

A Strategy to Prevent Signal Losses, Analyte Decomposition, and Fluctuating Carbon Contamination Bands in Surface-Enhanced Raman Spectroscopy

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Signal losses and fluctuating carbon contamination bands are “bottle-necks” in the application of surface-enhanced Raman spectroscopy (SERS) for reliable chemical analysis. They originate mainly from prolonged laser irradiation of the sample during data collection, which causes analyte decomposition and/or loss of the enhancing capabilities of the adsorption site. In this work, a laser illumination/signal collection technique, the “multiple points collection” (MPC) method is introduced to circumvent these problems. The MPC method is based on the use of a pair of galvanic mirrors to scan the laser beam rapidly and steadily across the sample surface. Each position is irradiated for $<10 \mu\text{s}$, at a rate of ~ 0.5 Hz. The SER spectrum is obtained by summing the signals collected from a large array of non-overlapping sample points. The MPC is compared with the conventional “single point collection” method, in which the laser beam is statically focused onto a particular spot and the scattered signals acquired. The MPC has the following advantages: (1) illumination and collection efficiencies are not compromised, (2) signal losses originating from analyte decomposition and/or alteration of the enhancing capabilities of the adsorption site are avoided, (3) high-quality SER spectra for analytes such as biomolecules and dipicolinic acid (a common marker for bacteria spores) can be easily obtained, and (4) the occurrence of broad amorphous carbon bands and the commonly observed temporal fluctuations in SERS are prevented. The success of the MPC is attributed to the reduction of local sample heating, as the time interval between the laser irradiations of a spot is much longer than the actual irradiation time itself.

Index Headings: Chemical analysis; Surface-enhanced Raman spectroscopy; SERS; Laser scanning confocal microscope; Amorphous carbon.

INTRODUCTION

Surface-enhanced Raman spectroscopy (SERS) has gained prominence as a powerful tool for the chemical analysis of molecules adsorbed on noble metal nanostructures.^{1–6} As a result of its sensitivity heading towards reproducible single molecule detection, great efforts have been made to develop SERS platforms for chemical and biological sensing. These advances are crucial for rapid monitoring of harmful biological agents and explosives and will also lead to progress in proteomics and drug discovery.

A common way of performing chemical analysis using SERS is to deposit the analyte on a roughened Ag substrate and

illuminate the sample with a visible wavelength laser. When the wavelengths of the laser and metal surface plasmons are in resonance, strong local electromagnetic fields are created that will amplify the Raman signatures of the molecules. The scattered signal can then be analyzed spectroscopically. The most popular setup is the Raman microscope because of its superior detection efficiency.^{4,5,7–9} High numerical aperture (N.A.) objectives are generally used not only to improve spatial resolution, but more importantly to optimize the SERS enhancements (through the angle of the incident beam) and light collection.^{10–13} This reduces the signal collection time, which is crucial for the optimum performance of fast sensors.

The usefulness of SERS for chemical analysis lies in its ability to give a high-quality vibrational spectrum of the analyte. However, this aim is often frustrated by SERS signal losses during the experiment. The reasons underlying the losses are still points of contention in the SERS community. A commonly invoked factor is the decomposition of the analyte triggered either by laser heating during the experiment, by catalytic activity of the substrate, or from contaminants adsorbed on the metal surface during substrate preparation.^{7,9,10,14,15} As a result, amorphous carbon, manifested as two broad bands at $\sim 1360 \text{ cm}^{-1}$ (D-band) and $\sim 1580 \text{ cm}^{-1}$ (G-band), or as rapidly fluctuating peaks, is frequently observed (spectral fluctuations). The latter is more problematic as the peaks occur randomly in duration, Raman shift, and intensity. They cannot be easily identified and subtracted away from the signal of the analyte.¹⁶ Morphological and chemical changes of the enhancing sites due to laser irradiation may also deteriorate their SERS activity and cause signal losses.^{9,17} These interferences create problems for routine chemical analysis by SERS.

To circumvent these problems, low N.A. objectives, low incident laser powers, or non-resonance Raman spectroscopy can be used.^{7,10} These plausible solutions reduce the incident photon flux or mitigate the effects of laser-induced damage on the sample. However, their application will generally result in a longer spectra collection time. Carbon can be removed by chemical displacement using self-assembled thiol monolayers, rinsing samples in solvents, and/or O_2 and Ar plasma cleaning, etc.^{18,19} However, these cleaning methods are neither suitable for all types of metal surfaces nor do they assure cleanliness of the metal substrates for an extended period of time.

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Contamination can still appear during the analysis. The lack of suitable remedies for SERS carbon contamination has led to its classification as an “omnipresent” problem.¹⁰

In this work, a strategy to circumvent the problems highlighted above is presented, namely to prevent SERS signal losses and to suppress the fluctuating carbon contamination signals. A micro-Raman system based on a laser scanning confocal microscope (LSCM) is used for collecting the spectra. The heart of the instrument consists of a pair of galvanic mirrors that scan the laser beam rapidly and steadily across the sample plane.^{20,21} Vapor-deposited Ag thin films are used as SERS substrates due to their popularity and ease of preparation, but this methodology is applicable to any type of SERS substrate. To acquire a SER spectrum using a LSCM, we first obtain an image of the Ag surface (with detection of the backscattered light by a photomultiplier tube (PMT)). The laser is then focused onto a particular spot, from which a SER spectrum is collected. This method is equivalent to focusing light onto the sample using a commercial Raman microscope, widely used by the SERS community.^{2,4,5,7-9} It will be termed here the single point collection (SPC) method.

The method presented here for the acquisition of a SER spectrum involves confocal imaging of the surface during the active collection time, but transmitting the backscattered light into the Raman spectrometer rather than into the PMT of the LSCM. As opposed to the SPC mode, the laser beam is scanned rapidly over a larger area, with the signal being continuously collected to give a single spectrum. This method will be termed as the multiple points collection (MPC) method. This approach leads to a reduction of local sample heating because the time intervals between the laser irradiations of a spot are much longer than its actual irradiation time. It will be demonstrated that the illumination/collection efficiency of the MPC and SPC methods is quantitatively identical. SERS signal losses, analyte decomposition, and fluctuating carbon contamination signals are shown to be easily prevented using the MPC method. High-quality SER spectra of several molecules, including, for the first time, the antibody immunoglobulin M, are presented here.

EXPERIMENTAL

Chemicals and Sample Preparation. For the SERS experiments, glass slides (Paul Marienfeld GmbH & Co. KG, Germany) were cleaned in piranha solution (concentrated H₂SO₄:H₂O₂, 3:1) for 10 minutes. After rinsing with methanol and blowing them dry using N₂ gas, these were mounted into a vapor-coating chamber (MED 020, Bal-Tec, Liechtenstein). Then, 6 nm Ag (Bal-Tec, 99.99%) was deposited on to the slides at a rate of 0.05 nm/s. The pressure of the system is $<1 \times 10^{-5}$ mbar during the coating process.

The analytes used were brilliant cresyl blue dye (BCB, Fluka), pinacyanol chloride (PC, Aldrich), dipicolinic acid (DPA, 99%, Aldrich), cytochrome c (Cc, from bovine heart, $\geq 95\%$, Fluka), and immunoglobulin M (IgM, from human serum, $\sim 95\%$, Sigma). The two dyes were dissolved in methanol, while DPA and Cc were dissolved in H₂O. IgM was purified of its buffer salts using desalting columns (Micro Bio-Spin with Bio-Gel P-30, Bio-Rad, Hercules, CA) and re-solvated in H₂O. The analyte was spin coated onto the Ag coated glass slides immediately after removing the slides from the coating chamber.

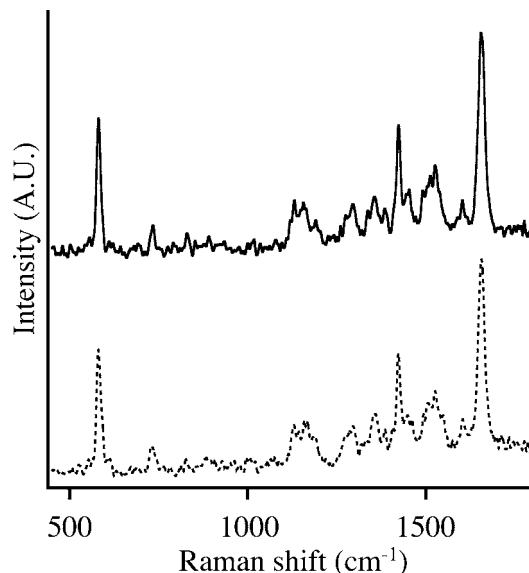


Fig. 1. Raman spectra of BCB on a glass slide collected using MPC (solid trace) and SPC (dotted trace). The incident laser power is 7 μ W and the spectrum collection time is 10 s.

Instrumentation. Our home-built setup has been described elsewhere.²² It consists of an inverted LSCM (IX 70, Olympus, Japan) and a Raman spectrograph/charge-coupled device (CCD) detector (Kaiser HoloSpec, Ann Arbor, MI). A 1.4 N.A. oil-immersion objective was used to focus light from a 532 nm diode-pumped solid-state laser (Ventus, Laser Quantum, UK) onto the samples. The laser spot size is ~ 400 nm. Back-scattered light was collected through the same objective. By means of a flipping mirror, the photons can be fed either into the Raman spectrograph through fiber optics or to the PMT (through a pinhole) to give an image of the surface. The aperture of the fiber and the pinhole maintain confocality whether light is channeled to the spectrograph or to the PMT. All the experiments were done under ambient conditions.

For the MPC method, the LSCM is made to scan an area of $\sim 210 \times 210 \mu\text{m}^2$ with a resolution of 512×512 pixels. Hence, each pixel corresponds to $\sim 410 \times 410 \text{ nm}^2$, roughly equivalent to the size of the laser focus. A scan time of ~ 2 s was used for each frame, i.e., each pixel is scanned at ~ 0.5 Hz, with an exposure of $<10 \mu\text{s}$. No additional modification of the instrument is required. Alternatively for the SPC method, the laser is focused onto randomly chosen points. The MPC and SPC methods were always performed consecutively on the same sample.

No background subtraction or smoothing of the spectra was carried out. The data points were processed using Igor Pro Version 4.09A Carbon (WaveMetrics, Inc., Portland, OR).

RESULTS

Validation of the Multiple Points Collection Method. For the MPC method to be useful, the illumination/collection efficiency of this method should be the same as that for the conventional SPC method. This is an important consideration that can be inadvertently neglected in the quest for scanning methods that better maintain sample integrity. For example, spinning a sample during an experiment may prevent it from heating up and decomposing, but mechanical instability from

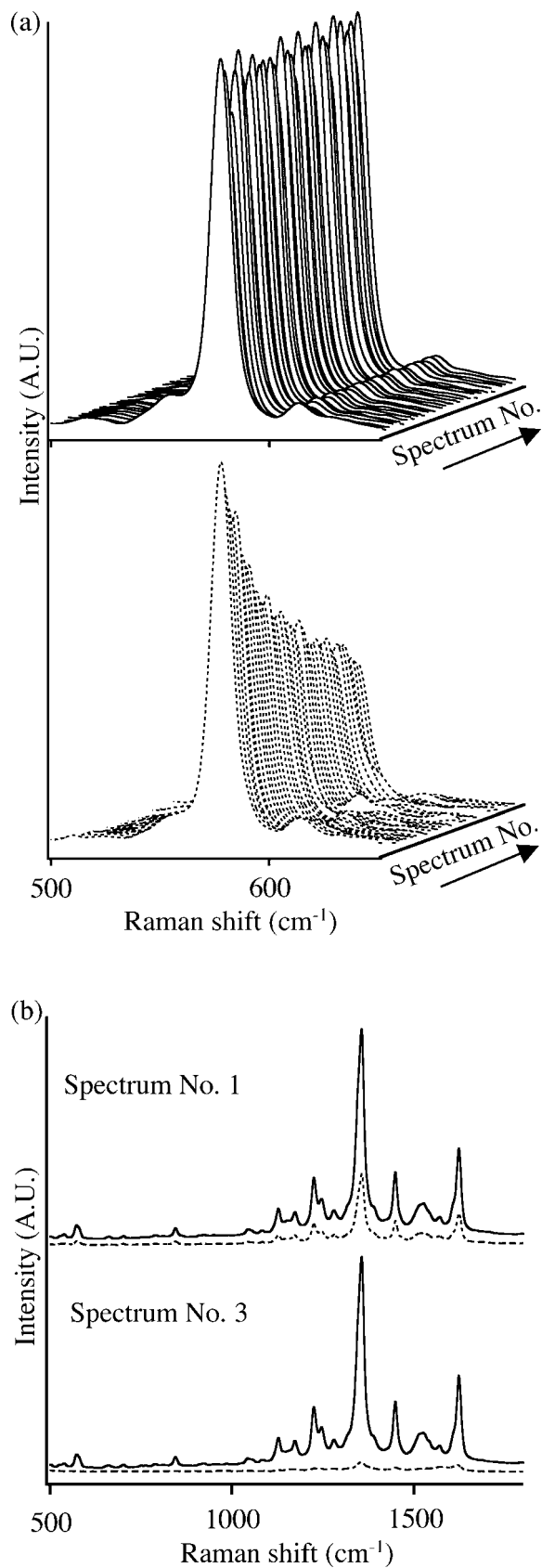


FIG. 2. (a) Time-sequence plots of SER spectra of BCB on a Ag surface collected using MPC (solid traces) and SPC (dotted traces). Forty consecutively acquired spectra are shown. (b) SER spectra of PC on a Ag surface collected using MPC (solid traces) and SPC (dotted traces). The upper traces are the first set of spectra collected when the laser irradiation is first initiated, while the lower ones (the third set) are obtained ~ 6 s later.

its movement can also lead to laser defocusing and hence signal losses. Figure 1 shows the Raman spectra of a homogeneous BCB thin film spin coated on a glass slide collected by both the MPC and SPC methods. BCB was chosen as the sample as it is completely stable at low laser irradiation; $7 \mu\text{W}$ of incident laser was utilized. Normal Raman spectroscopy instead of SERS was performed in order to eliminate unknown experimental parameters such as surface distribution of enhancing hot spots, which may invalidate the quantitative aspects of this test. It could be observed that the band intensities and signal-to-noise ratios (S/N: defined as the ratio of intensity of the desired signal over the background noise) are the same for both spectra, regardless of whether the MPC or SPC is used.

Preventing Surface-Enhanced Raman Spectroscopy Signal Losses: Essential for Quantitative Analysis. Quantitative analysis using SERS surfaces is generally difficult because the prolonged focusing of the laser beam on a single spot leads to photo-induced and thermal decomposition of the molecules.⁷ Physical/chemical changes of the adsorption sites may also cause them to lose their enhancing capabilities.⁹ In addition, randomly situated SERS hot spots will give locally very highly enhanced Raman signals that do not reflect the actual physical concentration of the analyte.

An example of the MPC and SPC methods applied to BCB deposited on Ag films is presented in Fig. 2a. BCB was again chosen as the analyte because we found that its decomposition products do not give strong amorphous carbon bands that will interfere with the quantitative aspects of this analysis.²³ This is in contrast with analytes such as malachite green isothiocyanate.¹⁵ One of BCB's vibrational peaks at 578 cm^{-1} was used to monitor the intensity changes. The sample was illuminated with a high laser power of $150 \mu\text{W}$ and SER spectra were continuously acquired with a collection time of 1 ms (with 2–3 s intervals). The BCB signals collected with the MPC method can be observed to be stable, while those using the SPC method have dropped to $<1/2$ of their original value in less than 2 minutes.

A more dramatic example is shown in Fig. 2b. Here, SERS of PC, a photosensitive dye, is investigated with the same laser power and collection time as the example presented above for BCB. In the very first set of SER spectra obtained, the signals collected by the SPC method have less than half of the intensity compared to those collected by the MPC method. This can be attributed to any of the three factors mentioned above. More importantly, by the third set of spectra acquired (in <8 s of laser irradiation), the signal intensities in the SPC spectrum are almost at the background level, while those acquired with the MPC method have not changed. Rapid signal losses of PC caused by analyte decomposition and/or loss of SERS activity of the substrate when the SPC method is used is clearly exhibited here.

These two experiments demonstrate that the MPC method better ensures the integrity of the analyte and the substrate, hence facilitating quantitative analysis using SERS. The MPC method of collecting spectra also gives ensemble information over the entire scanned surface. Unlike the SPC method, it avoids strong dependence on randomly located hot spots present on the metal surface or inhomogeneous analyte distribution. This ensures a more accurate measurement of the quantity of analyte present.

Preventing Analyte Decomposition: Essential for Qualitative Analysis. The SER spectra of DPA (a marker for

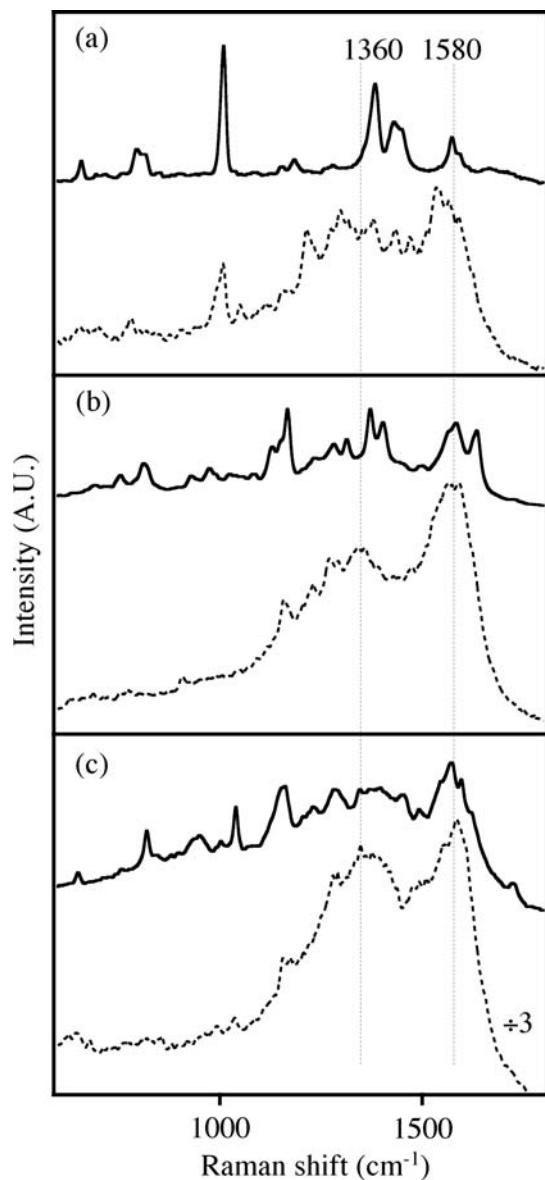


FIG. 3. SER spectra of (a) DPA (incident laser power 150 μW , collection time 1 s), (b) Cc (incident laser power 150 μW , average of three spectra, collection time 10 s each), and (c) IgM (incident laser power 65 μW , average of six spectra, collection time 10 s each). The solid and dotted traces denote the spectra collected using the MPC and SPC methods, respectively. Note that for DPA, its degradation occurs even within a very short laser irradiation time.

bacteria spores, including those of *Bacillus anthracis*) and two biologically important molecules, Cc and IgM, are presented in Fig. 3. Cc exhibits an optical resonance at 532 nm, while DPA and IgM do not. Using the MPC method, high-quality SER spectra were obtained for all three analytes. The spectra of DPA and Cc are in excellent agreement with previous studies,^{24,25} while to the best of our knowledge, this is the first reported SER spectrum of IgM. In contrast, when the SPC method is applied, analyte signal losses, most probably originating from decomposition of the analytes, can be observed. This is corroborated by the presence of vibrational features attributable to amorphous carbon bands (e.g., broad peaks at $\sim 1360\text{ cm}^{-1}$ and $\sim 1580\text{ cm}^{-1}$).

However, the carbon signals may not originate solely from

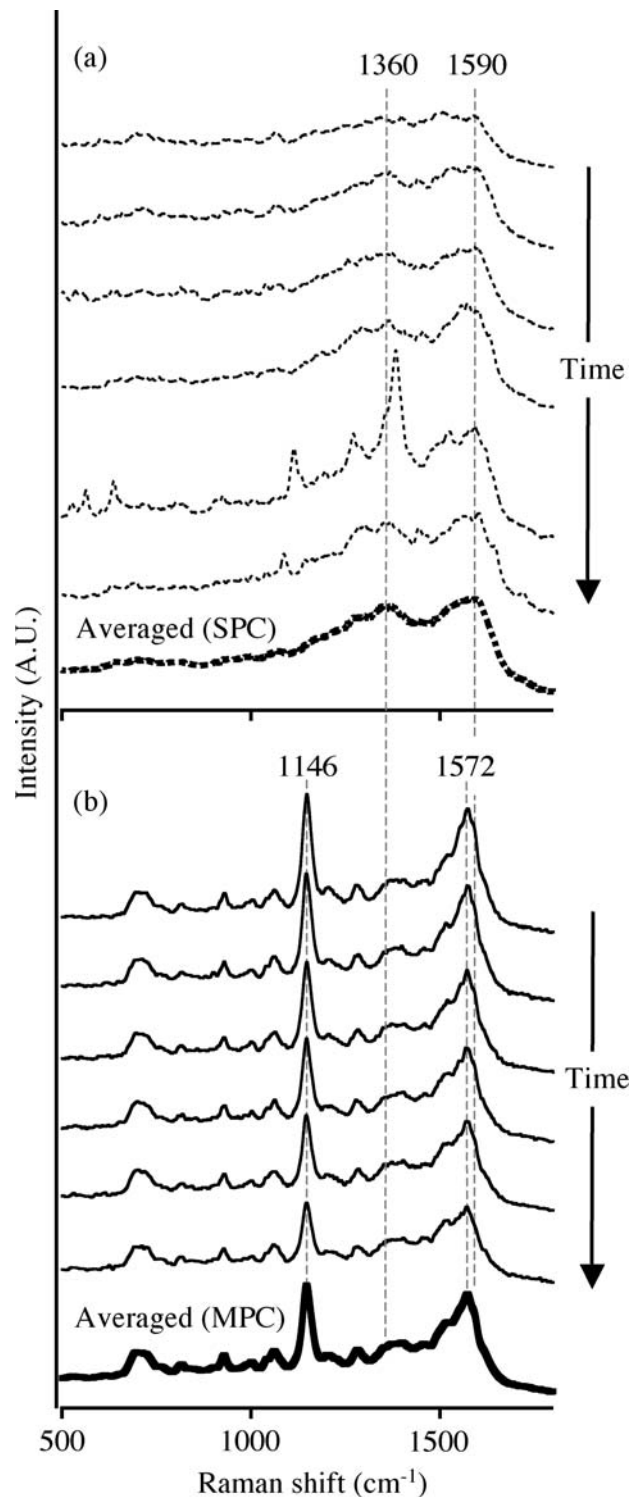


FIG. 4. SER spectral changes of a Ag surface as a function of irradiation time. Spectra are collected using (a) SPC (dotted traces) and (b) MPC (solid traces). Every 5th spectrum acquired is presented. The averaged SER spectra (bold traces) calculated from 28 recorded spectra using either the SPC or MPC method are presented at the bottom of each set of spectra. The incident laser power is 150 μW and the spectrum collection time is 1 s.

the decomposed analyte. They could also be from the decomposition of residual contaminants present on the Ag substrate even before analyte deposition.⁹ Amorphous carbon has a Raman cross-section four orders of magnitude larger than

that of benzene and is also known to have broad molecular resonance from the UV to the near-infrared.^{1,26} Thus, signals from amorphous carbon will overwhelm those from the analyte even if the latter had not decomposed. This renders any chemical analysis difficult; a solution is presented in the next section.

Preventing the Occurrence of Amorphous Carbon Bands and Spectral Fluctuations. The most difficult issue facing SERS analysis is the ubiquitous carbon contamination.¹⁰ Figure 4a shows a series of spectra collected with the laser focused (SPC) onto a Ag film without any analyte. The spectra are collected with 1 s accumulation time and show temporally fluctuating signals. When they are averaged, two broad bands centered at 1360 cm^{-1} and 1590 cm^{-1} are obtained. These are assigned to amorphous carbon. This observation is not unexpected because carbon contamination cannot be completely avoided in the vapor-deposition process.⁹ In contrast, when the MPC method is applied on the same Ag film, SER spectra with well-defined peaks with fairly constant intensities are found (Fig. 4b). Most importantly, they do not exhibit random and temporal shifts in Raman frequencies. The vibrational features of the averaged spectrum do not resemble the broad amorphous carbon bands shown in Fig. 4a. The two intense peaks at 1146 and 1572 cm^{-1} can be respectively assigned to the C–C and C=C stretching modes of carbon polyenes containing six conjugated double bonds, while the weaker ones are ascribed to graphitic sheets.^{27,28}

An insight into the physical origin of the commonly observed amorphous carbon SER bands can be obtained from this experiment. The two characteristic broad bands at 1360 and 1590 cm^{-1} were observed only in the averaged spectrum for the SPC method. This reveals that prolonged focusing of the laser beam at a particular spot (SPC) plays a role in generating these bands, probably through decomposition of the distinct carbonaceous molecular precursors such as polyenes (polyenes have been spectroscopically recorded as intermediates during the laser-induced conversion of polydiacetylene to amorphous carbon on Ag surfaces).²⁷ This is corroborated by the absence of these bands when the MPC method was used. If amorphous carbon had been present prior to sample irradiation, then it should be more easily observed with the MPC method because a larger area is sampled.

Spectral fluctuations ceased in the present work when the MPC method was utilized. For contaminants that are firmly adsorbed on the surface, e.g., through strong Ag–S bonds, the implication here is that their vibrational signatures can be first identified and subsequently subtracted from the analyte spectrum. This was hitherto not possible because of the frequent presence of rapidly fluctuating signals when the SPC was used. The MPC will be particularly helpful in the study of analytes at low concentrations or compounds that have very weak Raman scattering cross-sections.

DISCUSSION

Multiple Points Collection for Chemical Analysis Based on Surface-Enhanced Raman Spectroscopy. Surface-enhanced Raman spectroscopy signal losses, analyte decomposition, and amorphous carbon bands are great obstacles in SERS-based chemical analysis. However, rather little effort has been made to overcome these problems. At present, the common practice for collecting a good SER spectrum in a reasonable time is to tediously balance the excitation laser

power and collection time or to use low N.A. objective/lenses. These measures usually sacrifice excitation/collection efficiency.^{7,10,15}

The effectiveness of the MPC method is attributed to the reduction of local sample heating, as the time interval between the laser irradiations of one pixel is much longer than the actual irradiation time itself. This scanning configuration distributes sequentially the total photon dose over space, which facilitates heat dissipation. The amount of power a sample can tolerate also increases when light is distributed on it through many non-overlapping spots (MPC), rather than through one big spot (both with a fixed total area).²⁹ As a further illustration of the negative effects of prolonged laser heating, the quality of some of the SER spectra in this work deteriorated (e.g., signal intensity losses and/or band broadening were observed) when the μs irradiation time of each pixel was lengthened by just one order of magnitude.

In the light of the present results, the effect of prolonged vs. intermittent laser irradiation of a sample was investigated. The fluorescence and Raman intensities of Rhodamine 6G and PC thin films were respectively measured. A sample spot was irradiated either continuously (A s) or intermittently (A/B s \times B number of times), while maintaining the total photon dose. If a sample is degraded purely through photo effects, no intensity differences would be expected regardless of the way the photons are temporally distributed. This is because each absorption–emission cycle has the same probability of causing photobleaching. From our preliminary studies, increasing the intermittent irradiation of a sample enhances its stability by 30%. This confirms the destructive effects of continuous laser irradiation, i.e., heating, on the integrity of a sample. Investigations to elucidate the thermal and photo contributions towards the degradation of a molecule are now being pursued in our laboratory.

An alternate setup for the MPC method could be realized with a rapid, stable translation of the sample (on a scan stage) with respect to a stationary laser beam.³⁰ However, stages that meet the requirements for speed and stability presented in this work are not commercially available. Mechanical stability is crucial for the reproducible focusing of the laser beam onto the sample surface, especially when tight focusing of the laser spot and confocality of the microscope are used. In general, movements between the sample and focusing objective should be avoided, as even small perturbations may cause laser beam defocusing and hence loss of signals. There are other scanning configurations designed for normal Raman spectroscopy that could be useful in solving the problems of SERS signal losses and spectral fluctuations, e.g., line and global illumination scan modes.^{31–33} These alternatives can be home-built or commercially purchased. However, the MPC method enjoys advantages over some of these apparatuses, such as a faster scan speed, better signal collection efficiency, no loss of confocality (which occurs for some instruments using line illumination scans), etc.

Full Raman imaging of samples could in principle be done in the MPC mode by the use of a CCD detector that can collect and read out at μs speed. A summation of signals collected from a particular pixel (after a certain number of scans over the entire area) could then be obtained to give a better S/N spectrum. To the best of our knowledge, such CCD detectors are not yet commercially available. It is, however, emphasized here that chemical identification by SERS for biological and

chemical applications usually do not require high spatial resolution detection. This is because the analyte is *randomly* spread over the SERS substrate during sample preparation or when flowing through the sensing device.^{4,6,34}

A final remark on highly robust SERS sensors: metallic nanostructures are often used to build such sensors and prolonged laser illumination can lead to alteration of their optical properties. We predict that the MPC strategy, demonstrated through this work to be effective in preventing signal losses, will play an important role in the wider usage of SERS sensors in research, in industry, and in the consumer market.

CONCLUSION

In this work, common problems in SERS such as signal losses and fluctuating carbon contamination bands have been overcome using the multiple points collection method. Illumination and detection efficiencies are not compromised by the MPC method. High-quality and clean SER spectra of DPA, Cc, and IgM have been obtained. The MPC method also prevented the occurrence of fluctuating carbon bands. This allows the identification of pre-adsorbed contamination, whose signals can be subtracted from the SER spectrum after adding the analyte. The MPC method is more than an effective collection method: the physical origin of amorphous carbon SER bands has been better understood by using it. The success of the MPC method is attributed to the reduction of local sample heating, as the time interval between the laser irradiations of one pixel (~ 2 s) is much longer than the actual irradiation time (< 10 μ s) itself. Higher laser power can also be more easily used with the MPC method, which will lead to a shorter spectrum collection time. The use of galvanic mirrors to scan the laser beam assures stable focusing of the laser spot on the sample.

Finally, from the series of experiments performed for this work, the signal intensity of the analytes collected by the MPC was observed either to be the same (when low incident laser power or a very robust sample is used) or often higher compared to that using the conventional SPC method. Carbon contamination from analyte decomposition was hardly observed when the MPC method was employed. This demonstrates that signal losses, sample decomposition, and contamination, which are bottlenecks hindering the development of SERS, can be more easily overcome than commonly held. Since the MPC method can be easily implemented, we hope that it will soon add to the arsenal of experimental techniques that will render SERS a highly reliable and quantitative chemical analysis method.

1. R. Aroca, *Surface-Enhanced Vibrational Spectroscopy* (John Wiley and Sons, Ltd, West Sussex, 2006), 1st ed.
2. J. A. Dieringer, A. D. McFarland, N. C. Shah, D. A. Stuart, A. V. Whitney, C. R. Yonzon, M. A. Young, X. Y. Zhang, and R. P. Van Duyne, *Faraday Discuss.* **132**, 9 (2006).
3. T. H. Reilly, S. H. Chang, J. D. Corbman, G. C. Schatz, and K. L. Rowlen, *J. Phys. Chem. C* **111**, 1689 (2007).
4. G. Braun, S. J. Lee, M. Dante, T. Q. Nguyen, M. Moskovits, and N. Reich, *J. Am. Chem. Soc.* **129**, 6378 (2007).
5. D. R. Ward, N. K. Grady, C. S. Levin, N. J. Halas, Y. P. Wu, P. Nordlander, and D. Natelson, *Nano Lett.* **7**, 1396 (2007).
6. X. Y. Zhang, J. Zhao, A. V. Whitney, J. W. Elam, and R. P. Van Duyne, *J. Am. Chem. Soc.* **128**, 10304 (2006).
7. I. Khan, E. Polwart, D. W. McComb, and W. E. Smith, *Analyst* (Cambridge, U.K.) **129**, 950 (2004).
8. R. M. Stöckle, V. Deckert, C. Fokas, and R. Zenobi, *Appl. Spectrosc.* **54**, 1577 (2000).
9. M. L. Jacobson and K. L. Rowlen, *J. Phys. Chem. B* **110**, 19491 (2006).
10. N. P. W. Pieczonka and R. F. Aroca, *Chem. Phys. Chem.* **6**, 2473 (2005).
11. A. Wei, B. Kim, B. Sadtler, and S. L. Tripp, *Chem. Phys. Chem.* **2**, 743 (2001).
12. K. A. Bosnick, J. Jiang, and L. E. Brus, *J. Phys. Chem. B* **106**, 8096 (2002).
13. I. Baltog, M. Baibarac, and S. Lefrant, *Phys. Rev. B* **72**, 2452402 (2005).
14. A. Kudelski and B. Pettinger, *Chem. Phys. Lett.* **321**, 356 (2000).
15. K. F. Domke, D. Zhang, and B. Pettinger, *J. Phys. Chem. C* **111**, 8611 (2007).
16. Y. C. Liu and R. L. McCreery, *J. Am. Chem. Soc.* **117**, 11254 (1995).
17. W. Zhang, T. Schmid, B.-S. Yeo, and R. Zenobi, *J. Phys. Chem. C* **112**, 2104 (2008).
18. K. L. Norrod and K. L. Rowlen, *Anal. Chem.* **70**, 4218 (1998).
19. Y. Saito, J. J. Wang, D. N. Batchelder, and D. A. Smith, *Langmuir* **19**, 6857 (2003).
20. R. A. Farrer, M. J. R. Previte, C. E. Olson, L. A. Peyser, J. T. Fourkas, and P. T. C. So, *Opt. Lett.* **24**, 1832 (1999).
21. S. W. Paddock, *Mol. Biotechnol.* **16**, 127 (2000).
22. C. Vannier, B. S. Yeo, J. E. Melanson, and R. Zenobi, *Rev. Sci. Instrum.* **77**, 023104 (2006).
23. W. H. Zhang, B. S. Yeo, T. Schmid, and R. Zenobi, *J. Phys. Chem. C* **111**, 1733 (2007).
24. P. Hildebrandt and M. Stockburger, *J. Phys. Chem.* **90**, 6017 (1986).
25. S. E. J. Bell, J. N. Mackle, and N. M. S. Sirimuthu, *Analyst* (Cambridge, U.K.) **130**, 545 (2005).
26. J. C. Tsang, J. E. Demuth, P. N. Sanda, and J. R. Kirtley, *Chem. Phys. Lett.* **76**, 54 (1980).
27. K. Itoh, I. Kudryashov, J. Yamagata, T. Nishizawa, M. Fujii, and N. Osaka, *J. Phys. Chem. B* **109**, 271 (2005).
28. H. E. Schaffer, R. R. Chance, R. J. Silbey, K. Knoll, and R. R. Schrock, *J. Chem. Phys.* **94**, 4161 (1991).
29. D. M. Zhang, J. D. Hanna, Y. N. Jiang, and D. Ben-Amotz, *Appl. Spectrosc.* **55**, 61 (2001).
30. M. A. De Jesus, K. S. Giesfeldt, and M. J. Sepaniak, *Appl. Spectrosc.* **57**, 428 (2003).
31. N. Zimmerer and W. Kiefer, *Appl. Spectrosc.* **28**, 279 (1974).
32. S. Mamedov, A. Kisliuk, S. Loheider, and D. Quitmann, *Appl. Spectrosc.* **49**, 1199 (1995).
33. J. J. Andrew, "Raman Microscopy and Imaging", in *Encyclopedia of Analytical Chemistry*, R. A. Meyers, Ed. (John Wiley and Sons, Ltd., Chichester, 2000), vol. 15, p. 13078.
34. D. J. Anderson and M. Moskovits, *J. Phys. Chem. B* **110**, 13722 (2006).