Imaging with Raman photons: a novel use of mixed-mode spectroscopy

K N Prajapati, Anoop A Nair, Jervis Fernandes, S Ravi P Silva and J Mitra

1 School of Physics, Indian Institute of Science Education and Research, Thiruvananthapuram 695551, India
2 School of Biology, Indian Institute of Science Education and Research, Thiruvananthapuram 695551, India
3 Advanced Technology Institute, University of Surrey, Guildford GU2 7XH, United Kingdom

* Authors to whom any correspondence should be addressed.
E-mail: krishnagkp12114@iisertvm.ac.in and j.mitra@iisertvm.ac.in

Keywords: Raman spectroscopy, plasmonics, imaging, surface engineering, pattern recognition

Abstract
Surface enhanced Raman spectroscopy is today an established technique used for chemical fingerprinting. Here, we showcase an engineered hierarchical substrate, in which the plasmonically active regions, restricted to a micron scale, two dimensional hexagonal pattern are examined. Spatial variation of the enhanced Raman signal from any analyte, uniformly coating the substrate, consequently bears a high registry with the underlying pattern. This spatially contrasted enhancement allows optical imaging of the 2D pattern solely using the Raman scattered photons from the analyte. While the pattern brightness and contrast determine analyte identification and detection sensitivity, hyperspectral imaging can be exploited for increasing specificity. Proof of concept demonstration of the technique is carried out via the acquisition of Raman images with rhodamine and fluorescein dyes and then applied to detect glucose in 40 mM concentration. The large area optical imaging and the requirement of long-range uniformity in the detected patterns for positive analyte detection, is implemented using a machine learning based pattern recognition protocol which also increases the statistical confidence of detection. This simultaneous, large area signal detection sacrifices continuous spectral information at the cost of speed, reproducibility and minimising human error via automation of detection in the hyperspectral imaging technique presented here.

1. Introduction

The field of chemical sensing and analyte detection by Raman spectroscopy has been significantly advanced with the advent of plasmonics, and the discovery of surface enhanced Raman spectroscopy (SERS) [1]. Today, SERS is a powerful technique with increasing infiltration in fields such as food safety, medical diagnostics, and especially in areas requiring trace analyte detection like forensics and security. In SERS, the Raman signal enhancement originates from collective charge oscillations, i.e. plasmons, induced in metal nanostructures by electromagnetic excitation. It results in amplification and localisation of the incident electric field into subwavelength volumes within the close proximity of the nanostructures, yielding stronger Raman scattered signals from molecules lying therein. These effects have been extensively investigated, employing geometric hot-spot and antennae effects leading to unprecedented sensitivity and single molecule detection [2, 3]. Plasmonics is a platform that is enriched by allowing the extreme interplay of electrons, photons and phonons across a large bandwidth, ensuring applications well beyond spectroscopy, from enhancing reactions to process kinetics that were hitherto deemed impractical [6, 7]. The scientific and industrial importance of the field is evidenced by the journal publications over the last decade with ever increasing research spin-offs dealing with SERS related products. In parallel, improvements in optical detection, including spectrometers, has led to increased adoption of the platform beyond research to wider sectors like agriculture, environmental monitoring, security, forensics,
biomedical diagnostics etc. Yet, beyond the research laboratories, the technique remains unaffordable due to the high latent cost of the experimental setup and the specialised expertise involved in the subsequent analysis of the Raman spectra that lead to chemical identification.

In spite of the significant advances made in the technical aspects of SERS, there remains a veritable gap in quantitative understanding of the origin of SERS enhancement, especially the dichotomy between chemical and electromagnetic modes of signal enhancement [8–10]. Previous investigations have demonstrated that random metal nanostructure clusters often offer higher SERS enhancement compared to engineered, lithographed nanostructures, i.e. the exact shape and size of the nanostructures are of lesser importance than the statistical abundance of ‘hot-spots’ created therein. In fabricating SERS substrates, the above observation tilts the balance away from engineered nanostructures that are ordered in the nanoscale towards disordered nanostructure clusters, which are easier to make and scalable with significantly lower fabrication costs. Further, the present investigation aims to translate the SERS technique such that it becomes more pervasive, reduces cost and involves a chemical identification protocol that is amenable to modern data analysis techniques like machine learning. Fundamentally, in SERS, the ‘substrate’ remains the most critical component which benefits from intelligent scientific and technical design. Here we showcase a SERS substrate that is adorned with a generic μscale hexagonal metalized pattern, onto which ZnO nanorods (diameter ~200 nm) are electrodeposited and subsequently decorated with Au nanoparticles (NPs) of diameter ~30 nm, creating a hierarchical SERS substrate that is disordered in the nanoscale but ordered in the micron scale. Expectedly, the Raman signal of analytes (Rhodamine B and Fluorescein) coated onto this substrate shows large signal amplification due to the plasmonic Au NPs, enabling detection of analytes in nanomolar concentrations, as has been reported earlier [11–13]. But more importantly, the signal enhancement is restricted to the hexagonally patterned region, i.e. at the ZnO nanorods (ZNRs) decorated with Au NPs, even though the analyte is uniformly coated across the entire substrate. The enhanced Raman signal and its spatial registry with the 2D μscale pattern makes it possible to ‘image’ the surface with Raman scattered photons through an optical microscope, thereby translating Raman spectroscopy to microscopy. The efficacy of image acquisition and analysis can be extended by using suitable filters at selected Raman band(s) of the suspect analyte. Operationally, successful imaging and identification of the characteristic pattern (above a threshold intensity) at multiple known wavenumber bands will trigger the positive identification of an analyte, which is demonstrated here. The quantum efficiency (QE) of Raman scattering, which is an inelastic scattering process, is notoriously low (< 10^-6). Thus, recording a Raman image would require extreme signal enhancement, well above the background light from all possible sources. Unlike Raman spectral analysis (peak identification), the imaging route precludes any background subtraction. We show that the hierarchical nature of the substrate and the material properties of the Si, ZnO and Au combination is such that the background does not dominate the overall emission within the imaging spectral bandwidth, which remains responsive only to the intensity of the Raman scattered photons of the analyte. Further spatial variability of SERS intensity across a substrate, due to analyte or substrate inhomogeneity, becomes irrelevant in this widefield imaging technique that records information from a large area at once.

We present the motivation and methodology of the development of the 2D patterned hierarchical SERS (2D-SERS) substrate and imaging cum analysis protocol for chemical identification of analytes as an alternative to the standard spectral analysis. The methodology developed here is not tailored for trace chemical detection or the ultrasensitive single molecule detection, but for detection of analytes available in μM concentrations, in volumes that provide uniform coverage of the substrates developed here. The developed methodology is standardised using Raman images on known analytes and then applied to detect glucose in biologically relevant concentrations. Feasibility of hyperspectral imaging is demonstrated in spectral windows of 10–15 nm that may be potentially exploited to engineer specificity of detection in future. Finally, we outline an optimised machine learning based large area pattern detection protocol that leverages the inherent advantages of the Raman imaging methodology to detect the presence of known analytes with >90% confidence. The 2D-SERS substrates presented here are further amenable for surface functionalization to selectively bind molecules in a fluid or flow cell for the chemical identification of specific species.

2. Experimental methods

2.1. Materials
All chemicals including zinc nitrate hexahydrate, hexamethylene-tetramine (HMTA), Au nanoparticles (NPs) (#741973), Rhodamine B (RhB, C28H31ClN2O3) (#83689), Fluorescein (FSN, C29H12O3) (#46955) and D-(-)-Glucose (#G8270) of purity > 99.5% were supplied by Sigma-Aldrich and used as supplied. Deionized water has been used throughout for all experiments.
2.2. Fabrication of 2D-SERS substrates

The 2D-SERS substrates were fabricated on Si substrates that were photolithographically patterned with a micron-scale hexagonal pattern and metallized with 50 nm Au film (optical image of a pattern after metallization shown in inset of figure 1(a)). See supplementary material section A1 for further information. The metallized pattern acted as the electrode for electrodeposition of ZNRs. The ZNRs were synthesized in an electrochemical bath of 5 mM equimolar aqueous solution of zinc nitrate hexahydrate and HMTA at 85 °C, using platinum wire as the counter and reference electrode. The patterned substrate was sequentially biased at 2.25 V and 2.73 V for 1 min and 30 min, respectively [14]. After ZNR growth, the substrate was rinsed in deionized water and dried at 65 °C for 1 h. The SEM images in figures 1(b) and (c) show selective growth of randomly oriented ZNRs at the hexagonal patterned electrodes. Finally, to prepare the plasmonically active substrates, 30 μl of Au NPs suspension was drop-casted on top of ZNRs grown substrate to realise the hierarchical SERS substrates AuNP/ZNR/Si. In substrates were developed on 10 mm × 10 mm sized Si pieces. For conducting the Raman studies, the various concentrations of the dyes, RhB and FSN were drop casted on the various test and control substrates. Both RhB and FSN were chosen for characterising the substrates developed since (i) both dyes do not absorb any light at the primary Raman excitation wavelength 633 nm employed here and consequently does not have any luminescence in their respective Stokes shifted Raman spectral bandwidth and (ii) they have multiple Raman active modes within the Raman shift wavenumbers 600–1600 cm⁻¹ which corresponds to the wavelength range 650–715 nm for 633 nm excitation. Absorption and emission Spectrum of RhB and FSN with their corresponding chemical structure are shown in figure S5.

2.3. Characterization

Morphological imaging of the substrates and their material components was performed using Nova Nano SEM 450 field-emission scanning electron microscope (SEM) and 300 kV FEI TECHNAI G2-TF-30 transmission electron microscope (TEM). X-ray diffraction (XRD) analysis was carried out using a powder x-ray diffractometer (Empyrean, PANalytical) with reference radiation of Cu Kα = 1.540 Å at an operating voltage of 45 kV. Photoluminescence (PL) spectra were recorded using a spectrophotometer (Fluorolog 3, Horiba Jobin-Yvon) at room temperature. The various absorption spectra were obtained using Perkin Elmer Lambda 900 spectrophotometer. All the Raman spectra and Raman maps (figure 4(a)) were acquired using Horiba Scientific XploRa Plus with a 100x objective, with 633 nm laser excitation, laser power ~1% of the total power (~18 mW), spot size ~1 μm, typical acquisition time ~1s per spectrum, grating ~1800 grooves mm⁻¹, unless otherwise specified. A typical map covers 30 μm × 30 μm area, with a laser spot size of ~1 μm and step size of ~1 μm.

2.4. Raman imaging and image analysis

The optical Raman images were recorded using a confocal microscope (Carl Zeiss LSM 880) with 405 nm (5 mW) and 633 nm (laser power ~5 mW) laser sources. The images are typically 85 μm × 85 μm in area with a resolution of 1024 × 1024 and were acquired with a wide pinhole of ~60 μm. Pattern recognition: the various algorithms for detecting and validating the hexagonal clusters were trained and executed on a quad-core i3–5005U CPU and by utilizing online resources like Google Colab [15].
Figure 2. (a) Development flowchart of the SERS substrate from bare Si (A₀) to AuNP/ZNR/Au-grid/Si (E₁). Their analyte (Rhodamine B) coated counterparts have subscript 1. (b) Background corrected Raman spectra of selected bare and analyte coated substrates. The spectra have been vertically shifted for clarity and all spectra (barring E₁) have been amplified by a multiplication factor for comparison.

3. Results and discussion

3.1. 2D-SERS substrate development

Figure 1(a) shows the scanning electron microscope (SEM) image of ZNRs, electrodeposited [14] on pre-patterned Au hexagonal grid on SiO₂/Si substrate (ZNR/Au grid/Si), with an optical image of bare Au grid/Si substrate in the inset. Figure 1(b) shows a magnified SEM image of a hexagonal ZNR/Au grid/Si pattern with the inset showing a close up of the ZNRs. The ZNRs with an average diameter ~ 200 nm and length of ~ 2 μm uniformly cover the Au grid. Figure 1(c) displays the high resolution transmission electron microscopy (HRTEM) image of a ZNR decorated with Au NPs where the lattice fringes of ZNR with spacing ~ 0.26 nm confirm the orientation and crystallinity of the ZNRs and the lattice fringes of an Au NP with a spacing ~0.23 nm confirming the Au(111) plane [16]. A low resolution TEM of a ZNR attached with Au NPs is shown in the inset of figure 1(c). The XRD spectrum of as grown ZNRs on Au grid/Si substrate is shown in figure 1(d), where peak indexing confirms the wurtzite structure of ZNRs. The Au (111) and Si peaks arise from the underlying Au layer and the Si substrate. Figure 1(e) shows a set of photoluminescence (PL) spectra for ZNR/ Au-grid/Si system for various excitation wavelengths (λ exc). For the excitation energies greater than the ZnO bandgap energy (E exc > 3.3 eV), the emission spectra show a sharp UV peak at 377 nm corresponding to the near band edge (NBE) emission and a broad visible emission centred at 645 nm attributable to oxygen vacancy related defect states [14, 17]. Importantly, the PL spectra for excitations with energies less than the bandgap, i.e. at λ exc = 405 nm and 633 nm, show negligible emission beyond 650 nm. Figure S1 (available online at stacks.iop.org/NANOX/3/035007/mmedia) in supplementary material shows the extinction spectra of Au NPs (dispersed in deionized water) displaying the characteristic maximum around 525 nm with (FWHM ~68 nm) due to plasmonic absorption with the inset showing the TEM image of the Au NPs of average diameter ~ 30 nm.

The schema in figure 2(a) shows the steps of development of the 2D-SERS substrate through its levels of nanostructure incorporation and hierarchy, with the subscripts 0 and 1 demarcating the bare and Rhodamine (RhB) coated samples. A₀: bare Si, A₁: RhB/Si, B₀: Au-grid/Si, B₁: RhB/Au-grid/Si, C₀: AuNP/Au-grid/Si, C₁: RhB/AuNP/Au-grid/Si, D₀: ZNR/Au-grid/Si, D₁: RhB/ZNR/Au-grid/Si and finally the 2D-SERS substrate configured as E₀: AuNP/ZNR/Au-grid/Si with its RhB coated counterpart E₁: RhB/AuNP/ZNR/Au-grid/Si. Figure 2(b) shows the background corrected Raman spectra from some of the above samples. Spectra with subscript 1 correspond to substrate coated with 200 μM aqueous solution of RhB, with all other experimental parameters remaining the same. The Si Raman peak at 514 cm⁻¹ dominates the acquired spectra in all cases...
barring that labelled E₁ from the final 2D-SERS substrate coated with RhB. In the latter RhB's plasmonically amplified Raman peaks dominate the overall spectrum. The identical nature of the A₀ and A₁ spectra reflects the low QE of the Raman scattering process such that no signature of RhB is detectable in A₁. The spectrum for B₁ (RhB/Au-grid/Si) shows the first signatures of the Raman peaks associated with RhB due to SERS enhancement from the roughened Au-grid layer. However, the signals are weak since the Au-grid covers less than 20% of the substrate area. The same patterned substrate, when decorated with Au NPs from the ligands attached to the Au NPs. The spectrum from sample E₁ (RhB on E₀) fully resolves the Raman signature of RhB, displaying all the known peaks of the analyte. The spectrum for E₁ was acquired at the hexagonal grid on the substrate. The spectra have been numerically scaled by the factors mentioned for comparison with that from E₁. Table (S1) in the supplementary material lists all the peaks and their origin, benchmarking them against known datasets.

Figure 3(a) shows a series of SERS spectra obtained from RhB of different concentrations varying from 200 μM to 0.2 μM on the 2D-SERS substrate. Evidently, the intensity of the Raman peaks decreases with the decreasing concentration of RhB with almost no detectable change in the Raman shift (peak wavenumber). Figure 3(b) shows the log-log plot of the variation of peak intensity with concentration at four peak wavenumbers 614 cm⁻¹, 1275 cm⁻¹, 1354 cm⁻¹, and 1505 cm⁻¹ along with the variation for total Raman intensity i.e. area under the background corrected Raman spectra in the concentration range 20 nM–200 μM. The plots exhibit linear dependency between the intensity (I) and analyte concentration (C) in the logarithmic scale, indicating that the quantities are related via an empirical relation of the form \( I = \alpha C^\rho \). Where \( \alpha \) and \( \rho \) are fit parameters that vary slightly between the peaks and the nature of the dependent variable. Table S4 in supplementary material lists the best-fit parameters, \( \alpha \) and \( \rho \), obtained from the linear fits shown in figure 3(b). Importantly, the values of the exponent \( \rho \) obtained across these 5 plots are comparable with that for the area under the Raman spectra variation expectedly being the least. These dependencies are useful as calibration parameters for quantifying unknown analyte concentration [11–13]. The above results demonstrate the quality of the hierarchical 2D-SERS substrate, which may be further quantified by calculating the SERS enhancement factor (EF) for the substrate according to the following standard equation.

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

Where \( I_{\text{SERS}} \) and \( I_{\text{Raman}} \) are the intensities of a SERS and the corresponding normal peak in a Raman spectrum and \( N_{\text{SERS}} \) and \( N_{\text{Raman}} \) are the number of analytes (i.e. RhB) molecules illuminated within the incident laser spot [13, 19]. Comparing the above numbers corresponding to the 0.2 μM RhB peak at 614 cm⁻¹ on substrate E₁ against that obtained for 200 mM RhB on bare Si, we obtain \( EF \sim 10^6 \), with comparable EF calculated for the 1645 cm⁻¹ peak. Spatial uniformity in the Raman enhancement and between the Raman spectra recorded across the substrate is confirmed by the series of spectra presented in figure S2 of supplementary material, which were recorded at 3 different positions, uniformly distributed across the 10 mm × 10 mm substrate.
3.2. Raman maps

Having established the basic performance of the 2D-SERS substrate, figure 4(a) shows a series of spatially resolved Raman intensity maps for 200 μM RbB at 12 wavenumbers between 1200 cm\(^{-1}\) and 1550 cm\(^{-1}\). The maps were generated from Raman spectra acquired over a 30 μm × 30 μm area with a laser spot size of ~1 μm and step size of 1 μm, i.e. over 900 points, which were post-processed to yield the maps. The 12 maps correspond to high, middle and lowest signal intensity across multiple peaks of the Raman spectrum, blue arrows shown in figure 4(b). It is important to note that though the spectrum in figure 4(b) is background corrected the Raman maps in figure 4(a) record the total intensity, i.e. without any background correction. Evidently, the contrast in the Raman maps shows a high degree of registry with the hexagonal pattern of the substrate that harbour the ZNRs decorated with Au NPs. The signal enhancement arises from the plasmonic Au NPs which were coated across the entire substrate, in spite of which the hierarchical nature of the substrate at the regions of ZNRs, i.e. on the hexagonal pattern, most effectively enhance and localise the Raman signal therein—which is the central tenant of this investigation and to be exploited in due course. The Raman maps at the three peak wavenumbers 1274 cm\(^{-1}\), 1354 cm\(^{-1}\), and 1505 cm\(^{-1}\) (high intensity) spatially resolve the hexagonal patterns with reasonably uniform intensity patterns. By contrast maps at the wavenumbers with low intensity, i.e. 1225 cm\(^{-1}\), 1311 cm\(^{-1}\), and 1397 cm\(^{-1}\), the contrast between the substrate and grid decrease significantly making the grid pattern are markedly less differentiable. Figure 4(a) also shows the Raman maps at six other wavenumbers corresponding to the middle intensities of the peak maxima at 1267 cm\(^{-1}\), 1284 cm\(^{-1}\), 1345 cm\(^{-1}\), 1361 cm\(^{-1}\), 1493 cm\(^{-1}\) and 1518 cm\(^{-1}\) where the contrast in the Raman maps are midway between those at the highest and lowest intensities in the spectrum, where the hexagonal grid are resolved. Figure S3 in supplementary material shows the Raman maps and spectrum from the same sample as in figure 4 but recorded after 70 days of storage in the dark. The spectral position of the peaks remains unchanged though their maximum intensities decrease by more than a factor of 5. The reduced contrast in the Raman maps makes the hexagonal grid pattern barely identifiable but are nevertheless there. Comparison of the data presented in figures 4 and S3 demonstrate the robustness and stability of the substrate developed here. Raman mapping for 0.2 μM RbB adsorbed on the 2D-SERS substrate with corresponding Raman spectrum are shown in figure S4. Though the spectrum adequately resolves the main Raman peaks, all the three maps at 1274 cm\(^{-1}\), 1505 cm\(^{-1}\) and 1645 cm\(^{-1}\) indicate that spatial contrast between the grid and the substrate is highly compromised, in which the hexagonal patterns are difficult to distinguish.

3.3. Raman imaging

The Raman spectrum of 200 μM RbB on the 2D-SERS substrate (figure 2) shows that the analyte’s major Raman peaks typically lies between 600 cm\(^{-1}\) to 1700 cm\(^{-1}\), corresponding to a 100 nm wavelength window \(\Delta \lambda_{\text{Raman}} \sim 650–750\) nm, corresponding to 633 nm excitation. Figures 5(a)–(c) shows an optical image recorded with Raman scattered photons in the \(\Delta \lambda_{\text{Raman}}\) spectral window, for three different laser powers. The Raman images gain clarity and contrast with increasing 633 nm laser power from 2%–60%, with figure 5(c) fully resolving the hexagonal grid, the regions from which the highest intensity of the scattered photons originate. To confirm that the brightness and contrast in these images dominantly arise from the Raman scattered photons from RhB, the Raman image from the bare 2D-SERS substrate, i.e. without RhB, at 60% laser power was recorded as shown in figure 5(d). This image quantifies the background illumination of all the Raman images, which originate from all inelastic scattering or emission sources inherent to the substrate, under 633 nm excitation. Of particular relevance is the low luminescence of ZNRs under 633 nm excitation (figure 1(e)). Figures S6 and S7 shows two further sets of Raman images on freshly prepared substrates demonstrating the overall reproducibility and robustness of the observations.
Figure 6. (a)–(d) Raman images of fluorescein on 2D-SERS substrate under 633 nm excitation, for three different laser powers. Images in (c) and (d) were recorded at different places on the same sample. Images recorded in the range 650–750 nm.

Figure S8 shows the Raman spectra from 200 µM fluorescein (FSN) adsorbed on the 2D-SERS substrate. Table S2 (supplementary material) lists the characteristic Raman wavenumbers of FSN from literature and present work. Figure 6 shows the Raman images recorded with the FSN dye on the 2D-SERS substrate, again evidences the registry of the recorded pattern with the underlying hexagonal patterning of the substrate. Figure 6(c) and (d) were recorded at different places on the same substrate demonstrating possible variability of response across the substrate. This also demonstrates that the Raman imaging technique discussed here is applicable to multiple analytes. To further quantify the change in the brightness of the images shown in figures 5, S6, S7 and 6, a frequency distribution of pixel brightness in these images are shown in figures S9 and S10, along with the variation in cumulative brightness with laser power. The brightness range in each image is identically restricted to 0–100. Evidently, engineering adequate brightness and contrast in the images is central to the Raman imaging technique and the choice of material and their functional form is central to the efficacy of the 2D-SERS substrate. Unlike Raman spectroscopy analysis, this imaging techniques does not allow for background correction. Thus, minimising photons scattered or emitted from the bare substrate, under illumination, within the spectral range of imaging is essential to ensure minimal interference with the enhanced Raman signal from the coated analyte. Thus, the use of Si as a substrate, with ZnO nanorods as the scaffold to localise and concentrate the Au NPs are specifically chosen after due experimentation [20]. Section A2 in supplementary material discusses the roles played by these various constituents, with their individual role displayed in the development of 2D-SERS substrate in figure 2(a) and the corresponding Raman spectra in figure 2(b).

3.4. Spectrally resolved Raman imaging
It is quite likely that within the wide spectral window of detection (∆λ_{Raman} ∼ 100 nm), multiple analytes have different spectral peaks that contribute towards the pattern brightness and its generation. Thus mere detection of the 2D hexagonal pattern with photons detection over the large wavelength range will not result in positive analyte identification with ‘high confidence’. To increase the confidence level of positive identification of any analyte with a known Raman signature, it will be advantageous to conduct hyperspectral imaging within multiple narrower ∆λ. Figures 7 (a)–(e) further shows a series of spectrally filtered 2D-SERS images that are recorded with different spectral filters, each showing the hexagonal pattern but with different intensity corresponding to the intensity of the Raman signal in the respective spectral range of the spectrum in figure 7(f). Though these images were acquired with user selectable monochromators, they demonstrate that for identifying a specific analyte, multiple narrow bandwidth optical filters (∆λ_{filter} ∼ 10 nm) at designated wavelengths may be employed for positive chemical identification with high confidence. Through a microscope, the detector (human eye or camera) then looks for the hexagonal pattern in the acquired images recorded with various filters. Positive pattern recognition with above threshold brightness and contrast across multiple filters characteristic of the analyte would result in positive analyte identification. Identification of the presence of multiple analytes in a sample is afforded by the small FWHM of Raman peaks, which avoids overlap of peaks to a large extent enabling detection of a variety of molecules with high confidence. This is the biggest casualty of sacrificing continuous
spectral information in the Raman imaging technique, which can be mitigated to some extent via hyperspectral imaging. Though the possibility of hyperspectral imaging is demonstrated with spectral windows of 10–15 nm, their efficacy lies in discriminating between multiple analytes in a single sample which has not been addressed here.

3.5. Comparing Raman spectroscopy with Raman imaging

Raman imaging based chemical identification has both advantages and disadvantages over the established method of spectral analysis. Firstly, the throughput of this technique is far higher than that of acquiring individual spectra at multiple positions across a sample surface for obtaining cumulative response or to generate a 2D map. For example, to generate a set of Raman maps as shown in figure 4(a), the typical time taken for data acquisition on a 30 μm × 30 μm area is 2–3 h with additional 2–3 h spent by a trained expert to process the data and generate the plots. By comparison, the time required to obtain the full set of spectrally resolved Raman images (85 μm × 85 μm area) as shown in figures 7(a)–(e) was typically 15 min followed by computerised pattern recognition and analysis. Adoption of the Raman imaging technique can thus speed up the analyte screening process. Secondly, one of the challenges of SERS based detection and quantification has been reproducibility of enhancement of the Raman signal and is also relevant to the present technique. In the proposed Raman imaging technique, the chemical fingerprint of the analyte across a large area of the 2D-SERS substrate is obtained at once, with the long-range uniformity parameter and averaging built into the detection protocol. That is unless the entire pattern over the whole optical field of view of the sample ‘uniformly’ generates Raman scattered photons and above a threshold intensity, registry of the acquired image with the micron-scale 2D pattern will not be obtained. Here, positive analyte detection will be determined by identification of a widefield pattern, an image with spatially variable image brightness and contrast, and not on the analysis of individual spectra. In other words, spatial averaging of the Raman signal across the entire image area is inbuilt into the detection protocol. The imaging technique does sacrifice spectral information, a functionality that can be partially restored and exploited as discussed above. Figure 3(b) also plots the variation in mean brightness of Raman images obtained with RhB for various concentration in the range 100–2000 μM. While the mean brightness again shows a systematic variation with concentration, its dependence on the latter via the exponent ρ is muted compared to that observed in the variation of spectral intensity, see figure 3(b). The best fit parameters for mean and cumulative brightness variation provided in table S4 shows that ρ = 0.26 for image brightness in contrast to ρ ~ 0.4 for spectral intensity. Section A3 is supplementary material provides further details on the reproducibility of the acquired Raman images along with sample wise variation in mean brightness at selected concentrations of the analyte (figure S11). Thus, though Raman imaging displays lower sensitivity of detection, a positive detection will be more robust than that obtained from Raman spectroscopy technique. Further, since positive identification of the analyte is possible by the positive correlation of multiple Raman images with a known pattern, the entire process can be readily automated utilising standard image processing and subsequent pattern recognition via 2D correlation between the images obtained and the expected pattern—paving the way to automation, further economising the entire process of chemical fingerprinting. An important drawback of Raman imaging is that prior knowledge of the Raman spectrum is necessary to define the wavelength windows for hyperspectral imaging with Raman scattered photons and the availability of suitable optical filters or monochromator. Thus, it can only be employed to detect known analytes and is of limited use in identifying unknown species. Further, the imaging protocol is conceptualised around processing and recording
large area images of a periodic pattern precluding detection of analytes in trace quantitates or in low concentrations.

Finally, to test the proposed detection technique on a diagnostically relevant analyte, we investigated the detection of dextrose (d-glucose) in deionised water. Non-invasive [21–23] and easy detection of glucose in humans has remained a challenge, with several investigations suggesting electrical detection of abnormal levels of glucose in the range 500 $\mu$M–50 mM will be highly beneficial for monitoring type II diabetes. The typical Raman signal of glucose is inherently compromised due to the low scattering cross section of the process. Though SERS based detection amplifies the signal, it is disadvantaged due to low adhesion between glucose and Au or Ag nanostructures. Figure S12(a) shows the Raman spectra acquired from various concentrations of glucose solution in deionised water. Up to 40 mM concentration, the spectra are dominated by the features of deionised water, and the characteristic signatures of glucose are manifest only for concentrations in excess of 1000 mM. The detected peaks match published data [27] confirming the presence of D-glucose. Figure S12(b) shows the Raman spectra from 4 mM and 40 mM glucose solutions drop-casted and dried onto the 2D-SERS substrate. Importantly, the spectral features are quite different between those obtained from glucose in solution and the solution dried onto the 2D-SERS substrate. Comparison between the spectra from 2D-SERS substrates between 4 mM and 40 mM glucose solutions show differences in spectral peak positions. Though the dominant peaks in each of the two spectra match or lie close to those reported [27–29], the relative strength of the peaks varies significantly. However, both spectra show that the majority of the dominant peaks lie in the range 1100–1800 cm$^{-1}$. Figures 8(a)–(c) shows hyperspectral images of the 2D-SERS substrate, coated with 40 mM glucose solution (after drying), recorded with 633 nm excitation. Their correspondence to the spectral range of detection of each image is indicated in the Raman spectrum shown in figure 8(d). Evidently, the image recorded in the 680–715 nm spectral window (figure 8(c)) shows the highest brightness, though the hexagonal pattern is present in all the images.

**3.6. Pattern recognition using machine learning**

Overall, the acquired Raman images demonstrate that hyperspectral imaging of analyte coated 2D-SERS substrate via Raman scattered photons poses a viable route to analyte detection. To explore possible routes towards automating the process of chemical identification via automated detection of the 2D grid pattern, we have explored two protocols that allow quantifiable pattern recognition. Figure 9(a) shows a filtered (contrast enhanced) 2D-SERS image recorded for 40 mM glucose solution, over the spectral range 650–715 nm. The hexagonal closed packed (hcp) unit cell is demarcated in red. The 2D fast Fourier transform (FFT) of the image, after suitable thresholding is shown in figure 9(b), indicating that the Fourier space image of the hexagonal grid is another hexagonal lattice with lattice constant given by $4\pi/\sqrt{3}a$ and rotated through 30° about the perpendicular to the 2D real space plane, where $a$ is the lattice constant of the real space lattice (side of the hcp unit cell).
unit cell in figure 9(a). Figure 9(c) then shows the inverse 2D FFT of figure 9(b), reproducing the hexagonal pattern of the original image that was acquired with Raman scattered photons. Thus, 2D FFT protocol allows adequate filtering and enhancement of the acquired image, which followed by pattern recognition, offers a straightforward and controllable option of automated detection of the 2D-SERS pattern imaged by Raman microscopy.

A second, more efficient and topical protocol in automated pattern recognition was explored utilizing a deep learning-based method, trained to identify the hexagonal pattern in the Raman images via a sequence of detection, clustering, and analysis algorithms. Across the various stages, the protocol was optimised by training on approximately 500 actual images, both with and without analytes constituting the positive and negative training sets and later tested on a new set of 150 positive and negative images for determining the accuracy of identification (as detailed in supplementary material). The Haar Cascade [30] trained on the images of interest was employed for initial detection of individual hexagons (figure 10(a)), followed by clustering and analysis using the K-means clustering [31] and Convolutional Neural Nets (CNN) [32] (figure S13 shows the workflow). Overall, the percentage of true positives (PTP) detected by the Haar cascade ranged from 55%–100% (figures 10(a), S16(a)–(c)), with the percentage of false positives (PFP) detected being less than 10% (figure S16(b)) in a majority of the images. The PFP exceeded 40% in few images (figure S16(c)), typically associated with regions of high contrast noise (figure S16(j)), substrate deformities and the presence of hexagonal fragments (figure S16(i)). The problem of high PFP in such images was overcome by utilizing an intermediate shallow neural network (Shallow–CNN) trained to reject fragments and noisy patches selectively, with an accuracy of 95% (figure S16(k)). Since the Shallow CNN was trained on an unbiased dataset, the accuracy was considered to be the sole indicator of performance. The incidence of false positives in negative images is further minimised by utilising the clustering procedure. It required the identification of cohesive groups of hexagons with a minimum cluster size of 5, with smaller clusters being rejected from further analysis, as shown in figure 10(b). Positive images that surpassed all the above checks were assigned a confidence value using a Deep Convolutional Neural Network (Deep–CNN) [see section S2.5.2 in supplementary material]. Figure 10(c) plots the evolution of accuracy with epochs demonstrating the stability of the detection protocol. Figure 10(c) inset shows the values of the confusion matrices corresponding to the validation and test set images, showing overall detection accuracies of 94.6% and 99.3%, respectively. The accuracy values were calculated using equation (S16) from the True Positive (TP), True Negative (TN), False Positive (FP), and False Negative (FN) values of the confusion matrices. The associated F1 scores of 0.9487 and 0.9934, close to 1, for the validation and test sets (Table S5) further validates the Deep–CNN’s optimal performance. It is worth noting that the algorithm is not specific to detecting hexagonal structures and is readily retrained on images with other periodic structures and symmetries, albeit of aspect ratio ~ 1. However, it is also well recognized that deep learning based pattern recognition protocols are
easy to be misguided by generic noise and random areal contrast, fool-proofing which remains unexplored in the above scheme. It is anticipated that integration of the Fourier filtering scheme outlined earlier can further increase the overall accuracy of detection. Lastly, we have not attempted to discriminate between multiple analytes, which would be the next significant step change towards translating the above to applications.

4. Conclusion

This study presents a novel methodology in translating Raman scattering based analyte detection from spectroscopy to microscopy. A hierarchical substrate, patterned in the 2D on the micron-scale, is developed to showcase the methodology, central to which is the plasmonic enhancement of the Raman signal, spatially localised to the 2D pattern. Later, an imaging cum analysis protocol based on hyperspectral imaging of the substrate under electromagnetic excitation is shown to yield optical images of the 2D pattern, acquired solely with Raman scattered photons. The 2D-SERS substrate is also shown to detect analytes down to few nM concentrations with spectral signal enhancement $\sim 10^6$, in the spectral range corresponding to 633 nm excitation. While the specific combination of the materials employed in the development of the 2D-SERS substrate viz. Si, ZnO nanorods and Au nanoparticles ensure minimal background signal within the detection window investigated here, the results do not limit the scope of exploring other materials and nanostructures in replicating the concept. Indeed, 2D-SERS substrates prepared employing specific material nanostructures would make them suitable for surface functionalization to selectively bind molecules in a fluid or flow cell for chemical identification of specific species and across different spectral windows corresponding to specific excitation wavelengths. Here, the performance of the developed methodology is established through recording Raman spectra, maps and finally optical images of the substrate coated with two dyes, rhodamine and fluorescein and then applied to detect glucose as the analyte under investigation is demonstrated, showing that the methodology may be further explored to enable detection of multiple analytes in a sample. Observation of the hexagonal pattern across multiple windows then increases the confidence of analyte detection and also ensures specificity, though the later has not been investigated. In all cases, the spectrally filtered images record the spatial localisation of the enhanced Raman signal to the hierarchical grid, demonstrating the conceptual basis of the method. Finally, an automated pattern recognition protocol is developed via a workflow using cascade, clustering and neural net algorithms to identify and analyse the quality of hexagonal objects in optical microscope images. The protocol trained and tested on a large set of positive and negative images demonstrates positive detection capability with 95% accuracy. It is anticipated that this investigation will help translate the SERS technique such that it becomes more pervasive, involving lower instrument costs wherein the chemical identification protocol is dominantly machine recognizable via image processing.

Acknowledgments

The authors acknowledge financial support from the Royal Academy of Engineering, Newton Bhabha Fund, UK (IAPPI_77) for enabling international exchange. JM acknowledges financial support from SERB, Govt. of India (CRG/2019/004965).

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Author contributions

The project was conceptualized by JM with the methodology developed by SRPS and JM. KNP conducted the sample preparation and experimental data acquisition. KNP and JF acquired optical Raman images. AAN developed the machine learning protocol for automated pattern recognition. Data analysis performed by KNP, AAN and JM. The manuscript was written by all authors.
Funding sources

This work was supported by the Royal Academy of Engineering, Newton Bhabha Fund, UK (IAPPI_77) and SERB, Govt. of India (CRG/2019/004965).

Notes

An Indian patent with reference number 202041051763 has been filed based on this work.

ORCID iDs

K N Prajapati © https://orcid.org/0000-0003-2566-3233
J Mitra © https://orcid.org/0000-0002-6633-9862

References

[1] Fleischmann M, Hendra P J and McQuillan A J 1974 Raman spectra of pyridine adsorbed at a silver electrode Chem. Phys. Lett. 26 163–6
[2] Nie S and Emory S R 1997 Probing single molecules and single nanoparticles by surface-enhanced raman scattering Science 275 1102–6
[3] Darby B L, Etchegoin P G and Le Ru E C 2014 Single-molecule surface-enhanced raman spectroscopy with nanowatt excitation Phys. Chem. Chem. Phys. 16 23895–9
[4] Kalathigil V, Dawson P and Mitra J 2017 Scanning tunneling microscope light emission: finite temperature current noise and over cut-off emission Sci Rep. 7 3530
[5] Kalathigil V, Dawson P and Mitra J 2016 Scanning tunneling microscope light emission: effect of the strong dc field on junction plasmons Physical Review B 94 035443
[6] Lenyk B, Schöps V, Boneberg J, Kabdulov M, Huhn T, Scheer E, Offenhäusser A and Mayer D 2020 Surface plasmon-enhanced switching kinetics of molecular photochromic films on gold nanohole arrays Nano Lett. 20 5243–50
[7] Zhan C, Chen X, Li Y, Li J-F, Wu D-Y and Tian Z-Q 2018 From plasmon-enhanced molecular spectroscopy to plasmon-mediated chemical reactions Nature Reviews Chemistry 2 361–36
[8] Doherty M D, Murphy A, Pollard R J and Dawson P 2013 Surface-enhanced raman scattering from metallic nanostructures: bridging the gap between the near- and far-field responses Phys. Rev. 3 011001
[9] Henry A I, Ueltschi T W, McAnally M O and Van Duyne R P 2017 Sipers Memorial Lecture. Surface-enhanced Raman spectroscopy: from single particle/molecule spectroscopy to angstrom-scale spatial resolution and femtosecond time resolution Faraday Discuss. 205 9–30
[10] Langer J et al 2020 Present and future of surface-enhanced raman scattering ACS Nano 14 28–117
[11] Karadan P, Aggarwal S, Anappara A A, Narayana C and Barshilia H C 2018 Tailored periodic Si nanopillar based architectures as highly sensitive universal SERS biosensing platform Sensors Actuators B 254 264–71
[12] Kumar G S, Shrestha R G, Li Q, Hill J P, Ariga K, Acharya S and Shrestha L K 2018 Hierarchical heterostructure of Ag-nanoparticle decorated fullerene nanorods (Ag-FNRs) as an effective single particle freestanding SERS substrate Physical chemistry chemical physics : Physical chemistrychemical physics : Physical chemistry chemical physics : Physical chemistry chemical physics : Physical chemistrychemical physics 20 18873–8
[13] Xu X, Guo Y, Li C, Li Z, Li D, Zhang C, Jiang S, Liu A, Man B and Zhang C 2018 High-performance 3D flexible SERS substrate based on graphene oxide/silver nanoparticles/pyramid PMMA Opt. Mater. Express 8 644
[14] Bandopadhyay K and Mitra J 2015 Zn interstitials and O vacancies responsible for n-type ZnO: what do the emission spectra reveal? RSC Adv. 5 23540–7
[15] https://colab.research.google.com/notebooks/
[16] Fageria P, Gangopadhyay S and Pande S 2014 Synthesis of ZnO/Au and ZnO/Ag nanoparticles and their photocatalytic application using UV and visible light RSC Adv. 4 42492–72
[17] Vempari S, Mitra J and Dawson P 2012 One-step synthesis of ZnO nanostructures: a blue-white fluorophore Nanoscale Res. Lett. 7 165202
[18] Ramon Casco E, Lia, Jordi I and Luis A 2007 Temperature dependence of raman scattering in ZnO Physical Review B 75 165202
[19] Yin H, Chan Y F, Wu Z L and Xu H J 2014 Si/ZnO nanocomb arrays decorated with Ag nanoparticles for highly efficient surface-enhanced Raman scattering Opt. Lett. 39 4184–7
[20] Prajapati K N, Johns B, Bandopadhyay K, Silva S R P and Mitra J 2020 Interaction of ZnO nanorods with plasmonic metal nanoparticles and semiconductor quantum dots J. Chem. Phys. 152 064704
[21] Makaram P, Owens D and Aceros J 2014 Trends in nanomaterial-based non-invasive diabetes sensing technologies Diagnostics 4 27–46
[22] Ferrante do Amaral C E A-L, Jordi I and Luis A 2007 Temperature dependence of raman scattering in ZnO Physical Review B 75 165202
[23] Bruen D, Delaney C, Florea L and Diamond D 2017 Glucose sensing for diabetes monitoring: recent developments Science Advances 17 01866
[24] Lee H, Song C, Hong Y S, Kim M S, Cho H R, Kang T, Shin K, Choi S H, Hyeon T and Kim D-H 2017 Wearable/disposable sweat-based glucose monitoring device with multistage transdermal drug delivery module Science Advances 3 1601314
[25] Botta R, Rajamanikkah A and Bansal C 2016 Silver nanoclusters films for glucose sensing by Surface Enhanced Raman Scattering (SERS) Sensing and Bio-Sensing Research 9 13–6
[26] Gupta S, Sandhu S V, Bansal H and Sharma D 2015 Comparison of salivary and serum glucose levels in diabetic patients J. Diabetes Sci. Technol. 9 91–6
[27] de Veij M, Vandenabeele P, De Beer T, Remon J P and Moens L 2009 Reference database of Raman spectra of pharmaceutical excipients J. Raman Spectrosc. 40 297–307
[28] Mathlouthi M and Vinh Luu D 1980 Laser–raman spectra of d-glucose and sucrose in aqueous solution Carbohydr. Res. 81 203–12
[29] Shao J, Lin M, Li Y, Li X, Liu J, Liang J and Yao H 2012 In Vivo blood glucose quantification using raman spectroscopy PLoS One 7 e48127
[30] Viola P and Jones M 2001 Rapid object detection using a boosted cascade of simple features Proc. of the 2001 IEEE Computer Society Conf. on Computer Vision and Pattern Recognition, CVPR 2001 pp I-I (https://doi.org/10.1109/CVPR.2001.990517)

[31] MacQueen J 1967 Some methods for classification and analysis of multivariate observations Fifth Berkeley Symp. on Math. Statist. and Prob 5.1 281 – 97

[32] Lecun Y, Bottou L, Bengio Y and Haffner P 1998 Gradient-based learning applied to document recognition Proc. IEEE 86 2278–324