Statistical Correlation Between SERS Intensity and Nanoparticle Cluster Size

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ABSTRACT: Surface enhanced Raman scattering (SERS) mapping of biomarkers has shown great promise in determining the distribution of proteins of interest in cells and tissues. Metallic nanoparticle (NP) probes are generally used in such mapping. Since SERS intensities from NPs are dependent on size, shape, and interparticle distance/distribution, it is unclear if this method can provide reliable biomarker quantification. To address this problem, we investigated a statistical approach to the quantification of SERS from SERS probe clusters. The investigation began by considering multiple biotinylated surfaces that had been exposed to pegylated NPs (designed for biological SERS mapping) functionalized with streptavidin (defined as SERS probes). The surfaces were imaged with a scanning electron microscope and SERS-mapped with a Raman microscope. Statistical distributions of the SERS probe clusters and mapped SERS intensities on the surfaces were developed. It was found that there was a smooth polynomial relationship between SERS intensity and probe cluster size. Our result is in contrast to the sharp, highly variable intensity increases observed in studies of unmodified NPs. Based on the polynomial relationship found, it is clear that pegylated NP SERS probes might be useful for quantification in the SERS mapping of biological material, as the SERS intensity can be potentially related back to the number of probes at the acquisition point.

INTRODUCTION

Surface enhanced Raman scattering (SERS) has proven to be a very useful technique in the field of cellular imaging and bioanalytics due to its ability to detect low-level concentrations of molecules with a high degree of specificity.1−12 SERS-based immunoassays have been used for the detection of biomarkers in samples such as cells,13−20 tissue,21−23 and human blood serum.24−29 Metallic nanoparticles (NPs; typically silver or gold) are the SERS-enabling platform in those assays. Typically, the NPs are coated with a Raman reporter molecule and functionalized with an antibody for a particular biomarker of interest to impart selectivity to the assays.14,16,21−23,28 The NP-reporter-antibody platforms used in these types of applications are known as “SERS probes”. In order to be comparable to more traditional immunoassay techniques, such as the enzyme-linked immunosorbent assay (ELISA), quantification of the detected biomolecules is necessary, and has been attempted in SERS with promising results in many cases.1,5,6,8,11,14−16,22,28,30,32,34−37,39 Normalization using an internal standard to correct for changes in the sample properties, SERS substrate, or laser beam intensity during the acquisition period is also used for quantification.26,29,33,41

However, signal averaging might not be appropriate in attempts to use SERS probes to map an analyte that is nonuniformly distributed over a surface, as in the case of a planar assay platform or in the in vitro detection of cell membrane proteins. When mapping a surface, the SERS probed area all have a strong influence on the observed SERS intensity.45,51−53,56,57

While modern techniques allow for a great degree of control over the size and shape of NPs,55,59 the random degree of aggregation of SERS probes during an assay can potentially lead to wild variations in SERS signal. If the SERS probes are introduced to a sample that is in solution and assumed to have a relatively uniform target molecule (analyte) concentration, the variations in measured SERS intensity can be addressed by simply performing spectral averaging throughout the sample.8,14,22,28,30,32,34−37,39 Normalization using an internal standard to correct for changes in the sample properties, SERS substrate, or laser beam intensity during the acquisition period is also used for quantification.

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Stabilized the resulting clusters by individually encasing them in SiO₂ shells. After identifying individual clusters with dominating the overall enhancement provided by the cluster dependent on the size of the interparticle gap. Crevices formed that of a NP heptamer, with the enhancement much more showed that a NP dimer can provide SERS intensities as high as and can result in signal over 10⁸ times larger than that of SERS mapping. However, a popular type of NP probe normal Raman scattering. Typically used in SERS imaging of cells and tissues has a NP

The variation in SERS intensity with NP aggregation is well-known, and previous studies have monitored changes in intensity caused by the chemically induced aggregation of nanorods.⁶¹ and aggregation due to the deposition of multiple NP monolayers.⁶²−⁶⁴ In general, it was found that an increase in particle aggregation leads to an increase in the average SERS intensity. A few groups have studied the SERS enhancement provided by individual NP clusters of varying size.⁵¹−⁵³,⁵⁵ For instance, Wustholz et al.⁵³ achieved a degree of control over the NP aggregations by using field flow fractionation⁶² and stabilized the resulting clusters by individually encasing them in SiO₂ shells. After identifying individual clusters with transmission electron microscopy (TEM) and acquiring SERS and localized surface plasmon resonance (LSPR) measurements, they discovered that there is not necessarily an increase in SERS enhancement with the cluster size.⁵³ The results showed that a NP dimer can provide SERS intensities as high as that of a NP heptamer, with the enhancement much more dependent on the size of the interparticle gap. Crevices formed in the junction between adjacent NPs act as “hot spots” dominating the overall enhancement provided by the cluster and can result in signal over 10⁸ times larger than that of normal Raman scattering.

In practice, the strong dependence on the interparticle gap can be very problematic when attempting to quantify the results of SERS mapping. However, a popular type of NP probe typically used in SERS imaging of cells and tissues has a NP core protected by a coating of polyethylene glycol (PEG),¹⁷ which should control the gap distance between the Au-NP cores upon aggregation, leading to smaller variations in SERS intensities. The potential of these SERS probes to reduce SERS intensity variation warrants further investigation. Furthermore, despite the success of Wustholz et al.⁵³ in isolating and analyzing a variety of NP aggregates, their sample size of 89 individual nanoclusters represents only a small fraction of the NP cluster sizes that could be encountered in, for instance, a cell imaging experiment. In order to more completely characterize the relationship between SERS intensity and NP cluster size, we have developed a statistical approach that enables sampling of thousands of NP aggregates. Also, this approach helps to mitigate the time-consuming efforts of attempting to obtain images of the probe clusters (taken via scanning electron microscope (SEM),⁵⁰ atomic force microscopy (AFM),⁵² or TEM⁵⁰) and SERS data from the exact same regions of the sample. Through the analysis of SEM images and SERS maps acquired from randomly chosen regions of a gold surface coated with SERS probe clusters of varying size, a relationship between SERS intensity and cluster size is determined.

By implementing this statistical approach, a large sample size is achieved with as many as 20 000 SERS intensities and 1500 individual clusters included in the analysis for a single incubation time, and four different NP incubation times considered in all. Furthermore, the use of pegylated SERS probes, instead of uncoated Au NPs, provides information on how SERS intensity varies for probes that are specifically designed for target applications involving SERS imaging of biological cells. As a result, analysis of the SERS intensities of clusters formed by these SERS probes will provide information that could be directly applicable in vitro, allowing, for example, the identification and quantification of protein cancer-markers within cells.¹⁷

EXPERIMENTAL METHODS

Chemicals. Ultrapure water (UP-H₂O) was used throughout the experiment (Barnstead Nanopure ultrapure water purification system, 18.2 MΩ·cm resistivity; Thermo Scientific, Ottawa, Ontario, Canada). Gold(III) chloride trihydrate (HAuCl₄·3H₂O; ≥99.9+% trace metals basis), sodium citrate dehydrate (≥99%, FG), sodium azide (ReagentPlus, ≥99.5%), Nile Blue A perchlorate (dye content 95%), streptavidin (essentially salt-free, lyophilized powder, ≥13 units/mg protein), bovine serum albumin (BSA; ≥98% (agarose gel electrophoresis), lyophilized powder), Polysorbate 20 (Tween20), and acetone (ACS reagent, ≥99.5%) were purchased from Sigma Aldrich (Saint Louis, MO, USA). Polyethylene glycols (PEG) were obtained from Rapp Polymere (Tuebingen, Germany; HS-PEG-COOH, molecular weight = 3000 Da) and Jenkem Technology (Allen, Texas, USA; Methoxy PEG-Thiol, molecular weight = 5000 Da). Ethyl dimethylaminopropyl carbodiimide (EDC) and sulfo-N-hydroxysuccinimide (sulfo-NHS) were purchased from ProteoChem (Cheyenne, Wyoming, USA). Anhydrous ethyl alcohol was purchased from Commercial Alcohols (Brampton, Ontario, Canada); HPLC grade methanol from Caledon Laboratory Chemicals (Caledon Laboratories Ltd., Georgetown (Halton Hills), Ontario, Canada). Biotinylated tri(ethylene glycol) undecanethiol was purchased from Whitesides Monothiol (Nanoscience Instruments, Inc., Phoenix, AZ, USA).

Synthesis of Au NPs. Colloidal gold nanoparticles were synthesized by the reduction of HAuCl₄ with sodium citrate in a glass beaker.⁶²,⁶⁶−⁶⁸ To begin, 1 mL of a 1% solution of HAuCl₄·3H₂O was added to 99 mL of UP-H₂O, and the resulting solution was brought to a boil while stirring. Once boiling, 1 mL of a 1% trisodium citrate dihydrate solution was added dropwise to the mixture. After boiling for another 5 min, the heat was turned off, and 1 mL of a 5% sodium azide solution was added dropwise to the beaker. The mixture continued to stir as it was allowed to cool for about 30 min, after which point it could be stored in the refrigerator.

Preparation of SERS Probes. SERS probes were produced by modifying the NPs of the preceding section according to an amended version of the method described by Qian et al.¹⁷ The nanoparticles were first coated with NBA dye, followed by a PEG shell to which a targeting molecule was attached (see the schematic in Figure 1a). The PEG coating acts to reduce the nonspecific binding of the SERS probes to sites other than the target.

To begin, 250–300 μL of a 5 μM NBA solution was slowly added, dropwise, to 2 mL of a vigorously stirring NP suspension. The dye was allowed to mix with the NPs for 15 min, after which time 320 μL of a mixed PEG solution was added to the rapidly stirring mixture. The mixed PEG solution
was comprised of 0.5 mL of a 200 μM solution of HS-PEG-COOH (MW = 3000 Da) and 0.25 mL of a 200 μM methoxyl PEG-Thiol solution (PEG-SH, MW = 5000 Da), diluted to 1 mL with UP-H2O. The heterofunctional linker HS-PEG-COOH was included to attach to the streptavidin (targeting molecule), while the PEG-SH was to coat any areas of the gold NPs not covered by the linker.

After stirring for 20 min, the NP/NBA/PEG suspension was centrifuged twice at 13 000 rpm for 7 min each time and resuspended in 2 mL of UP-H2O. To activate the COOH groups on the NP surfaces, 40 μL of a 20 mg/mL solution of EDC was added, rapidly followed by 40 μL of a 55 mg/mL solution of sulfo-NHS. Following 40 min of stirring, 0.5 mL of a PBS buffer containing 1% Tween20 (PBST) was added to the NP mixture to improve its solubility, and the suspension was stirred for a further 5 min. Excess reagent was then removed by two rounds of centrifugation (13 000 rpm for 7 min) and resuspension in 2 mL of PBST. At this point, 140 μL of a 0.5 mg/mL streptavidin solution was added to the nanoparticle suspension, resulting in a protein concentration of approximately 35 μg/mL. The mixture was left to react overnight in the refrigerator at 4 °C.

After 24 h, 0.5 mL of PBST was added to the suspension before centrifuging (13 000 rpm for 7 min) and resuspending in 2 mL of a PBS buffer with 0.1% Tween20 and 100 μg/mL BSA. The purpose of the BSA was to cap any unreacted activated COOH groups. After reacting for 20 min, the solution was centrifuged once more and resuspended in 2 mL of PBST.

Characterization of the SERS Probes. Particle characterization is vital to guarantee that samples from different batches used in the experiments had comparable physical and chemical properties. In fact, several characterization techniques must be employed in this type of research to also ensure that enough information about the samples are available for eventual assessment of the results by other laboratories. The SERS probes were characterized at diverse stages during the synthesis by dynamic light scattering (DLS), ultraviolet/visible (UV/vis), and surface enhanced Raman spectroscopy (SERS). These measurements were performed at four stages during the probe production: on unmodified Au NPs, NPs after the addition of NBA and PEG, NPs with NBA/PEG/streptavidin, and NPs with NBA/PEG/streptavidin/BSA. Results of the characterization process are available as Supporting Information.

Particle diameter was measured by DLS using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY). The DLS technique yields the hydrodynamic diameter, which included contributions from molecules attached to the NP surface, so it was possible to observe increases in the effective probe diameter as the NBA, PEG, and BSA were attached to the NP core.

The stability of the NPs during synthesis was monitored by UV/vis spectroscopy, performed on the probe sample at each stage, using a Varian Cary 50 scan spectrophotometer (Agilent Technologies Canada Inc., Mississauga, Ontario). Unmodified NPs exhibited a characteristic spectral peak at approximately 530 nm. The probes were discarded at any stage if the 530 nm peak presented large red-shifts or broadening, since these are indicative of particle aggregation.

SERS of the probe suspension was carried out in all stages after the addition of the NBA to ensure that the spectral characteristics of the dye, including a strong peak at 600 cm⁻¹, were always present. Raman spectroscopy was performed using an InVia Renishaw microscope (Renishaw Inc., Hoffman Estates, IL) with a 5X dry objective (Leica Microsystems, Wetzlar, Germany) and 1200 lines/mm diffraction grating. A 633 nm Helium Neon Laser (Renishaw plc, Transducer Systems Division, Gloucestershire, UK) was used for sample excitation and was focused on a drop of the SERS probe suspension centered on a glass slide. Spectra were acquired with a 5 s exposure and a laser power of ~1 mW at the sample. Laser power through the objective was measured using a Coherent FieldMax-TOP Laser Power/Energy Meter (Coherent Inc., Portland, Oregon, USA).

Biotinylation of Gold Slides and SERS Probe Incubation. Glass slides coated with 100 nm gold films through a 5 nm chromium (Cr) adhesion layer (EMF Corporation, Ithaca, NY, USA) were chemically modified with biotin in order to provide a surface to which the streptavidin-modified SERS probes could attach. Each slide was first annealed, thoroughly rinsed with acetone and ethanol, and placed in a clean 50 mL glass beaker. A 1 mM solution of biotinylated tri(ethylene glycol) undecane thiol was then prepared in 2 mL of ethanol and added to the beaker. The beaker was sealed with Parafilm M (Bemis Company, Inc., Neenah, WI, USA) as a monolayer self-assembled on the Au surface for 12 h.

The slide was then removed from the biotin solution, rinsed thoroughly with ethanol and UP-H2O, and placed in a sealed
beaker containing the SERS probe suspension for either 1, 2, 3, or 12 h. After the incubation, the surface was washed thoroughly with PBST and UP-H2O before being left to air-dry.

**SERS Mapping and SEM Imaging.** Raman spectroscopy was performed with the InVia Renishaw microscope using a 100× dry objective (N.A. = 0.9; Leica Microsystems, Wetzlar, Germany), 1200 lines/mm grating, and 633 nm helium neon laser. Maps of three randomly chosen regions were acquired on the gold slide corresponding to each SERS probe incubation time. To ensure rapid scanning without compromising signal-to-noise ratio, Streamline Plus Raman imaging (Renishaw Inc., Hoffman Estates, IL) was used. The resolution of the maps was 1 μm × 0.5 μm, with each point exposed to ∼1 mW of laser power for 3 s. Each map covered an area of about 4000 μm² and took approximately 15 min to complete. Two-dimensional SERS map data was presented with each pixel corresponding to the area under the NBA dye peak at 600 cm⁻¹. The three SERS maps for each slide were also combined and presented as a histogram of SERS intensity. In these histograms, SERS intensity refers to the area of the NBA dye peak at 600 cm⁻¹.

**SEM Imaging.** A Hitachi S-4800 field emission scanning electron microscope (FESEM; Hitachi High-Technologies Canada, Inc., Toronto, Ontario) was used to acquire high resolution, high magnification images of the SERS probes on the surface of the gold slides. All images were obtained using an acceleration voltage of 1.0 kV and a magnification of 20 000×, covering an area of about 30 μm². A sampling of images of the SERS probes was acquired on the slide corresponding to each incubation time. At least five images were taken of each slide, with any differences in the imaged area of the slides accounted for in the analysis.

**Data Analysis and Statistical Approach.** An overview of the statistical approach is provided in Figure 1. A detailed description of each step is provided as Supporting Information. The SERS probes were pegylated gold NPs, coated with SERS-active NBA dye, and functionalized with streptavidin in order to target a biotin monolayer deposited on a gold slide (Figure 1a). Several different incubation times were used to allow for the production of SERS probe clusters of a great variety of sizes, from single probes to clusters as large as 15 probes or more. The clusters were identified and categorized through the analysis of SEM images and the SERS intensities were determined from two-dimensional SERS maps of multiple regions on the slide (Figure 1b). Previous investigations in the correlation between SERS intensities and NP cluster sizes relied on using the same region for imaging (AFM, SEM, TEM, and SEM²) and SERS and a direct, individualized, comparison between SERS intensities and cluster sizes. In contrast, we developed an approach where the correlation between the SERS intensity mappings and the SEM images was based on the distributions of SERS intensities under each laser illuminated spot and the distribution of SERS probe cluster sizes under a similar area. Representative histograms of the SERS probe cluster size and SERS intensity for a given incubation time were generated (Figure 1c), and the two distributions were related through a series of equations describing the total SERS intensity acquired at a given point (Figure 1d). Solutions of the empirical formulas provided a relationship between SERS probe cluster size and the average SERS intensity resulting when the cluster was probed with the Raman microscope (Figure 1e).

### RESULTS

**SERS Mapping and Histograms.** Figure 2 shows the SERS mapping of the gold slides coated with SERS probes for four different incubations times (1–12 h). The SERS probes (illustrated in Figure 1a) included streptavidin proteins that specifically attached to the gold surface decorated with biotin. The SERS mapping at low incubation times (Figure 2a) shows a large spatial variation in SERS intensities, due to the sparse coverage of the gold surface by SERS probes. The intensity maps become more uniform as the incubation time increases (Figure 2, panels b–d). In those cases, the overall SERS probe surface coverage increases, as does the number of particles illuminated in each mapped spot. These observations are corroborated in Figure 3, where the distributions of SERS intensities corresponding to each incubation time are represented as histograms. Each count in the histogram corresponds to the SERS intensity (integrated SERS peak) obtained under a laser-illuminated area during mapping. The histograms were generated by combining the data from three large area (76 × 53 μm²) maps obtained from random positions in each slide, leading to a large sample size of SERS intensities. Zero intensity events (defined by a cutoff) were discarded in the construction of the histograms; hence, the sample sizes varied from approximately 7000 for the 1 hr data to over 23 000 for the 12 h data. Following a common treatment of error on histograms, the measured number of counts in each bin (Nbin) is assumed to be the mean of a Poisson distribution, with the error bars corresponding to ±Nbin^{1/2} (1σ).⁵⁹,⁷⁰

The results for the shortest incubation time (Figure 3a) show a skewed distribution, with a skewness of 10.1 (skewness values can provide a quantitative assessment regarding the symmetry of the distribution⁵⁹), and a high number of low intensity events. These correspond to illuminated areas that contained low efficiency probes that produced a Raman signal near the detection limit of the system. Even at this low coverage regime, however, a small number of events presented a relatively high SERS intensity (∼1000 counts). The distribution clearly spread to higher intensities as the incubation time increased (Figure 3b) and the number of regions with zero-intensity events...
decreased. When the incubation time reached 3 h (Figure 3c), the number of low signal events became small, and a peak clearly develops in the distribution. This result is consistent with a larger coverage and a higher probability of SERS probes being found under each laser illuminated area during mapping. Finally, in Figure 3d, the least skewed distribution, a peak forms at higher intensities than in Figure 3c. The distribution in Figure 3d can be said to be more symmetric than the others (Figure 3, panels a−c), based on its low skewness value, appearing almost Gaussian in shape. This result is consistent with a higher surface coverage of SERS probes and, consequently, a larger probability of several NPs being interrogated by the laser in each particular mapped spot.

**SEM Imaging and Histograms.** Sample SEM images of the gold slide incubated with SERS probes for different times are shown in Figure 4 (SEM images of individual clusters are provided in the Supporting Information). The scale of the images in Figure 4 was chosen so as to provide the largest surface area possible, displaying a large number of clusters while still allowing for the identification of individual probes. Single SERS probes were identified, and a histogram of their pixel areas (using data from all SEM images) was generated and fit with a Gaussian. From the Gaussian, the average size of a single SERS probe was obtained, and using this value it was then possible to produce a histogram, presented in Figure 5, of the SERS probe cluster size distribution for the slides corresponding to each incubation time. The histograms in Figure 5 were obtained using a different number of SEM images for each incubation time. Therefore, the number of clusters was normalized using the imaged area. The histograms in Figure 5 clearly show an increase in the number of SERS probes in the aggregated clusters with the incubation time, although some large clusters, with more than 10 SERS probes, were also observed in the 1 h incubation experiment. Notice that the number of single particles and small clusters (less than five SERS probes) still dominates the distribution even after 12 h. This is a strong indication that the PEG-layer efficiently protects the SERS probes against aggregation; meaning that the
SERS probe adsorption is possibly driven by the specific interaction with the surface, rather than by particle-to-particle attraction.

Our final goal is to correlate the SERS intensity from each illuminated spot obtained from the SERS map histograms (Figure 3) with the number of SERS probe clusters at that spot. In order to achieve that, a new set of histograms, shown in Figure 6 were obtained from the SEM images. In the case of

![Figure 6](image_url)

**Figure 6.** Histograms showing the distribution of illuminated SERS probe clusters per acquisition for the data corresponding to the (a) 1, (b) 2, (c) 3, and (d) 12 h probe incubation times. Error bars correspond to ±1σ(N^{1/2}).

Figure 6, the histograms considered the number of SERS probes clusters illuminated per acquisition (i.e., the number of SERS probe clusters in a 1 × 1 μm² SEM imaged area). As expected, the histograms in Figure 6 shows that each laser-illuminated area is mostly populated with either 0, 1, or 2 SERS probe clusters for the lower incubation time (1 h). On the other hand, most of the mapped SERS intensities were generated by illuminating two to five SERS probe clusters when the incubation was 12 h.

Finally, by sampling the histogram in Figure 3, a SERS intensity from a random 1 × 1 μm² spot is obtained. The probability that the intensity is from a certain number of clusters is generated from Figure 6, and the possible size of these clusters (number of SERS probes per cluster) is revealed by sampling the distribution in Figure 5. Therefore, using the total SERS intensities of Figure 3 and the histograms of Figures 5 and 6, a system of equations was then produced corresponding to the relationship between the SERS intensity and the number of clusters. This approach allowed the use of thousands of sampled values from all histograms, generating a statistically robust result. A more detailed description of this procedure, including examples, is available as Supporting Information.

**Solving Systems of Equations for the SERS Intensity Ratios.** As discussed in the previous section, the SERS intensity at each illuminated area was correlated to the number of SERS probe clusters and their size in that same area. In other words, the total SERS intensity from a particular spot was treated as a linear sum of SERS intensity contributions from each cluster. The individual intensities from each cluster were unknown. Therefore, for each incubation time, the process was repeated to generate enough equations that would allow the unknown SERS intensities, attributed to each SERS probe cluster size, to be evaluated. The system of equations for each SERS data set was then an \( m \times m \) matrix, with \( m \) being the maximum cluster size for a particular set. Due to the fact that very large SERS probe clusters (>15 probes) were rarely detected, particularly for the shorter probe incubation times (see Figure 5), it was decided that the total SERS intensity distributions could be described by only considering SERS probe clusters up to a maximum cutoff value, \( m_{\text{max}} \). For each incubation time, a different value for \( m_{\text{max}} \) was chosen, based on the cluster size distributions of Figure 5.

Starting with the 1 h incubation time, a value of \( m_{\text{max}} = 6 \) was chosen (based on where the histogram begins to approach zero in Figure 5a), and a \( 6 \times 6 \) equation matrix was generated. This procedure is illustrated in Figure 7. The solutions to the \( 6 \times 6 \) matrix provided values for \( I_1 \) to \( I_6 \) corresponding to the contributions to the total SERS intensity from one SERS probe \( (I_1) \) and a cluster containing six SERS probes \( (I_6) \), respectively. To account for variability in these solutions, 500 \( 6 \times 6 \) equation matrices were generated for the 1 h data set and solved, giving 500 independent sets of solutions (Figure 7b). All solutions for \( I_1 \) to \( I_6 \) were then averaged and the uncertainties on the mean values were calculated (Figure 7c). To provide an idea of the variability in the values of these intensities, seven independent calculations of \( I_1 \) to \( I_6 \) for the 1 h incubation experiment have been overlaid in Figure 8. In Figure 8, the solution for \( I_6 \) is seen to have a high uncertainty compared to the first five SERS intensity solutions due to the fact that it contains contributions from the relatively small number of NP clusters larger than six that form after a 1 h incubation. As a result, \( I_6 \) was recalculated from the equations for the 2 h incubation, while \( I_1 \cdots I_6 \) were treated as known values.

For the 2 h incubation, a system of equations was generated with an \( m_{\text{max}} \) set to be equal to nine (based on Figure 5b), and using the intensity solutions for clusters of one to five SERS probes as known values, solutions for clusters up to nine probes large were found (Figure 7d). The process was again repeated 500 times, as discussed in Figure 7e. The 3 h probe incubation data was then similarly used to find intensity solutions for clusters up to 12 probes large (this time with \( I_1 \) to \( I_9 \) as known values), and the 12 h probe incubation data was used to find solutions for SERS intensities produced by clusters up to 15 probes large (with \( I_1 \) to \( I_{15} \) as known values). The final results of the calculations are found in Figure 9, where the average SERS intensities normalized to the intensity due to a single probe \( (I_1/I_1) \) are plotted against the number of SERS probes per cluster.

High reproducibility of the calculated SERS intensities of Figure 9 is suggested by the strong agreement (\( p < 0.05 \)) between the seven sets of independent calculations of the SERS intensities for the first six cluster sizes shown in Figure 8.
When using NPs as a substrate in SERS, the specific enhancement to the Raman signal of the sample provided by the NPs can be highly variable and difficult to control. In general, it is known that an irregular surface allows for the greatest enhancement and that a collection of NPs provides greater enhancement than a single NP. Studies that observe the change in the SERS intensity as a function of random NP aggregation have shown an increase in intensity with aggregation, and the polynomial relationship shown in Figure 9 supports this idea. However, the gradual increase in SERS intensity with SERS probe cluster size is somewhat unexpected considering that studies of multiple depositions of NP monolayers show a more rapid increase in average SERS intensity as the number of layers (and thus NP aggregation) increases, eventually reaching a plateau. Similarly, studies of individual NP clusters found a very sharp increase in intensity (as much as 4 orders of magnitude) between single and dimer NPs. Although the results recently reported by Pazos-Peres et al. showed a smaller enhancement of the dimer relative to the single particle, the importance of interparticle spacing when it comes to SERS enhancement was also evident. However, an increase in the cluster size beyond the dimer does not necessarily result in a much larger increase in SERS efficiency. In fact, Wustholz et al. suggest that a pair of NPs with a particular spacing can produce a “hot spot” which can potentially result in a SERS enhancement as large as that provided by bigger clusters.

The slowly rising curve in Figure 9 seems to suggest that the type of “hot spot” that would result in a 4 orders of magnitude increase in SERS intensity between a single and dimer SERS probe was not observed in this work or at least did not occur.
frequently enough to influence the average SERS intensities for each cluster size. Likely, this is due to the fact that the distance between the Au-NP cores of the SERS probes used to generate Figure 9 was controlled by the PEG coating. The pegylation of the Au-NPs and their functionalization with streptavidin to form the SERS probes provided a relatively “thick” coating that limited the distance of the closest approach between the Au-NP cores which was not present on the Au-NPs used in the work of Wustholz et al.\textsuperscript{53} Due to this fundamental difference, we cannot expect that our work will compare directly with that of Wustholz et al.\textsuperscript{53} However, the systematic attempt to correlate SERS intensities to NPs cluster sizes performed by Wustholz et al.\textsuperscript{53} does mirror the study described here. In our case, the molecular coating provided by the PEG seems to have prevented the conditions necessary for the formation of the type of “hot-spots” formed in between the NPs that provided such significant rises in SERS intensity\textsuperscript{53,57} and likely had an effect on the rapid increase in SERS intensity found in the studies of multiple depositions of NP monolayers\textsuperscript{62–64} and of nanorod aggregation.\textsuperscript{65} Indeed, in high magnification SEM images of clusters of the SERS probes (as can be seen in the Supporting Information), there is a clear separation of 10–20 nm between many of the probes within the clusters. It is possible that the separation between the NPs in the SERS probe clusters causes the probes to provide surface-enhancement somewhat independently of each other, and the intensity due to a cluster is the cumulative result of the intensity due to each probe in the cluster. However, this would suggest a linear relationship, which is not what is seen in Figure 9. While the exact reason for the nonlinear nature of the relationship in Figure 9 is unclear, it does imply that there may be some degree of “hot-spot” interaction between the probes in the cluster. Similarly, it is possible that the SERS intensity produced by the clusters is affected by a relationship between the gold substrate and the SERS probes, whether that be due to a “hot-spot” interaction, or simply reflection of Raman scattered photons toward the objective that would otherwise have been lost. Nonetheless, the polynomial relationship between SERS probe clusters and their resultant SERS intensity is extremely well-defined ($R^2 = 0.99812$) and reproducible (see Figure 8), and a nonlinear component to the relationship is entirely possible.

Given confidence in the reproducibility and accuracy of the results shown in Figure 9, the polynomial relationship that the figure displays will prove to be useful in experiments that use PEG NPs as SERS probes. The exhibited relationship between cluster size and the resultant SERS intensity is unique to the PEG NP probes described in this work, and these probes, based on those in the paper by Qian et al.\textsuperscript{17} are specifically designed for targeted SERS imaging, such as in the identification of proteins within cells and tissues. As discussed in the introduction, determining the quantity and distribution of the target within the SERS maps of a sample remains challenging. However, considering the simple relationship between cluster size and SERS intensity shown in Figure 9, PEG NP SERS probes seem ideally suited for quantifiable SERS mapping. For example, using a calibration curve similar to Figure 9, the SERS intensity at a given point would be known to be a multiple of the intensity due to an individual SERS probe. It would then be simple to determine the number of clusters contributing to the total SERS intensity at the given point and thus the number of targeted sites could be determined at that location.

**CONCLUSION**

This work provides a novel technique that relates 2D SERS intensity maps to SEM images of SERS probes on a biotinylated gold surface, allowing for the calculation of SERS intensities due to SERS probe clusters of varying size. In order to examine a variety of cluster sizes, targeted SERS probes were incubated with biotinylated surfaces for several time periods prior to the acquisition of SEM and SERS images. Using the SEM images and SERS maps, statistical descriptions of both the SERS probe cluster distribution and SERS intensities on the surface were developed. Based on the statistical relationships, systems of equations for the total SERS intensity measured at a given point were devised. Solutions to the equations provided the SERS intensities resulting from enhancement due to clusters from 1 to 14 SERS probes in size.

A simple polynomial relationship between SERS probe cluster size and the resultant SERS intensity was found, suggesting the utility of pegylated SERS probes in targeted SERS mapping. By simply controlling the probe incubation time to limit the distribution of cluster sizes formed in a targeted SERS sample, quantification of targeted sites within a sample would be relatively straightforward. The ability to quantify targeted sites in a sample would make targeted SERS mapping a viable technique for imaging protein distribution within cells and tissues, providing a quantifiable, high-resolution alternative to methods such as immunohistochemistry.

**ASSOCIATED CONTENT**

Supporting Information

Information regarding the characterization of the SERS probes; high magnification SEM images of individual probe clusters; a detailed description of the data analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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