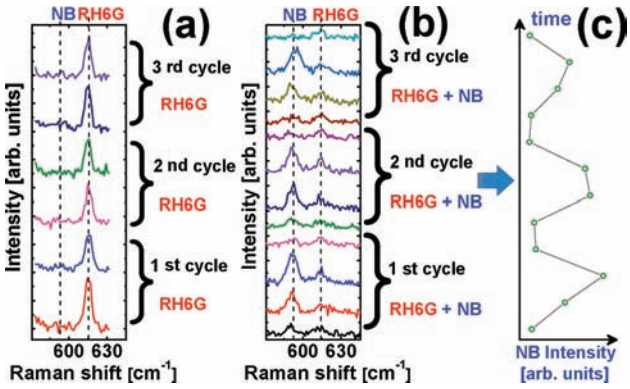

Figure 2. Electrochemically modulated bianalyte SERS spectra. (a) Single RH6G event, where the 610 cm^{-1} mode of RH6G was observed over three electrochemical cycles at a scan rate of 1 V s^{-1}. (b) "Mixed" event (NB + RH6G) with fingerprint modes at 590 (NB) and 610 cm^{-1} (RH6G). (c) Time evolution 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 tradeoff 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.

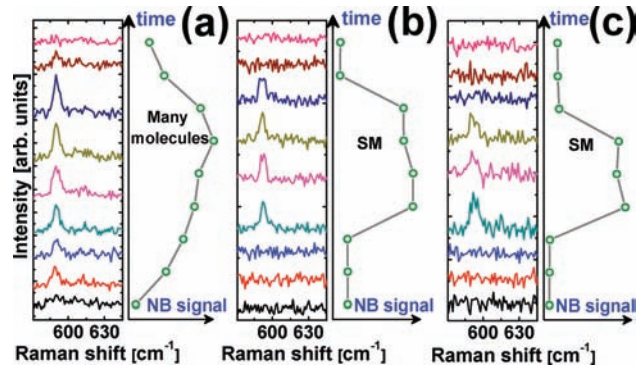

Figure 3. Many-molecule and single-molecule SERS electrochemistry. (a) Time evolution of SERS spectra over a full electrochemical cycle (scan rate = 0.3 V s^{-1}) for many molecules. (b, c) Two examples of SM events. A smoother time evolution of the signal was obtained for many molecules (25 nM NB with 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 protons[24] (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

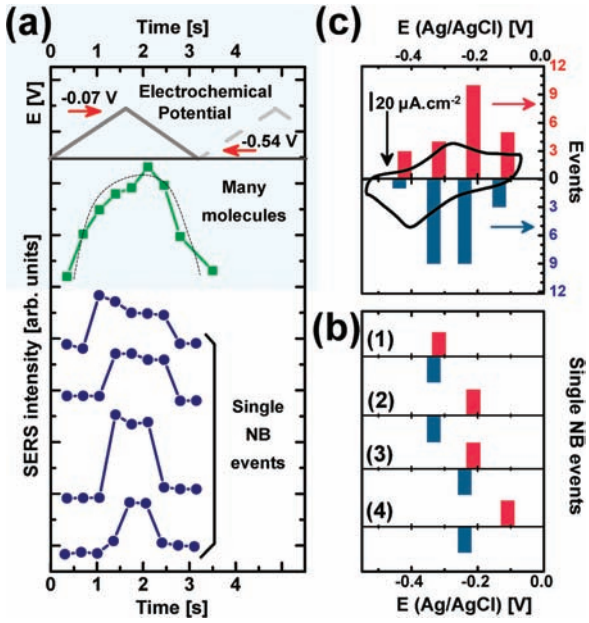

Figure 4. Variations in redox properties of different SM events. (a) Many-molecule and SM-SERS intensity cases for NB. The varied durations of the signals in the "on" (oxidized) state reveal the different redox potentials for that particular molecule. (b) Electrochemical potential for oxidation (red)−reduction (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 $E^{\circ\prime}$ derived from the histogram is $E^{\circ\prime} \approx -0.22$ V, which is ~0.10 V more anodic than that estimated from the voltammogram for adsorbed NB at full coverage ($E^{\circ\prime} = -0.32$ 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.

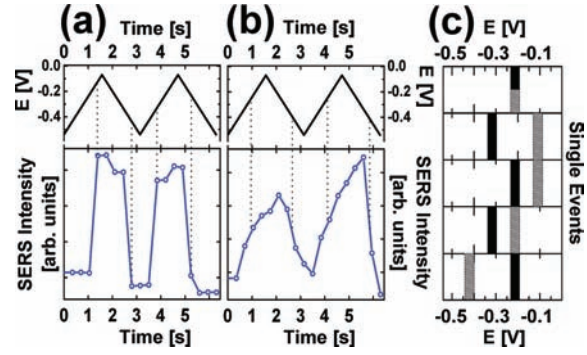

Figure 5. (a, b) Variations in redox properties for two consecutive cycles, as determined from the SERS intensity of the 590 cm^{-1} peak of NB along two voltammetric cycles: (a) single molecule; (b) many molecules. The electrochemical cycle shown at the top. Dashed lines are guides to the eye for the 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 bianalyte 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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