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Editorial

Quo vadis surface-enhanced Raman scattering?
DOI: 10.1039/b913171j

Perspective

Surface-enhanced Raman spectroscopy of dyes: from single molecules to the artists' canvas
DOI: 10.1039/b904733f

Communication

Tip-enhanced Raman scattering (TERS) of oxidised glutathione on an ultraflat gold nanoplate
Tanja Deckert-Gaudig, Elena Bailo and Volker Deckert, Phys. Chem. Chem. Phys., 2009
DOI: 10.1039/b904735b

Papers

Self-assembly of n, o-aliphatic diamines on Ag nanoparticles as an effective localized surface plasmon nanosensor based in interparticle hot spots
Luca Guerrini, Irene Izquierdo-Lorenzo, José V. Garcia-Ramos, Concepción Domingo and Santiago Sanchez-Cortes, Phys. Chem. Chem. Phys., 2009
DOI: 10.1039/b904631c

Single-molecule vibrational pumping in SERS
DOI: 10.1039/b904638k

Silver nanoparticles self assembly as SERS substrates with near single molecule detection limit
DOI: 10.1039/b904744a

Gated electron transfer of cytochrome c, at biomimetic interfaces: a time-resolved SERR study
DOI: 10.1039/b904434e

Investigation of particle shape and size effects in SERS using T-matrix calculations
DOI: 10.1039/b905645a

Plasmon-dispersion corrections and constraints for surface selection rules of single molecule SERS spectra
DOI: 10.1039/b905846j

Redox molecule based SERS sensors
DOI: 10.1039/b905600a

Controlling the non-resonant chemical mechanism of SERS using a molecular photoswitch
DOI: 10.1039/b904745j

Interfacial redox processes of cytochrome b_{562}
DOI: 10.1039/b904926f

Surface-enhanced Raman scattering of 5-fluorouracil adsorbed on silver nanostructures
Mariana Sardo, Cristina Ruano, José Luis Castro, Isabel López-Tocón, Juan Soto, Paulo Ribeiro-Claro and Juan Carlos Otero, Phys. Chem. Chem. Phys., 2009
DOI: 10.1039/b903823j

SERS imaging of HER2-overexpressed MCF7 cells using antibody-conjugated gold nanorods
DOI: 10.1039/b904592a
Nanospheres of silver nanoparticles: agglomeration, surface morphology control and application as SERS substrates
DOI: 10.1039/b904712c

SERS enhancement by aggregated Au colloids: effect of particle size
Steven E. J. Bell and Maighread R. McCourt, Phys. Chem. Chem. Phys., 2009
DOI: 10.1039/b906049a

Towards a metrological determination of the performance of SERS media
DOI: 10.1039/b904621f

Ag-modified Au nanocavity SERS substrates
DOI: 10.1039/b904685m

Surface-enhanced Raman scattering studies of rhodanines: evidence for substrate surface-induced dimerization
DOI: 10.1039/b905008f

Characteristics of surface-enhanced Raman scattering and surface-enhanced fluorescence using a single and a double layer gold nanostructure
DOI: 10.1039/b903819c

High performance gold nanorods and silver nanocubes in surface-enhanced Raman spectroscopy of pesticides
Jean Claudio Santos Costa, Rômulo Augusto Ando, Antonio Carlos Sant’Ana, Liane Marcia Rossi, Paulo Sérgio Santos, Márcia Laudelina Arruda Temperini and Paola Corio, Phys. Chem. Chem. Phys., 2009
DOI: 10.1039/b904734d

Water soluble SERS labels comprising a SAM with dual spacers for controlled bioconjugation
DOI: 10.1039/b905092b

Chromic materials for responsive surface-enhanced resonance Raman scattering systems: a nanometric pH sensor
DOI: 10.1039/b904747f
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Surface-enhanced Raman spectroscopy of dyes: from single molecules to the artists’ canvas

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This perspective presents recent surface-enhanced Raman spectroscopy (SERS) studies of dyes, with applications to the fields of single-molecule spectroscopy and art conservation. First we describe the development and outlook of single-molecule SERS (SMSERS). Rather than providing an exhaustive review of the literature, SMSERS experiments that we consider essential for its future development are emphasized. Shifting from single-molecule to ensemble-averaged experiments, we describe recent efforts toward SERS analysis of colorants in precious artworks. Our intention is to illustrate through these examples that the forward development of SERS is dependent upon both fundamental (e.g., SMSERS) and applied (e.g., on-the-specimen SERS of historical art objects) investigations and that the future of SERS is very bright indeed.

I. Introduction

Dyes that produce vibrant colors have been treasured since antiquity. For example, the red dye obtained from crushed cochineal insects was so desired by Europeans following the discovery of the Americas that it directed cultural trends, world economy, and international politics for centuries.1 Cochineal profoundly influenced merchants, sovereigns, dyers, artists, and scientists. Early microscopists sought to determine the mysterious source of the colorant: was it plant, animal, or corpuscular in nature? Indeed, long before the advent of modern spectroscopy, artists and scientists were united by their fascination with dyes. This perspective focuses on contemporary studies of chromophores using surface-enhanced Raman spectroscopy (SERS). In particular, we describe applications of SERS to the fields of single-molecule spectroscopy and art conservation—from individual dye molecules to ancient Peruvian dyed textile fibers.

Working from small to large, we begin by reviewing recent progress in single-molecule surface-enhanced Raman spectroscopy (SMSERS). Since its discovery in 1997 SMSERS2,3 has generated considerable interest, with > 100 research articles as well as several reviews on the subject. In this portion of the perspective, we seek to contextualize current SMSERS experiments without providing an exhaustive review of the literature, which can be found elsewhere. 4–9 Significant advances in SMSERS including: proof of single-molecule existence, geometry of single-molecule “hot spots”, and the excitation-wavelength dependence of SMSERS are highlighted. Furthermore, we discuss outstanding questions and future challenges in the context of developing a practical and informative single-molecule technique. To complement this perspective on new frontiers in SERS, recent progress toward applying SERS to
art conservation and restoration efforts is included. We highlight recent work as well as future challenges associated with transitioning the identification of colorants in artworks using SERS from the laboratory to the museum setting. Ultimately, the experiments described herein exemplify our ongoing fascination with dyes and demonstrate the utility of SERS from fundamental experiments on single dye molecules to applied investigations of the artists’ canvas.

II. Brief background on SERS

Raman spectroscopy provides the unique vibrational signatures of molecules. However, the Raman effect is notoriously weak with cross-sections of \( \sim 10^{-29} \text{ to } 10^{-30} \) cm\(^2\) as compared to typical absorption cross-sections of chromophores that are \( \sim 10^{-15} \text{ to } 10^{-16} \) cm\(^2\). Therefore, it is not surprising that the original observations of surface Raman signals from pyridine on electrochemically-roughened Ag films,\(^{10}\) followed by the realization that they were enhanced by a factor of \( 10^6 \) generated considerable excitement.\(^{11,12}\) SERS promises ultrasensitive detection and identification of molecules with otherwise weak inelastic scattering probabilities—even down to the single-molecule level. In the years following the discovery of SERS,\(^{11,12}\) much attention was devoted to understanding the origins of surface enhancement. Since Raman intensity scales as the product of the squared molecular polarizability derivative (\( \alpha \) or \( \beta \)) and the incident field intensity (\( |E_0|^2 \)), the observed signal enhancement in SERS was explained by two mechanisms: chemical and electromagnetic. The chemical mechanism is considered an enhancement in polarizability due to chemical effects such as optically-excited charge transfer (e.g., transitions between the molecule and metal), while the electromagnetic mechanism (EM) is enhancement in the local field intensity as a result of localized surface plasmon resonance (LSPR) excitation. The relative contribution of chemical and electromagnetic mechanisms in SERS is the subject of numerous investigations, and in general, chemical enhancement is modest (\( \sim 10^1 \text{ to } 10^2 \)) as compared to EM effects (\( \sim 10^7 \text{ to } 10^{10} \)). Therefore, we focus next on a brief description of the EM enhancement that is largely responsible for SERS.

The EM enhancement is a consequence of electromagnetic excitation of the surface plasmon resonance of nanostructured coinage metals.\(^{11}\) Detailed theoretical treatments of the EM enhancement are presented elsewhere.\(^{13-17}\) Briefly, the incident electromagnetic field excites a collective oscillation of the conduction electrons in a noble metal, resulting in enhanced fields close to the surface. The Raman scattering intensity is proportional to \( |E_0|^2 \), which is enhanced by the presence of the metallic surface. In particular, for a small metallic sphere, the electromagnetic field intensity at the surface of the nanoparticle is given by:

\[
|E_{\text{out}}|^2 = 2|E_0|^2 / (\varepsilon_{\text{in}} - \varepsilon_{\text{out}}) (\varepsilon_{\text{in}} + 2\varepsilon_{\text{out}})^2
\]

where \( \varepsilon_{\text{in}} \) and \( \varepsilon_{\text{out}} \) are the dielectric constants of the metal nanoparticle and external environment, respectively. From this equation it is apparent that the maximum enhancement occurs when \( \varepsilon_{\text{in}} \approx -2\varepsilon_{\text{out}} \), a resonance condition that is satisfied in the visible region for Au and Ag nanoparticles. Likewise, the Raman scattered field may also be enhanced, though generally by a different amount than the incident field, since the frequency is dissimilar.\(^{16}\) The overall enhancement associated with the incident and inelastically scattered fields is considered to be the EM contribution to SERS and is typically expressed as an enhancement factor (EF) relative to normal Raman scattering. EFs are evaluated by comparing SERS to normal Raman measurements of an analyte as:

\[
EF = \frac{N_{\text{SERS}}}{N_{\text{Raman}}}
\]

where \( N_{\text{SERS}} \) and \( N_{\text{Raman}} \) are the number of molecules contributing to the inelastic scattering intensity (evaluated on a surface for SERS and typically in solution for normal Raman measurements). Ultimately, the measured EF describes the average Raman enhancement at a single excitation wavelength and is often used to evaluate the quality of SERS substrates. At present, enhancements > \( 10^7 \) are routinely reported in the literature for plasmonic substrates such as Au and Ag nanoparticle aggregates,\(^{2,3,18}\) Ag film-over-nanospheres (AgFONs),\(^{19,20}\) and Ag periodic particle arrays (AgPPAs).\(^{21,22}\)

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These large enhancements, combined with the development of robust nanofabrication techniques and computational modeling of nanostructures have inspired widespread application of SERS. This perspective focuses on two different and yet fundamentally related applications of SERS: interrogating dyes from the single-molecule level to artist media. First, we discuss the development and outstanding questions for SERS measurements of single molecules, which are by and large limited to dyes.

III. The development of SMSERS

a Background

Twenty years after the discovery of SERS, the technique was taken to the ultimate limit—detection of single molecules. In particular, the groups of Nie and Kneipp reported the observation of SER spectra from individual molecules (i.e., rhodamine 6G (R6G) and crystal violet) adsorbed to Ag nanoparticles. The detection of inelastic scattering from a single molecule is remarkable considering that only \( \sim 10^{-4} \) photons s\(^{-1}\) molecule\(^{-1}\) is scattered in a typical Raman experiment. Indeed, the surface-enhanced Raman cross-section is substantially magnified with respect to normal Raman; original estimates put the single-molecule EF at \( \sim 10^{14–15} \) based on comparison of a typical Raman scattering cross-section (\( \sim 10^{-30} \) cm\(^2\)) to the absorbance cross-section of a typical chromophore (\( \sim 10^{-15} \) cm\(^2\)). As is well known from the single-molecule fluorescence (SMF) literature, the utility of single-molecule studies is that the effects of ensemble averaging and non-synchronicity are removed, revealing behavior otherwise obscured by the ensemble. Moreover, whereas SMF studies are limited to luminescent molecules, in principle any molecule can be interrogated using single-molecule SERS (SMSERS). It is therefore not surprising that the initial SMSERS observations prompted substantial interest; arguably, SMSERS holds the promise for a ubiquitous single-molecule technique. Unfortunately, the widespread application of SMSERS to problems in chemistry, materials science, and biology has yet to be realized. This is due, at least in part, to: (1) an incomplete picture of the SMSERS mechanism; (2) the uncertainties involved in using randomly aggregated nanoparticle assemblies as the substrates for SMSERS; and (3) difficulty in establishing a reliable method to prove single-molecule detection. In the following sections, we discuss these concerns as well as recent work that provides a positive outlook for the future of SMSERS.

b Proving single-molecule detection

At the crux of a single-molecule experiment is the proof that signal originates from just one molecule. In SMF several observations are used to demonstrate single-molecule behavior: (1) concentration studies to demonstrate the diffraction-limited spots decrease proportionally with analyte concentration; (2) the observation of “blinking”, a result of populating and depopulating non-emissive states; (3) eventual single-step photobleaching of the chromophore; (4) spectral diffusion; and (5) photon antibunching measurements, based on the fact that one molecule will not produce two photons at the same time. Likewise, several methods have been used to establish single-molecule SERS detection: (1) concentration (i.e., the observation of Raman signal from \( \sim 10^{-16} \) M analyte corresponding to 0–1 analyte molecule per nanoparticle); (2) polarization (i.e., single-molecule spectra are expected to be polarization sensitive); (3) blinking or spectral wandering (i.e., stochastic fluctuations in Raman intensity and/or band position over time); and (4) Poisson statistics (i.e., the observation of quantized SERS intensities corresponding to 0,1,2,... molecules). In the last few years however, it has been recognized that several observations used to establish single-molecule detection such as blinking and Poisson statistics are problematic. For example, Rowlen et al. demonstrated that the observation of blinking is not adequate evidence of SMSERS. Moreover, evaluating SMSERS blinking is often complicated by the appearance of new Raman bands corresponding to photodamage of the target molecule. Recent investigations by Pettinger et al. and Etchegoin et al. revealed that the observation of Poisson statistics corresponding to quantized intensities is impossible and more likely a consequence of insufficient sampling.

What, then, constitutes a reliable proof of single-molecule sensitivity? Initial insight was provided by Doering and Nie, who hypothesized that sudden exchanges between competing analytes (i.e., R6G and citrate present from nanoparticle synthesis) were occurring. Subsequently, the bi-analyte technique was developed as a simple and reliable method to establish single-molecule behavior. In this statistical approach two competing adsorbates (e.g., R6G and nile blue) are used to establish the detection of one analyte molecule. Pieczonka and Aroca demonstrated that combining the bi-analyte approach with a Langmuir–Blodgett technique provides two-dimensional SMSERS mapping capability as well as precise control of analyte concentration and spatial distribution. However, the downside of the bi-analyte approach is that the interpretation of SMSER spectra from two analytes is complicated by their differing Raman and absorbance cross-sections as well as surface chemistries. Recently, Dieringer et al. demonstrated that isotopically labeling the analytes (i.e., undeuterated and deuterated R6G) circumvents these issues, since the isotopologues exhibit identical optical and surface properties with the exception of their unique vibrational signatures. Fig. 1 displays the SMSER spectra of R6G-\(d_0\) and R6G-\(d_4\) as well as a histogram of the frequency that each isotopologue (or both) is observed. Analogous to a photon antibunching experiment in SMF, the histogram in Fig. 1b demonstrates that on average one or the other type of R6G is detected. The isotopologue and bi-analyte approaches have emerged as the most definitive proofs for single-molecule detection in SMSERS to date. Indeed, it is anticipated that the isotopologue approach in particular will have substantial impact on future SMSERS experiments.

c Structures and EFs of SMSERS-active sites

After the seminal SMSERS investigations, fundamental questions about the underlying mechanism were quickly raised. In particular, researchers sought to characterize the
measurements. These studies promoted the idea that gates as evidenced by polarization-modulation and AFM that the active sites are comprised of Ag nanoparticle aggregates have not been directly observed, although SMSERS-active structures comprised of spherical nanoparticles were characterized, since these studies were not performed on the structure of the SMSERS-active sites. Brus et al. demonstrated that the active sites are comprised of Ag nanoparticle aggregates as evidenced by polarization-modulation and AFM measurements. These studies promoted the idea that molecules were adsorbed to “hot spots”: regions of high EM enhancement located between adjacent particles. The notion of hot spots in nanoparticle aggregates was supported by transmission electron microscopy (TEM) and electrodynamic calculations. For example, correlated SMSERS, high-resolution TEM, computational modeling, and Rayleigh scattering measurements revealed the structure and EM enhancement of a real SMSERS-active junction. Fig. 2a presents the transmission electron micrograph of a SMSERS-active nanoparticle aggregate, with corresponding SMSER and calculated EM enhancement (Fig. 2c and d). The results from this investigation demonstrate that simple structures like anisotropic dimers could serve as a simple template for the rational design of SMSERS substrates. Interestingly, SMSERS-active structures comprised of spherical nanoparticle dimers have not been directly observed, although higher-order aggregates are commonly associated with SMSER spectra (Fig. 2b). Overall, a fundamental limitation of current SMSERS experiments is they lack well-defined, reproducible substrates.

Substantial attention has also been devoted to understanding the single-molecule EF that was originally estimated to be \( \sim 10^{15} \) based on comparison of a typical Raman scattering cross-section \(( \sim 10^{-30} \text{ cm}^2)\) to the absorbance cross-section of a typical chromophore \(( \sim 10^{-13} \text{ cm}^2)\). Although the notion of extremely high SMSERS EFs of \( \sim 10^{14} \) has pervaded the literature, it is now clear that these enhancements are largely overestimated. The absolute Raman cross-section of rhodamine 6G (R6G) on molecular resonance \((\lambda_{ex} = 532 \text{ nm})\) was recently determined to be \( \sim 10^{-23} \text{ cm}^2\) using femtosecond stimulated Raman spectroscopy. As a consequence, the resonance Raman contribution accounts for as much as \(10^7\) of the previously predicted \(10^{15}\) enhancement in the R6G/Ag system. Etchegoin and Le Ru also addressed this issue in detail, describing that the SMSERS EF “bridges the gap” between the normal Raman cross-section of a molecule and the minimum cross-section that is observable. Ultimately, these studies confirm that EM EFs of \( \sim 10^8\) and not \(10^{14-15}\), meet the minimum requirement to observe SMSERS from R6G (on the molecular resonance). That EFs of \( \sim 10^8\) are expected to support single-molecule detection with SERS provides us with additional enthusiasm about the future of the field, which is undoubtedly headed to nonresonant experiments.

d Excitation-wavelength dependence of SMSERS

Understanding the requirements for SMSERS is critical for the development of a broadly applicable single-molecule technique that employs a variety of analytes and experimental conditions. Therefore, much effort has been devoted to characterizing the relative contributions of the EM, chemical, and resonance Raman enhancement mechanisms that are responsible for single-molecule detection. As discussed previously, we now understand that the single-molecule EFs for R6G are \( \sim 10^8\) for excitation on molecular resonance. However, when excitation occurs away from molecular resonance, the contributions of resonance Raman and surface effects to SMSERS activity are less understood. Previous SMSERS studies on the R6G/Ag system have employed various fixed-frequency excitation wavelengths. For example, Vosgröne and Meixner measured SER spectra of R6G at five excitation wavelengths \((i.e., \lambda_{ex} = 632.8, 532.0, 514.4, 488.0, \text{ and } 457.9 \text{ nm})\) on samples with various dye concentrations, demonstrating that SER spectra obtained at 532 nm provided the maximum sensitivity \((\sim 10^{-13} \text{ M})\). Le Ru et al. showed that single-molecule SERS was observed with a SER cross-section of \( \sim 10^{-17} \text{ cm}^2\), corresponding to an EF \( \sim 10^9\) at \( \lambda_{ex} = 633 \text{ nm}\). These investigations suggest that the maximum SMSERS intensity occurs at the molecular resonance of R6G and that single molecules are observable far from resonance \((e.g., \text{ at } 633 \text{ nm})\). Yet, the nature of the excitation-wavelength dependence of SMSERS is still not well characterized, since these studies were not performed on the same nanoparticle aggregate nor the same molecule, and were limited to fixed frequency laser sources.

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**Fig. 1** Representative single-molecule SER spectra for R6G isotope logues (R6G-\(d_0\), and R6G-\(d_4\)), illustrating the frequency-domain proof for single-molecule detection \((\lambda_{ex} = 532 \text{ nm}, P_{ex} = 2.4 \text{ W cm}^{-2}, \tau_{ac} = 10 \text{ s}, \text{ grazing incidence})\). (b) Histogram of the events when only R6G-\(d_0\), only R6G-\(d_4\), or both vibrational modes were observed with \( \sim 1 \) molecule per nanoparticle under dry N\(_2\) environment. Adapted with permission from ref. 36.
Recently, the excitation-wavelength dependence of SMSERS was measured about the molecular resonance of R6G. Fig. 3 presents the surface-enhanced Raman excitation profiles (REPs) of a single R6G molecule on a Ag nanoparticle aggregate measured using a tunable optical parametric oscillator excitation source. The single-molecule surface-enhanced REPs for three vibrational modes of R6G were fit to single Lorentzian functions with FWHM of ~400 cm$^{-1}$. The single-molecule linewidths were narrower than the corresponding ensemble-averaged data as expected due to a reduction to inhomogeneous broadening (Fig. 3d). Overall, the structure of the single-molecule surface-enhanced REPs is dominated by the resonance Raman enhancement while the magnitude is dominated by EM enhancement, an observation that is supported by DDA calculations (Fig. 3e). These experiments provide insight for the excitation-wavelength dependence of SMSERS for R6G about the molecular resonance; but are these observations a general feature for all resonant molecules? Perhaps more importantly, what is the upshot of these measurements to nonresonant or preresonant SMSERS? Although there are reports in the literature of nonresonant SMSERS (e.g., Kneipp et al. reported the observation of SMSERS from crystal violet using near-IR excitation >200 nm from resonance) we are cautiously optimistic, as this has not yet been verified using the isotopologue or bi-analyte approaches. Ultimately, future SMSERS experiments (both resonant and nonresonant) should implement a robust proof for single-molecule detection.

IV. The future of SMSERS

In the previous sections we outlined three significant issues for SMSERS to overcome: (1) problematic proof of single-molecule detection; (2) understanding the SMSERS mechanism; and (3) lack of a reproducible and well-characterized SMSERS substrate. Fortunately, several of these issues have been addressed in recent years, most notably proof of single-molecule detection using the isotopologue approach and a refined understanding of the enhancements required to observe single-molecule signal. However, as we discuss next, the development of SMSERS as a robust, generalized single-molecule technique still requires that several outstanding issues be overcome.

How general is SMSERS?

Though several of the persisting questions about SMSERS have been addressed in recent years, there are considerable hurdles to overcome in order to realize its broad application. Perhaps the most profound question: how general is SMSERS? In order to outperform, or at least compete with existing SMF experiments, SMSERS must demonstrate widespread application. At present the overwhelming majority of SMSERS studies utilize the electronic resonance of analytes to facilitate single-molecule detection. In fact, just a handful of nonresonant SMSERS experiments have been reported (e.g., crystal violet at 830 nm excitation$^3$ and DNA base molecules$^{45-47}$) and these studies utilize the observations of Poisson statistics or blinking to argue for single-molecule behavior. Therefore, in our opinion, the generality of SMSERS from resonant to nonresonant conditions must be demonstrated using more reliable proof of single-molecule detection. If SMSERS of nonresonant molecules is indeed routinely observed, then single-molecule spectroscopy will no longer be limited to luminescent or fluorescently-labeled
molecules, an advance that holds particular promise for biological studies (e.g., DNA, proteins). The demonstration of molecular generality for SMSERS from resonant to non-resonant analytes using the isotopologue approach will provide a substantial contribution to the community. It should be noted, however, that if nonresonant SMSERS does not prove to be ubiquitous (or at least relatively common), the technique is still useful for a wide variety of problems involving resonant species. Another issue pertaining to generality is the SMSERS substrate, which is typically a structurally diverse set of Ag colloidal nanoparticles that aggregate to form hot spots of greatly varied structures (Fig. 2b). At present there is no reliable method for fabricating well-defined SMSERS-active substrates. However, we are emboldened by recent experiments that suggest alternatives to Ag-nanoparticle substrates are real possibilities (e.g., Langmuir–Blodgett deposition on Ag island films). Furthermore, EFs of $\sim 10^8$ are sufficient for SMSERS of resonant molecules like R6G, suggesting that single-molecule measurements on conventional SERS substrates like AgFONs and AgPPAs is a real possibility and such experiments are underway in our laboratory.

b What information can SMSERS provide?

Currently, fluorescence (less commonly absorbance) and SERS are the only optical methods for measuring individual molecules. It is well documented that SMF studies have applications from biology (e.g., FRET experiments of protein folding) to materials science (e.g., chromophore photophysics in dyed-composite materials). Yet, emission spectra are typically broad and featureless, providing little structural information about the dye and its local environment. On the other hand, SMSERS measures the unique vibrational fingerprints of analytes, where Raman intensities and frequencies are sensitive to changes in the equilibrium geometry of the molecule. Thus, in principle, SMSERS can provide information related to molecular structure, orientation, local environment, and reaction dynamics. The majority of SMSERS studies have focused on demonstrating single-molecule existence, exploring single-molecule EFs, and the structure of SMSERS-active sites. But an important question remains largely unaddressed: what information does a SMSERS experiment provide (other than the SER spectrum of one molecule)?

As previously discussed, SMSERS was recently used to interrogate the Raman excitation profiles (REPs) of a single molecule. In principle, one could measure a large set of single-molecule surface-enhanced REPs and the contributing nanostructure properties (e.g., LSPR scattering, morphology, chemical composition) in order to determine the effects of local environment on energy, linewidth, and population distribution. Moreover, a particularly useful demonstration of the utility of SMSERS is to measure single-molecule

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**Fig. 3** Single-molecule surface-enhanced Raman excitation profiles (REPs) of different Raman modes for an individual R6G molecule on colloidal silver. (a) $\nu = 610 \text{ cm}^{-1}$, (b) $\nu = 1504 \text{ cm}^{-1}$, (c) $\nu = 1647 \text{ cm}^{-1}$ ($P_{\text{ex}} = 0.050 \text{ W cm}^{-2}$, $t_{\text{ac}} = 10 \text{ s}$). Error bars represent one standard deviation from the mean obtained from multiple measurements at each excitation wavelength. The data were fit to single Lorentzian functions (solid lines) with linewidths $\sim 400 \text{ cm}^{-1}$; (d) Comparison of the total single-molecule surface-enhanced REP of R6G to the ensemble-averaged surface absorbance spectrum on Ag. The single-molecule REP (points) was fit to a Lorentzian function (solid line) where the error represents one standard deviation from the mean ($E_{\text{max}} = 18.563 \text{ cm}^{-1}$ and FWHM = $449 \text{ cm}^{-1}$). The molecular absorbance of R6G on 200 nm Ag film was fit by a sum of two Gaussians. As expected, the single-molecule data is significantly narrower than the ensemble-averaged analog ($\sim 1400 \text{ cm}^{-1}$). (e) EM enhancement ($E_o$ and $E_{\text{loc}}$ are the incident and local electromagnetic fields) calculated as a function of excitation energy from a model T-shaped nanoparticle aggregate inspired by previous experiments. Adapted with permission from ref. 44.
properties of a system that cannot be accessed using fluorescence spectroscopy. For example, SMER spectroelectrochemical experiments on nonfluorescent molecules (e.g., heme proteins) could probe the distributions of single-molecule electron transfer rate constants and standard reduction potentials.

Perhaps a more pressing issue, however, is to characterize the effect of the plasmonic substrate on SMSERS measurements. Whereas SMF relies on intrinsic fluorescence from the molecule to report on chromophore properties, dynamics, and local environment, SMSERS requires a plasmonic surface. The effect of the metallic substrate on the molecule and its environment has yet to be determined. Therefore, a natural first step is to perform single-molecule measurements on fluorophores in the presence and absence of a SERS substrate. Is the same information acquired from SMF and SMSERS experiments in a particular matrix (e.g., chromophores in a polymer matrix)? From an optimistic perspective, perhaps the sensitivity of SMSERS to intramolecular structure that is a consequence of binding and local environment will provide additional information relative to SMF measurements. However, obtaining single-molecule properties like molecular orientation using SMSERS will likely be nontrivial. Alternatively, it is now clear that tip-enhanced Raman spectroscopy (TERS) is an excellent tool to probe small numbers of molecules without the need for an internal plasmonic substrate. Recent work suggests that TERS can be extended to the few-molecule or single-molecule level, and we expect that single-molecule TERS measurements coupled to the isotopeologue approach will be an extremely promising avenue for future research.

As is often the case with emerging technologies, the recent SMSERS studies presented herein have raised new questions. Fortunately, the reliable isotopeologue proof for single-molecule detection in SERS enables us to begin to pursue these challenges. Here we have focused on future challenges of addressing the generality and utility of SMSERS experiments. In the following sections, we transition our discussion from fundamental single-molecule SERS studies to applied investigations at the ensemble-averaged level. In particular, we describe recent SERS studies that employ a minimally destructive approach to identify colorants in single fibers from historical artworks.

V. SERS for art conservation

a Motivation

Identifying organic colorants is exceedingly important for the conservation and long-term preservation of artworks as well as enhancing our understanding of their historical context. To this end several analytical techniques have been utilized, most notably high-performance liquid chromatography (HPLC), UV-Vis absorbance spectroscopy, 

and Raman spectroscopy. Although HPLC measurements have identified the largest number of natural organic colorants in artworks to date, the technique imposes a large sample requirement (i.e., ~5 mm of fiber) that is problematic for art conservation. Absorbance spectroscopy is commonly used to identify colorants that are difficult to analyze with HPLC such as the anthraquinone-based red lake pigments. However, UV-Vis spectra are typically broad and featureless such that pigment identification may be difficult. Colorants comprised of inorganic salts (e.g., Azurite—2CuCO3–Cu(OH)2) can be identified using Raman spectroscopy, but strong fluorescence precludes the measurement of Raman scattering from many organic dyestuffs, especially those from biological sources. Alternatively, SERS fulfills many of the requirements of an ideal analytical technique to detect and identify colorants in artworks. The SERS substrate not only provides enhanced Raman signals, such that exceedingly small sample sizes (i.e., ~ng) are measurable, but also quenches the fluorescence generated by many organic dyes. Several groups have demonstrated the applicability of SERS to the detection and identification of colorants with application to art conservation, the results of which are the subject of a recent review. However, initial SERS experiments on historical art objects utilized harsh chemical extractions to remove the colorant from the artist media, resulting in degradation of both the host material and dye. Although gentler extraction methods have since been developed, less effective color removal is reported. Ultimately, regardless of the particular extraction procedure the issue of limited sample size often prevents the analysis of actual historical artworks.

b Minimally-invasive SERS on historical art objects

To circumvent the problematic issues of dye extraction and sample size, recent work has used an on-the-specimen approach for obtaining SERS spectra from microscopic quantities of art sample, typically involving colloidal silver. In particular, Leona et al. reported a successful approach whereby hydrofluoric acid was used to pretreat (hydrolyze) dyed fibers in order to remove the colorant from the mordanted fiber. Next, Ag nanoparticles were applied to the treated fiber and the predominant dye (berberine) in a severely degraded 11th c. Byzantine textile was elucidated. Non-extractive non-hydrolysis SERS was first demonstrated by Chen et al. who measured SERS spectra of organic colorants from a Ag nanoparticle-treated dyed fiber taken from a mock artwork. Moreover, Jurasekova et al. performed in situ on-the-fiber detection of weld dyes on dyed reference fibers using photoreduced Ag nanoparticles. Van Elslande et al. reported direct non-extractive non-hydrolysis SERS on pigment grains taken from a Grecian archaeological site, allowing for the identification of the colorants 6,6′-dibromomindo and purpurin in real historical objects. These results showcase the unique advantage of SERS: identifying organic colorants from single pigment grains (i.e., ~ng quantities) is impossible using other techniques. More recently, we applied in situ on-the-specimen SERS to samples taken from actual historical textiles, pastels, and watercolors. Ultimately, these investigations demonstrate that SERS is ideally suited to identify dyes in actual artworks in a minimally-invasive manner, providing information that is crucial for their long-term preservation. In the next section we discuss a case study (an ancient Peruvian textile fiber) to illustrate minimally-invasive, on-the-specimen SERS.
c  Case study: red weft fiber from a pre-Columbian Peruvian textile

Some of the oldest and most technologically advanced examples of textile art are those originating from Peru.81 Evidence suggests that ancient Peruvian textile artists utilized ~200 different colors and implemented nearly every modern weaving technique in order to produce beautifully intricate and labor-intensive textile patterns. The wearers of these exquisite garments were highly esteemed in pre-Columbian civilizations such that textiles were more valued than precious metals. In these early Peruvian cultures, textiles were lavishly used in burial rituals; a single mummy would be wrapped in enough yarn to clothe hundreds of people. Typically the finest and most decorative textiles were restricted to the core of the mummy wrap, which, along with the arid desert environment of Peru, has contributed to preservation of these textiles throughout the centuries. Given the importance of such textiles to the history and culture of the region from which they were obtained, conservators are interested in their long-term preservation. In order to preserve ancient textiles, art conservators need to determine the colorants that are present in the textile, and assess the extent to which these colorants are subject to degradation after long-term exposure to light.

To highlight the application of SERS to the direct analysis of dyed historical textiles, we performed non-extractive SERS on a single fiber from an actual artwork. A small red weft fiber (0.5 mm × 2 μm) was removed from the textile pictured in Fig. 4. The band fragment dates to between 800 and 1350 A.D., and possibly originates from the Lambayeque region of Northern Peru. A repeating pattern of a richly clad human figure wearing an elaborate headdress, possibly a warrior, priest, or god, and flanked by two seabirds is depicted. The figures are surrounded by a chequer pattern, an exclusively geometric form that became prevalent during the Inca period (~1438 to 1533 A.D.) in central Peru.81 A challenge for any spectroscopic technique used to study art objects is to work with the minute amount of sample that can typically be obtained. The inherent sensitivity of SERS makes it a viable candidate for analyzing such a small sample, and recent work in our laboratory on such samples indicate that even in the presence of a complex matrix (e.g., binders, fillers, animal glue) the dyestuff components can be detected and identified using SERS, with very minimal sample preparation.78–80

As described elsewhere, Ag colloids were prepared via the standard Lee and Meisel procedure and concentrated 10× by centrifugation (36 000 relative centrifugal force, 15 min per cycle).78,82 A small volume (2 μL) of the resulting colloidal paste was applied to the fiber sample after it was visualized with a microscope. For SERS measurements, a Jobin-Yvon BXFM open microscope frame (Olympus), a holographic notch filter, and an 1800 grooves mm⁻¹ dispersive grating was used. Excitation from a HeNe laser (632.8 nm at 5 μW) was focused onto the sample and the Raman scattered light was back collected through an objective (100×, numerical aperture = 0.9). Once the silver paste dried onto the sample fiber, it was imaged under the microscope, and SER spectra were collected. Fig. 5a shows the photomicrograph of the fiber sample coated with colloidal paste, imaged using a 10× objective on the Raman microscope. The corresponding SER spectra of the historical textile fiber and a reference wool fiber dyed with alizarin, a major dyestuff component of madder (Rubia tinctoria L.), are shown in Fig. 5b. Major peaks corresponding to alizarin are observed at 1425 cm⁻¹ (ν(CC)/δ(COH)), 1321 cm⁻¹ (ν(C=C)), 1295 cm⁻¹ (ν(ν(C=O)), ν(C=C)), 1158 cm⁻¹ (ν(ν(C=O)), δ(CH)), 682 cm⁻¹ (ν(ν(C=O)), δ(C=O)), and 633 cm⁻¹ (ν(ring)).83,84 Remarkably, even though the historical textile fiber has undergone significant degradation during the many centuries since its creation, the dye used to produce red color in this fiber is a good spectral match to the reference SER spectrum of alizarin.

The Peruvian flora supplied ancient textile dyers with a large variety of colors. In addition, these early dyers implemented mordanting procedures on a handful of dependable resources in order to enhance their color palette. Red dyes, in particular, were obtained from several abundant and native sources including cochinille (Dactylopius coccus L.) and the roots of two plants: chapi-chapi (Relbunium microphyllum L.), a close relative of Old World madder (Rubia tinctoria L.), and Galium corymbosum L.85,86 Of these species, the only one which contains large amounts of alizarin is Galium, indicating that this plant was used to create the red color in the dyed band fragment.71 This finding is significant, as Peruvian textiles dyed with Galium species are exceedingly rare. Therefore, this work contributes to our understanding of the chronology and geographical distribution of this important colorant.

Fig. 4 Photograph of band fragment (possibly Lambeyeque) from the northern or central coast of Peru, A.D. 800–1350, 5.4 × 45.7 cm. AIC 1955.1613, obtained through the Kate S. Buckingham endowment.

Fig. 5 (a) Photomicrograph of the fiber obtained from the Peruvian band fragment (Fig. 4) coated with Ag nanoparticles. (b) SER spectra of the historical fiber (top) and a reference fiber dyed with alizarin (bottom) obtained with λex = 632.8 nm, Pex = 5 μW, tac = 1 s. Peaks labeled with an asterisk indicate bands due to citrate, present on the Ag colloids from chemical synthesis.
VI. Future outlook: SERS in the museum setting

An ideal analytical tool for studying art objects would be highly sensitive, selective, non-invasive, wide-spectrum (i.e., capable of identifying a wide range of organic and inorganic compounds), and easily adapted to a museum setting. This perspective has highlighted the fact that SERS possesses several of these qualities: selectivity and sensitivity. SERS measurements are now possible on a single grain or a few-microns length of fiber and provide unique chemical fingerprints of the dyes. In addition, recent work has demonstrated that harsh chemical extractions or sample pre-treatment are not required, thus preserving the integrity of the substrate. Simple citrate-reduced Ag colloids, easily synthesized from aqueous solution, function as an excellent and versatile SERS substrate for this application. These observations suggest that SERS studies on minute art samples can be readily accomplished at conservation departments with access to a Raman microscope.

However, extending SERS to encompass the ultimate quality of non-invasiveness remains elusive. SERS measurements require the presence of a noble metal surface, but applying Ag colloids directly to a work of art is out of the question. The nanoparticles would be nearly impossible to remove from the art object, and in any case, the cleaning procedures used to extract them would be invasive. For example, washing textiles is considered an invasive, irreversible treatment as it can lead to bleeding and staining of the dyes. An alternative to SERS for art conservation studies may come from recent advances in the field of tip-enhanced Raman spectroscopy (TERS), where the enhancing unit is an external tip. Another promising avenue for future SERS investigations on art objects is the development of SERS-active fiber optic probes. Although SERS experiments using these probes have not yet been demonstrated on actual artworks, we envision this technique to have real potential. However, given the short decay length of SERS (<5 nm), it is unclear whether the application of SERS-active fiber optic probes to art objects is truly non-invasive.

VII. Conclusion

It has taken more than thirty years for SERS to span the gulf between laboratory phenomenon and real technology, an achievement enabled by developing the basic science of SERS (e.g., SMSERS studies) and benefiting from applications of interdisciplinary nature. SERS has emerged as an excellent tool for the detection and identification of minute amounts of colorants used in precious artworks. While this knowledge is important for the conservation and long-term preservation of art objects, questions remain regarding other materials used and previous preservation treatments that may have been applied to the object. For example, the use of various binders and mordants can also provide clues as to the age and authenticity of historical artworks. In addition, art objects that have been treated with various pesticides are sometimes repatriated back to their originating society. Therefore, an important task is to characterize the residual pesticide concentrations and potential toxicity for humans, as these objects may be used during traditional ceremonies. The bottom line is that SERS of art objects has the potential to extend beyond dyes, toward the identification of other poorly characterized components in artist media. Likewise, we discussed that the future of SMSERS relies upon experiments that probe the generality and utility of the technique (i.e., nonresonant molecules). From our perspective, although SERS studies of dyes from single molecules to art objects have seen substantial progress, the future may lie beyond dyes.

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