1. Introduction

Raman spectroscopy is a widely used analytical tool for a range of applications.[1–3] In particular, Raman spectroscopy is useful for interrogation of biological samples, because of its insensitivity to water molecules and a working range within the visible and near-infrared regions.[4] Because the chemical fingerprints of biological samples can be measured with Raman spectroscopy, numerous clinical implementations have been investigated, such as histopathological and microbiological analysis, and diagnosis of the state of disease or inflammation.[5–8] Minimally invasive in situ monitoring of Raman scattering for in vivo diagnosis is one of the most desirable clinical applications and it can be achieved by integration of miniature fiber-optic Raman probes into existing endoscopes.[9–11] However, the intrinsically weak nature of Raman scattering (i.e., occurring for one in $10^7$ photons) hinders practical in vivo applications.

Surface-enhanced Raman spectroscopy (SERS) is a technique that is used to amplify inherent Raman scattering signals using roughened (nanostructured) metallic (e.g., Au and Ag) surfaces and/or metal nanoparticles (NPs).[12] Raman scattering is enhanced at the nanoscale tips and gaps on the roughened metallic surfaces and matrices of metal NPs due to highly confined electromagnetic fields created by plasmon excitation. These areas of signal enhancement are referred to as “hotspots” and various designs of NPs and surfaces have been reported to effectively create hotspots (e.g., diverse NP shapes, NP aggregates, nanostructure arrays, etc.).[13,14]

Two common approaches used to create SERS hotspots include chemical synthesis and traditional nanofabrication. The former involves chemical reduction of metal ions, followed by growth and decoration of NPs chemically in solvents. Photolithography, electron-beam lithography, and focused ion beam (FIB) milling are examples of nanofabrication techniques used to produce desired nanostructures and nanopatterns on silicon wafers. Despite being well established, several challenges remain in these methods especially in terms of structure uniformity, efficiency, controllability, repeatability, and complexity of fabrication processes.[15] Moreover, these methods often use toxic chemicals that may leave residual toxins, thus requiring extra purification steps before medical application. Most importantly, implementation of these approaches adds significant complexity when developing a miniature fiber-optic SERS probe (i.e., diameter $\leq 500 \mu m$) due to the size and positioning/handling of the optical fibers.[16–19]
A few alternatives have been proposed to overcome these limitations. For example, the use of highly ordered self-assembled polystyrene microspheres as a template for forming either nanoslands or nanocavities has been proposed as a simpler and cheaper option to obtain SERS hotspots on both wafer-scale substrates and the end-facets of optical fibers.[20] Despite attempts to alter the size and shapes of the microspheres by extra treatment (e.g., etching), fine control over the spacing distance and size of the nanostructures (key attributes for the SERS effect) is yet to be achieved. Furthermore, the fabrication efficiency and reproducibility can be low due to non-uniformities in layer thicknesses and microsphere distributions. Therefore, development of a new method for fabrication of SERS hotspots on the tips of optical fibers, which can overcome the previously mentioned limitations, is required.

Two-photon polymerization (2PP) is a promising candidate for this purpose. 2PP is a maskless microscale 3D printing technique with sub-diffraction limit resolution (down to 100 nm in lateral resolution and 300 nm in axial resolution).[21] 2PP not only allows for high resolution additive manufacturing, but also provides excellent versatility that enables fabrication of convoluted features that are not possible using conventional methods. Accordingly, 2PP has rapidly gained popularity in areas such as micro-optics and photonics, microfluidics, micromechanics, and micro-robotics.[22] Moreover, the 2PP technique is well suited to small-scale substrates, which is beneficial when applied to optical fiber end-facets.[23] Thus, fabrication of sophisticated structures on optical fiber tips has been successfully demonstrated using 2PP, with examples including micro-optics,[24–27] a miniature acoustic sensor,[28] a microscale force sensor,[29] and a force-sensitive micro-grasping tool.[30] In the field of SERS, 2PP has been utilized to build polymeric templates, supports, or masks for lift-off processes of metal layers, but these were mainly based on wafer-scale substrates.[31–36] Previously, we demonstrated the SERS effect and the potential biomedical sensing capability of some exploratory structures fabricated using 2PP on planar substrates.[17] To date, the 3D radar-like fiber-optic SERS probe developed by Xie et al. represents the only reported work using 2PP to generate SERS hotspots on optical fibers.[38] Although this probe exhibited promising SERS performance, the design of the device (with a small number of micrometer-wide openings) may not be practical for biological analyte sensing, and indeed only detection of non-biological analytes was presented.

Herein, we present a fiber-optic SERS probe ("SERS-on-a-tip") fabricated using a simple 2PP-based protocol that allows for a high degree of controllability and repeatability with all off-the-shelf components and materials. First, various SERS arrays were designed and fabricated on planar substrates and their SERS effects were characterized in order to optimize the geometry and alignment of the arrays. Assessment of the manufacturability of the SERS arrays was also conducted. Afterward, the optimized SERS arrays were replicated on the tips of optical fibers to evaluate their SERS sensing capability. Finally, to investigate a potential in vivo diagnostic application, SERS signals were collected and analyzed from live Escherichia coli (E. coli) cells using both SERS arrays on planar substrates and fiber-optic SERS probes.

2. 2PP Fabrication of SERS-On-a-Tip Optical Fibers

2PP is a photopolymerization process based on nonlinear two-photon absorption (2PA) that allows 3D printing on microscales. In this study, 2PP was used in a two-step process to fabricate SERS-on-a-tip (SOT) optical fibers in a highly repeatable manner, as illustrated in Figure 1. To align the optical fibers with respect to the focus of the near infrared (NIR) fs-laser of the 2PP system, an optical fiber holder was 3D-printed as shown in Figure S1, Supporting Information. Optical fibers were prepared as described in the Experimental Section and were clamped using the optical fiber holder. The fiber, photosensitive, and the microscope objective of the 2PP system were aligned (details in the Experimental Section), and 2PP was then directly accomplished on the end-facet of the optical fibers, which were immersed in the photosensitive in the dip-in laser lithography (DiLL) configuration (Figure S2, Supporting Information). Following 2PP fabrication, the unpolymerized photosensitive was removed using a developer (Figure 1c) to yield an optical fiber tip with the polymeric skeleton of the SERS array (Figure 1d). Finally, the SOT probes were completed by metallizing the tips of the optical fibers with a gold thin film (50 nm in thickness) to enable SERS hotspots (Figure 1e,f). Importantly, an analogous (but simpler) process can be used to fabricate SERS arrays on planar substrates by simply removing the fiber preparation steps and instead placing a planar substrate in the 2PP system as per regular printing procedures (Figure S2, Supporting Information).

Figure 1g illustrates the SOT sensing mechanism. When excitation light (\(\lambda = 785\) nm) transmitted through the optical fiber is incident on the SERS array, surface plasmon polaritons (SPPs) are excited and SERS hotspots are generated at nanogaps and nanotips in the SERS array designs.[39] The highly confined electromagnetic fields of these SERS hotspots act to enhance the Raman signal, which is then collected by the optical fiber and delivered to a spectrometer for detection.

3. Results and Discussion

3.1. SERS Arrays on Planar Substrates

In order to optimize the 2PP process and the SERS performance, the SERS arrays were first fabricated on planar glass substrates. We proposed different arrays of nano/microstructures from 2D to 3D structures to produce large quantities of SERS hotspots. Three types of structures were designed and printed: i) a hexagonally arranged single-voxel array (HSV, Figure S3, Supporting Information), which was intended to mimic an ideally fabricated self-assembled monolayer of microspheres; ii) a micropike array (cross spike array, CSA) to guide and concentrate SPP waves at the apexes; and iii) an array of fractal trees (fractal tree array, FTA) to generate both nanotips and nanogaps (Figure S4, Supporting Information). Additionally, smaller CSAs (Figure S4b, Supporting Information) and larger hemi-prolate spheroid arrays (Figure S4d, Supporting Information) were also designed to evaluate the size effect. The characteristic spacing distance, \(a\), for each of the SERS arrays was varied from a value just under the minimum separation resolution (where structures are partially merged) to larger
separation values in order to identify the optimal spacing distance. For evaluation of the SERS performance, spectra from 1 mm Rhodamine 6G (R6G) in aqueous solution were recorded. Further details of the fabrication process and data processing are described in the Experimental Section.

3.1.1. Optimization and Comparison of SERS Arrays

It was found that the HSVA with a 700 nm spacing distance exhibited the best enhancement performance among the SERS arrays fabricated on planar substrates (Figure 2). Strong peak intensity at 1510 cm$^{-1}$, up to 2000 a.u., was measured from HSVAs with the spacing distance of 700 nm (Figure 2a). In addition, the SERS effect was broadly homogeneous across the HSVAs, particularly with spacing distances of 400, 700, and 900 nm (Figure 2a). The area-averaged SERS spectra of R6G extracted from the mapping data displayed distinctive spectral features of the R6G molecule, with a signal-to-noise ratio (SNR) of over 20 at 614 cm$^{-1}$ (C-C-C ring in-plane vibration), 775 cm$^{-1}$ (C-H out-of-plane bending), 1186 cm$^{-1}$ (C-H in-plane bending), 1313 cm$^{-1}$ (N-H in-plane bending), 1363 cm$^{-1}$, 1510 cm$^{-1}$, 1605 cm$^{-1}$, and 1650 cm$^{-1}$ (aromatic C-C stretching) (Figure 2c). For all characteristic peaks, the HSVA with a 700 nm spacing distance gave the strongest response, for example, exhibiting up to 14 times stronger intensity than the HSVA with 1400 nm spacing distance (at 1605 cm$^{-1}$, Figure 2d).

Therefore, HSVA with 700 nm spacing distance was selected as an optimal structure for comparison with different SERS arrays (detail view in Figure 2b).
Figure 2. Optimization and comparison of various SERS arrays on a planar substrate. a) Raman mapping images of 1 mM R6G (intensity at $\nu = 1510$ cm$^{-1}$) on HSVAs with $\alpha$ from 300 to 1400 nm and SEM images of HSVAs at $\alpha = 300, 400,$ and 700 nm. Scale bars are 1 µm. b) Tilted close-up view of SEM image of voxels constituting the optimal HSVA ($\alpha = 700$ nm). c) Area-averaged SERS spectra of 1 mM R6G from the HSVAs displayed in (a). d) Intensities of characteristic peaks in R6G spectra from the HSVAs. e) Comparison of SERS spectra from different structures: i) HSVA, $\alpha = 700$ nm; ii) CSA1, $\alpha = 2.8$ µm; iii) CSA2, $\alpha = 3.2$ µm; iv) FTA, $\alpha = 4.8$ µm; v) CSA1 Small, $\alpha = 1.0$ µm; vi) CSA2 Small, $\alpha = 1.0$ µm; and vii) HSVA Big, $\alpha = 4.0$ µm. Non-SERS reference spectrum obtained on Al foil is also shown (black plot).
Considering the SERS enhancement effect as a diffraction grating-guided SPP excitation, it is possible to calculate the expected optimal spacing distance. In order to excite SPP by the diffracted rays, the momentum conservation between the SPP wave vector and the diffraction wave vector should be met and gives rise to the following equation:\(^{[42–44]}\)

\[
\lambda_{\text{ex}} = \frac{2\pi}{G_i} \sqrt{\varepsilon_m + \varepsilon_d}
\]

where \(\lambda_{\text{ex}}, G_i, \varepsilon_m,\) and \(\varepsilon_d\) are the wavelength that excites the SPP (i.e., the excitation wavelength of the Raman measurement system, 785 nm), the reciprocal lattice vector, and the dielectric constants of metal and the interface medium, respectively. The reciprocal lattice vector of a hexagonal array with lattice constant (i.e., spacing distance) \(a\) is written as \(G_i = (4 \pi / \sqrt{3} a) \sqrt{i^2 + j^2 + ij}\), and the first-order reciprocal lattice vector becomes \(G_i = 4 \pi / \sqrt{3} a\) (where the diffraction orders in \(x\)- and \(y\)-directions \((i, j) = (1, 0) \text{ or } (0, 1)\)), Equation (1) can be approximated as \(\lambda_{\text{ex}} = (2 \pi / G_i) \sqrt{n_d} = (2 \pi / G_i) n_d\), where \(n_d\) is the refractive index of the interface medium by considering that \(|\varepsilon_m| \gg |\varepsilon_d|\). Finally, the lattice constant where SPP is excited can be estimated as

\[
a_{\text{SPP}} = \frac{2\lambda_{\text{ex}}}{\sqrt{3} n_d}
\]

Therefore, the optimal spacing distance of the HSVA where SPP excitation is maximized for incident light of \(\lambda_{\text{ex}} = 785\) nm and in the aqueous environment \((n_d = 1.33)\) is found to be \(a_{\text{SPP}} = (2785 \text{ nm}) / \sqrt{3} (1.33) = 682\) nm. This is in good agreement with the observation of peak signal at \(a = 700\) nm (Figure 2a).

Figure 2e shows the average R6G spectra of different SERS arrays with optimal spacing distances (Figures S5 and S6, Supporting Information) alongside a reference (unenhanced) spectrum from R6G on aluminum foil. The strongest signal was observed from the HSVA, in agreement with previously acquired data.\(^{[37]}\) The analytical enhancement factors (AEFs) for the optimized SERS arrays were calculated and are listed in Table S1, Supporting Information. The highest AEF was over \(3 \times 10^3\) at \(614\) cm\(^{-1}\) for the optimized HSVA, although it is worth noting that the AEFs may have been underestimated because aluminum foil can exhibit a non-zero SERS effect.\(^{[45]}\)

### 3.1.2. Reproducibility of 2PP of HSVAs

Reliable repeatability and consistency of 2PP of HSVA was also demonstrated (Figure 3). Since HSVA was the most challenging array to fabricate, requiring the best precision and resolution, acquiring repeatability and consistency from HSVAs could reasonably guarantee that repeatability and consistency could
be achieved for the other SERS arrays. While each one of nine different panels of the optimized HSVAs fabricated in one batch was almost identical in the SEM and the optical microscope images (the first and the second rows in Figure 3a), a variation in the SERS effect (13% relative standard deviation (RSD) at 1510 cm$^{-1}$) was observed by Raman mapping (Figure 3a, bottom row). This variation in the averaged intensities, as well as the inhomogeneous intensity distribution within the HSVA, may be attributable to the uncertainty in the adsorption of the R6G molecules at the surface of the arrays, because the surfaces of the arrays were not chemically treated for better affinity to the R6G molecule. Nevertheless, the repeatability of 2PP of SERS arrays was deemed to be good when compared to the work of Cao et al.\[46\] Likewise, good repeatability was achieved for all HSVAs regardless of spacing distance (nine panels for each spacing distance, Figure 3b). Moreover, there was consistency across different batches of multiple substrates with the optimal spacing distance determined as 700 nm in the majority of cases (17% RSD, Figure 3c).

3.2. SOT Optical Fibers

3.2.1. SOT Fabrication Efficiency

Figures 4a,b show optical micrographs of SOTs with HSVA ($a = 700$ nm) and CSA (version 1, $a = 2.8$ µm), respectively. Again, repeatability in multiple fabrication of SOTs in one batch was accomplished. It is worth noting that the color observed at the fiber tips is due to diffraction from the SERS arrays. The diffraction also appeared when fibers were illuminated by an external white light source from the proximal end, which implied that the SPP excitation mechanism based on diffraction grating was still feasible in the SOT configuration. The CSA-SOT arrays exhibited stable attachment and well-defined structure (Figures 4c,d), whereas the fabrication efficiency of the HSVA-SOT was low as large areas of the arrays collapsed (Figure S7, Supporting Information). This was attributed to the error in the alignment between the focal plane of the 2PP system and the tilted end-facet of the optical fibers, which was more crucial to the HSVA than the CSA due to the heights of the two structures. The tilt was induced by the 3D-printed optical fiber holder and was thus consistent (ca. 1.3 degrees). Hence, this effect could be reduced by improving the design of the optical fiber holder.

3.2.2. Sensing Characterization of SOTs

Distinct SERS spectral features of R6G were collected through 30 cm long SOTs fibers, and the CSA-SOT demonstrated better sensing characteristics compared to the HSVA-SOT (Figure 5). The laser power transmitted through, and measured at the end of, the 30 cm long bare optical fiber was 4.7 mW and Raman spectra were acquired with 10 s integration times (averaged over ten repeat measurements in each case). The Raman peaks of R6G in the wavenumber region below 1300 cm$^{-1}$ were not detectable on account of the strong silica background of optical fibers (Figure 5a; Figure S8a–d, Supporting Information). In addition, the collection efficiency of the SERS scattering

Figure 4. Optical microphotographs and SEM images of SOTs. a and b) Four SOTs with HSVA ($a = 700$ nm) and CSA (version 1, $a = 2.8$ µm). c) SEM image of the tip of CSA-SOT. d) Detail view of CSA-SOT.
through the SOT was inferior compared to that of SERS on planar substrates due to the backscattering measurement configuration and the fact that there was no focusing of the light at the SERS array of the SOT. As opposed to the results from planar substrates, the CSA-SOT afforded a stronger SERS effect than the HSVA-SOT, so that even minor peaks at 1329, 1623, and 1671 cm\(^{-1}\) were distinguishable (Figure 5b).

This was again attributable to the fabrication efficiency of the SOTs as explained in the previous section. In addition, a red-shift in the locations of characteristic peaks of the R6G (by up to ca. 20 cm\(^{-1}\)) was observed when using the SOTs and bare optical fibers compared to the planar substrates (Figure 5b; Figure S9, Supporting Information). This red-shift was ascribed to differences in the calibration of the spectral detectors in the two measurement systems. This was further confirmed by recording spectra from ethanol using the two detection systems (i.e., the Raman imaging microscope and the benchtop spectrometer), which demonstrated a comparable red-shift (see Figure S10, Supporting Information).

From the demonstration of SOTs for R6G concentrations \(C_{\text{R6G}}\) ranging from \(10^{-6}\) up to \(10^{-3}\) \(\text{m}\), the weakest experimentally detectable signal was collected at \(10^{-6}\) \(\text{m}\) with SNRs of 6.6 and 10.7 for the HSVA-SOT and the CSA-SOT, respectively (Figure 5c,d and e,g). More importantly, a linear relationship between the peak intensity at 1529 cm\(^{-1}\) (\(I_{1529}\)) and the concentration in semi-logarithmic scale was observed for both the HSVA-SOT (\(I_{1529} = 119\log(C_{\text{R6G}}/10^{-7}) - 64, R^2 = 0.959\)) and the CSA-SOT (\(I_{1529} = 172\log(C_{\text{R6G}}/10^{-7}) - 100, R^2 = 0.983\)).
Table 1. Parameters for calculation of AEFs. Table shows: the peak intensities of R6G at $\nu = 1529\ \text{cm}^{-1}$ measured through bare fiber, HSVA-SOT, and CSA-SOT; the measurement parameters for calculation of AEFs; and the calculated AEFs of HSVA-SOT and CSA-SOT.

|                     | Bare fiber | HSVA-SOT | CSA-SOT |
|---------------------|------------|----------|----------|
| Peak intensity @ $\nu = 1529\ \text{cm}^{-1}$ [a.u.] | 448.78     | 51.59    | 117.13   |
| R6G concentration [m] | $5 \times 10^{-3}$ | $1 \times 10^{-6}$ | $1 \times 10^{-6}$ |
| Integration time [s] | 10         | 10       | 10       |
| AEFs                | $580$      | $1300$   |           |

$R^2 = 0.985$; Figure 5e,h. The slope (which represents the sensitivity) of the CSA-SOT was steeper than that of the HSVA-SOT, once again, due to poorer fabrication efficiency of the HSVA-SOTs. We also estimated the limit of detection (LOD) based on the equation, LOD = $3\sigma/m$, where $\sigma$ is the standard deviation of blank measurements and $m$ is the slope of the calibration curve.[47] The estimated LODs for the HSVA-SOT and the CSA-SOT in aqueous R6G solution were $4.8 \times 10^{-7}$ and $3.7 \times 10^{-7}$ m, respectively. Lastly, the AEFs of HSVA-SOT and CSA-SOT were calculated as $580$ and $1300$, respectively at $1529\ \text{cm}^{-1}$ (Table 1).

It should be noted that several previous studies on fiber-optic SERS probes have demonstrated sensing performances (i.e., LOD, enhancement factor (EF), etc.) based on settings that are not necessarily applicable in vivo. These include, for example, soaking of probes for several hours in the analyte solution (from 3 to 12 h) and drying of droplets at the fiber tip to maximize adsorption of molecules.[48–50] Moreover, some studies have demonstrated performance of the optical fiber probes only by externally examining the enhancement of the SERS structure on the sensing end-facet—i.e., by using the optical fiber as a substrate and not as a waveguide transmitting the illumination and SERS signal.[28] Hence, in this work, we only compared the achieved performance to those studies in the literature where characterization was performed through the optical fiber without any soaking or adsorption procedures.

Xie et al. reported a LOD of $10^{-6}$ m for crystal violet (CV) dissolved in ethanol using a 3D radar-like SERS structure fabricated by 2PP, but they used a larger core diameter fiber.[38] Xia et al. also reported a LOD down to $10^{-6}$ m for R6G in aqueous solution but double the integration time (relative to the work reported herein) was used. In addition, no estimation of EF was given.[51] Finally, Cao et al. reported an LOD of $10^{-9}$ m for 4-aminothiophenol (4-ATP) in aqueous solution, but they used an optical fiber taper and no estimation of the EF was given.[46] Despite not being directly comparable to previous works due to differences in integration time, laser power, fiber configuration, analyte, etc., the reported values for the LOD ($<10^{-6}$ m for R6G in aqueous solution) and the AEF (three orders of magnitude) nonetheless demonstrate excellent sensing performance for the SOT probe.

3.3. Rapid Bacteria Detection Capability

Finally, both SERS on planar substrates and SOTs were demonstrated to be effective for detection of live *E. coli* in suspension, with integration times as small as 2.5 ms (10 averages) and 1 s (no averaging), respectively (Figure 6; Figure S11, Supporting Information). Since the *E. coli* cells were neither immobilized nor deposited onto the SERS arrays on planar substrates, a diverse set of peaks intermittently appeared at different wavenumber positions on the spectra over the integration time caused by the bacteria swimming over the HSVA (Figure 6a). Nonetheless, there were some characteristic SERS bands in the fingerprint region that were observed commonly in multiple spectra (highlighted in Figure 6a). The most prominent peaks were found between 1440 and 1580 cm$^{-1}$ that can be assigned to proteins, lipids, and nucleobases such as E. coli in suspension. a) Four representative spectra obtained from HSVA on planar substrate. The highlighted regions in grey indicate where SERS peaks commonly appeared. b–e) Raw spectra (b,d) of background taken in PBS solution (dashed line) and *E. coli* suspension in PBS (solid line) from a representative CSA-SOT, and baseline corrected spectra (c,e) from multiple CSA-SOTs averaged over 100 spectra collected with 1 s (b,c) and 10 s (d,e) integration times.
guanine and adenine.\textsuperscript{[52,53]} The other characteristic regions were between 1080 and 1150 cm\(^{-1}\) (proteins), 1180 and 1250 cm\(^{-1}\) (amide III), and between 1310 and 1370 cm\(^{-1}\) (proteins, adenine, guanine). This was to be expected as \(E.\ coli\) is a Gram-negative bacterium with a cell wall that is rich in proteins and lipids. Furthermore, the spot size of the objective was below 1 \(\mu\)m, which is comparable to the size of an \(E.\ coli\) cell body (=1 \(\mu\)m in width by 2 \(\mu\)m in length) and therefore concurrent SERS signals from multiple cell organelles were also expected. Accordingly, it was interpreted from the results that the cell wall and nuclei of \(E.\ coli\) could be detected by SERS effect of the HSVAs arrays on planar substrates. Although acquisition could be detected by SERS effect of wall and nuclei of \(E.\ coli\), the limitations in the signal collection efficiency and the silica background signal addressed previously, spectra were obtained with highly concentrated bacterial suspensions with both 1 and 10 s integration times (100 averages, Figure 6b–e; no averaging, Figure S11, Supporting Information). Only a single Raman peak was observed using the SOT probes, but this major peak at 1569 cm\(^{-1}\) appeared in raw spectra (Figure 6b,d) and became further evident upon baseline correction (Figure 6c,e; also see the empirical spline fitted baselines shown in Figure S8e,f, Supporting Information). Noting that there was a red-shift of ca. 20 cm\(^{-1}\) between the SOT probes and the planar substrates (see Section 3.2.2), the SERS peaks of \(E.\ coli\) collected by the CSA-SOTs could be matched with the \(E.\ coli\) SERS spectra collected with SERS on planar substrates. Finally, while observation of only a single Raman peak clearly indicates that the system is at the threshold of detection for \(E.\ coli\) measurements (with only the brightest peak being detectable in a region with relatively low background signal), this still entails an important advance as, to the best of our knowledge, it represents the first demonstration of detection of live, unlabeled bacteria using a fiber-optic SERS probe.

4. Conclusion

We demonstrated competitive SERS performance of SERS arrays on planar substrates and SOTs fabricated in two steps—2PP of polymeric skeletons followed by metallization—for potential diagnostic applications. Importantly, this relatively simple fabrication procedure used all off-the-shelf components and materials. Fabrication of various SERS arrays with different degrees of complexity and controllability of the spacing distance was successfully conducted, enabling optimization of the SERS arrays for the best sensing performance. As a result, the HSVAs with a 700 nm spacing distance was shown to exhibit the best signal enhancement (AEF > 3 \(\times\) \(10^3\) at 614 cm\(^{-1}\)) followed by the CSA (version 1, 2.8 \(\mu\)m spacing distance). The fabrication technique was also confirmed to be repeatable and reproducible with RSDs of 13% and 17% for intra- and inter-experimental variance, respectively.

The results suggest that the proposed technique is a viable approach for fabricating SOTs with optimized SERS arrays on the end-facets of optical fibers in a direct and highly controllable manner. The fabricated SOTs exhibited excellent SERS sensing performance with a LOD of 3.7 \(\times\) \(10^7\) m and an AEF of three orders of magnitude, which are comparable to or exceed other previously reported fiber-optic SERS probes. Lastly, the feasibility for rapid bacteria detection was verified (for both the SERS arrays on planar substrates and the SOTs) using integration times as low as 2.5 ms (planar substrates, 10 averages) and 1 s (SOTs, no averaging). Notably, to the best of our knowledge, the bacteria detection capability of the SOT represents the first demonstration of label-free detection of live bacteria using a fiber-optic SERS probe. In conclusion, it was demonstrated that microstructure arrays can be tailored to achieve required SERS performance and can be incorporated onto fiber-optic probes via 2PP in a two-step process. Consequently, this fabrication technique—based on all off-the-shelf components and materials—is a reliable and practical method for the preparation of SOTs for diverse applications.

5. Experimental Section

Design and Fabrication of HSVAs: 2PP was performed for fabrication of the polymeric skeletons of the HSVAs with a Photonic Professional GT system (Nanoscribe GmbH, Germany). Prior to fabrication, 2D hexagonal coordinates were generated on an \(x-y\) plane by writing a command script in DeScribe (the software package provided with the Photonic Professional GT system) as illustrated in Figure S3, Supporting Information. The spacing distance \(a\) of HSVAs was varied from 300 to 1400 nm in 100 nm increments. Laser exposure parameters were also set within the script to provide a laser dose level just above the polymerization threshold (5 mW and 1 ms for the laser power and the integration time, respectively).

For the 2PP fabrication of HSVAs on planar substrates (fused silica, 25 \(\times\) 25 \(\times\) 0.7 (width \(\times\) length \(\times\) thickness) mm\(^3\)), the substrates were initially cleaned with acetone, isopropyl alcohol (IPA), and deionized water (DIW) followed by gentle drying under a nitrogen gas stream. Cleaned substrates were then fixed on the sample holder with adhesive tape. Afterward, a drop of the photosensit (IP-Dip, Nanoscribe GmbH) was applied onto the bottom-side of the substrates. Once complete, the sample holder was placed on the microscope (immersion objective; 63\(\times\); numerical aperture (NA) = 1.4; working distance = 190 \(\mu\)m, ZEISS, Germany) and the 2PP job was launched using the NanoWrite operating software (Nanoscribe GmbH). Multiple HSVAs (usually nine, 10 \(\times\) 10 \(\mu\)m\(^2\) per array) were fabricated on the substrates for each \(a\) value to allow for yield and repeatability testing. Once 2PP was complete, the samples were soaked in propylene glycol methyl ether acetate (PGMEA) for 20 min for development and then immersed in IPA for another 2 min to complete the removal of the non-crosslinked residue. Fully dried samples were then placed in an electron-beam evaporation chamber (HEX modular deposition system, Korvus Technology, UK) for metallization to create the plasmonic layer. A gold thin film (50 nm in thickness) was deposited on the polymerized structures under vacuum (3 \(\times\) \(10^{-5}\) mBar).

Design and Fabrication of CSAs: A command script including the geometry of a cross spike and patterns for CSAs was created in DeScribe. To achieve a sharp edge and tip for each spike, a \(z\)-axis stack of single-lined crosses that linearly decreased in size with respect to the \(z\)-axis was programmed (Figure S4a, Supporting Information). The width and height of a single cross spike for large and small CSAs (CSA and CSA...) were 4 and 5 \(\mu\)m, and 1 and 1.25 \(\mu\)m, respectively. For both CSAs, the arrays were also arranged in hexagonal patterns with two different rotational orientations (version 1 and version 2 as shown in Figure S4a, Supporting Information). The spacing distance of the large and small CSA arrays varied from 2.4 to 4.4 \(\mu\)m and from 0.6 to 1.6 \(\mu\)m, respectively. Fabrication of CSA arrays was carried out as described in section “Design and Fabrication of HSVAs.”

Design and Fabrication of Other 3D Microstructure Arrays: 3D CAD software, Solidworks (Dassault Systemes, France), was used for designing and generating other 3D geometries. The fractal tree for FTA

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**Figure S11, Supporting Information.**
was designed starting from a 3 μm wide trunk to evolve three branches separated by equal distances and angles. The tree was evolved up to the second generation as shown in Figure S4c, Supporting Information. For the large HSVA arrays (HSVA_Big), an elongated hemisphere of 2 μm diameter to mimic a single voxel was designed (Figure S4d, Supporting Information). The 3D microstructure arrays were arranged hexagonally with the spacing distance varying from 4.0 to 5.4 μm (FTA arrays) and from 2.0 to 4.0 μm (HSVA_Big arrays), respectively. Fabrication was carried out as described in section “Design and Fabrication of CSAs.”

Optical Fiber Preparation and SOT Fabrication: Low-OH multimode silica optical fiber (200/220 μm of core/cladding diameter, 0.22 NA, FC200LEA, Thorlabs, Inc., Germany) was cut into 36 cm long sections and the polymer coating at both ends was stripped off using a fiber stripper. The exposed ends were then cleaned with lens paper soaked with IPA and placed on an automatic fiber cleaver (CT-101, Fujikura Ltd., Japan). Approximately 3 cm was cleaved from each end to provide optically flat and clean facets. The cleaved 30 cm long fibers were placed, aligned, and cramped on the 3D-printed optical fiber holder as shown in Figure S1, Supporting Information. Once the optical fibers were ready, a droplet of IP-Dip photosresist was dropped on the objective lens of the 2PP system and then the optical fiber holder was carefully seated on one of the sample positions of the sample holder. Subsequently, the z-axis position of the objective was adjusted to find the focus at the end-facet of the fiber. At this point, it was important to make sure that the fiber end to be used was dipped within the photosresist droplet. A total of 100 × 100 μm² of SERS structure arrays (HSVA with a = 700 nm, CSA Ver1 with a = 2.8 μm) were printed on the end-facets of optical fibers. Development was carried out in the same manner as described above. Once the optical fibers with the polymeric skeletons of the SERS arrays were ready, they were mounted in an e-beam evaporation chamber (as clamped within the optical fiber holder) to deposit 50 nm thick gold layers as described above.

Rhodamine 6G Aqueous Solution Preparation: R6G aqueous solutions (1 μM) were prepared by dissolving R6G powder (Sigma–Aldrich, Germany) in DIW to allow comparison of the SERS structures and optimization of the spacing distances. To characterize the sensing capabilities at different concentrations, and to ascertain the LOD, serial dilutions were performed to produce R6G solutions with concentrations ranging from 10⁻³ M down to 10⁻⁷ M.

Bacteria Sample Preparation: Non-pathogenic E. coli K12 J53-1 (ATCC BAA-769, ATCC, USA) were cultured in autoclaved tryptic soy broth (Sigma–Aldrich, Germany) at 37 °C overnight with agitation. The saturated culture (cell concentration ca. 10⁹ cells mL⁻¹) was used for Raman imaging on the glass substrates. To achieve a higher concentration of cells for SOT experiments, 10 mL of the saturated culture was centrifuged (3 min, 4000 rpm, MiniSpin, Eppendorf, Germany) and the pellet was washed three times with phosphate buffered saline (PBS). All E. coli measurements made with the SOT fibers were then performed in PBS, and blank PBS spectra were recorded as background (see “Spectra Background Subtraction” section below).

Imaging using Raman Microscope: Droplets (~20 μL) of the chosen analytes—either R6G aqueous solution or saturated E. coli culture—were pipetted onto the glass substrates where SERS arrays had been fabricated. These samples were then secured by covering the droplets with a second glass coverslip (with no SERS structures) on top of a 3D imaging spacer (SecureSeal, Grace Bio-Labs, USA). Raman imaging was carried out using a DXRx2i Raman Imaging Microscope (Thermo Fisher Scientific, USA). A 785 nm frequency-stabilized single mode diode laser was used for excitation. A 50 μm slits aperture (the largest available aperture of the system with the lowest spatial resolution) was selected in order to mimic as far as possible the measurement conditions of the SOT configuration (diverging light, averaging both spatially and in depth). Samples were illuminated with 5 mW of excitation power through a 50x long working distance objective (Olympus, Japan) and data were collected using a 2.5 ms (400 Hz) integration time, 10 scans (averages), and a 0.5 μm pixel size. Droplets covered all nine SERS panels on the glass slides (which represented repeat fabrications of identical arrays with identical spacing distances—see “Design and Fabrication” sections above) allowing repeatability tests to be performed.

Non-SERS Reference Spectra Acquisition using Raman Imaging Microscope: In order to estimate the enhancement power of the fabricated SERS arrays, acquisition of pure Raman spectra free from SERS effects was required (i.e., spectra from non-SERS substrates were required). To this end, aluminum foil was used as the substrate and the concentration of the R6G solution was increased up to 10 mM. The integration time was increased to 0.333 s (3 Hz) and the averaging to 180 scans. A laser power of 5 mW was used, as for the characterization of SERS microstructure arrays (see above). Using these parameters, it was possible to collect unenhanced R6G Raman spectra with suitable SNRs, which were used to calculate enhancement factors.

Measurement using Raman Fiber Probe and Benchtop Spectrometer: In order to measure Raman spectra through optical fibers, a bench-top spectrometer system was designed as shown in Figure S12, Supporting Information. A previous version of this benchtop system was reported elsewhere.[56] The benchtop system measures 30 × 45 × 24.5 cm³ and is shielded by anodized aluminum plates for laser safety purposes. It comprises a continuous wave (CW) 785 nm laser diode (L785P090, Thorlabs, Inc., Germany) mounted within an integrated thermoelectric cooler and the same (LDM9T/M, Thorlabs, Inc., Germany), a laser diode driver (LDC202C, Thorlabs, Inc., Germany), and a spectrometer (QE Pro, Ocean Optics, Inc., the Netherlands). The laser light was first coupled into the excitation optical fiber of a commercial bifurcated fiber-optic Raman probe (InPhotonics RIP-RPB-785-FC-SMA, Ocean Optics Inc., the Netherlands) and the detection channel of this probe was used to direct the output signal to the spectrometer. The SOT fiber was clamped within a kinematic mount and the near end face was positioned at the focus of the bifurcated fiber-optic Raman probe. Light was coupled into the SOT fiber and the optical alignment was optimized by fine-tuning the translation (in both x- and y-axes) and tilt of the kinematic mount. The laser power measured at the distal end of 30 cm long bare optical fiber was 4.7 mW. The sensing tip (distal end) of the SOT fiber was then carefully dipped into test tubes for sample measurements. For data acquisition, spectra were collected with up to 10 s integration times (10 averages for R6G; 10 averages for E. coli).

Non-SERS Reference Spectra Acquisition using Bare Optical Fiber and Benchtop Spectrometer: The optical fiber used for SOT fabrication was cleaved into 30 cm sections and used for non-SERS reference spectra acquisition without any further treatment. Two cleaved optical fibers were dipped in DIW and the fiber silica background spectra were recorded. Raman spectra (10 averages) from 10 and 5 mW R6G aqueous solutions were also recorded with 4.7 mW at the distal end of the optical fiber and 10 s integration times (Figure S9, Supporting Information).

Spectra Background Subtraction: The built-in tool of the graphing software (OriginPro, OriginLab Corporation, USA) was used for background fitting and subtraction of collected spectra. Asymmetric least squares smoothing was carried out for the spectra obtained from the Raman imaging system for SERS on planar substrates (Figure S13cd, Supporting Information). However, empirical spline fitting based on the reference plots (DIW and PBS spectra for R6G and E. coli experiments, respectively) was performed for the SOT spectra (Figure S8, Supporting Information). Using this approach, the background levels were fitted to the spectral shapes from the measured reference spectra. This method was used for the SOT spectra because the inherent silica background spectra from the fibers were strong relative to the signal peak intensity (Figure S8a,c, Supporting Information) and were also too intricate to allow reliable numerical separation of the signal peaks from the background spectrum. In effect, this meant that the asymmetric least squares approach was not able to accurately remove the background, as sharp silica background peaks were often incorrectly identified as signal peaks. Thus, the above spline fitting approach was used as an alternative. While this required collection of separate background spectra, it provided reliable and accurate background subtraction.

Analytical Enhancement Factor Calculation: The analytical enhancement factor (AEF) was chosen to characterize the SERS microstructures and the SOTs. It is important to note that the AEF does not fully describe
the SERS effect since surface effects are ignored (e.g., molecular coverage and surface adsorption). Therefore, it is dependent on the molecular species and the sample preparation process. Nevertheless, the AEF provides a simple figure for the signal enhancement that can be expected in practical analytical measurements. The wavenumber and intensities of the major peaks were extracted from the baseline corrected spectra and put into the following equation.

\[
\text{AEF} = \frac{I_{SERS}}{I_{RS}} \cdot \frac{C_{SERS}}{C_{RS}}
\]

(3)

Here, \(I_{SERS}\) and \(C_{SERS}\) represent the peak intensity and the concentration of analyte for the SERS measurements, and \(I_{RS}\) and \(C_{RS}\), respectively, represent the equivalent values for non-SERS measurements. Different measurement parameters (e.g., the integration times) were further accounted for in order to calculate normalized AEFs as

\[
\text{Normalized AEF} = \frac{\left( \frac{I_{SERS}}{I_{RS}} \right)}{\left( \frac{C_{SERS}}{C_{RS}} \right)}
\]

(4)

where \(I_{SERS}\) and \(I_{RS}\) represents the integration times used in the SERS and non-SERS measurements, respectively. Where different laser powers, averaging values, and other parameters were used for the SERS and non-SERS measurements, identical correction calculations were also performed.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

bacteria detection, optical fibers, Raman spectroscopy, SERS, two-photon polymerization

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