Correlative SEM SERS for quantitative analysis of dimer nanoparticles†

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A Raman microscope integrated with a scanning electron microscope was used to investigate plasmonic structures by correlative SEM-SERS analysis. The integrated Raman-SEM microscope combines high-resolution electron microscopy information with SERS signal enhancement from selected nanostructures with adsorbed Raman reporter molecules. Correlative analysis is performed for dimers of two gold nanoparticles. Dimers were selected on the basis of the SEM images from multi aggregate samples. The effect of the orientation of the dimer with respect to the polarization state of the laser light and the effect of the particle gap size on the Raman signal intensity is observed. Additionally, calculations are performed to simulate the electric near field enhancement. These simulations are based on the morphologies observed by electron microscopy. In this way the experiments are compared with the enhancement factor calculated with near field simulations and are subsequently used to quantify the SERS enhancement factor. Large differences between experimentally observed and calculated enhancement factors are regularly detected, a phenomenon caused by nanoscale differences between the real and ‘simplified’ simulated structures. Quantitative SERS experiments reveal the structure induced enhancement factor, ranging from ~200 to ~20,000, averaged over the full nanostructure surface. The results demonstrate correlative Raman-SEM microscopy for the quantitative analysis of plasmonic particles and structures, thus enabling a new analytical method in the field of SERS and plasmonics.

Introduction

Noble metallic nanoparticles (nps) have attracted general interest due to their specific interaction with light. This interaction excites surface plasmons in the metal–dielectric interface. In nanostructures an intense focusing of light occurs with a high energy density that is far below the optical diffraction limit. Nano-plasmonics has been applied for nano-antennas,1 waveguides,2 and in sensing applications.3–6 Plasmonics is applied in Raman spectroscopy to obtain high signal enhancements of molecules in the near field, resulting in surface enhanced Raman spectroscopy (SERS). Low numbers of molecules can thus be detected with the specificity of Raman spectroscopy. The SERS enhancement factor (EF) depends strongly on the geometry of the metallic nanostructure, and EF up to $10^9$–$10^{10}$ have been reported in hot spot locations.7–9 The EF, averaged over the structure surface, is much lower, namely in the range of $10^4$–$10^6$.10 With a local EF of $10^7$–$10^8$ and higher single molecule, Raman spectroscopy has been performed.9,11 The most common materials used for SERS experiments are gold and silver. These materials give a field enhancement that is most prominent in the visible and near infrared spectrum.

Plasmonic dimers are of specific interest as a simple composite nanoparticle system. The interest in dimers arises from the high obtainable EF in the gap between the two particles, which we will address as the dimer gap. The field enhancement in the hot spot increases strongly with decreasing gap size and the gap size can be tuned by applying spacer molecules between the metallic nanoparticles.12–14 Multiple dimer particle systems have been composed, such as 2 spheres with and without separation by spacer molecules, 2 rod-shaped particles,12,15,16 and metallic tips and a gold (or metal) surface.17,18 Dimer structures are fabricated by deposition of nanoparticles or through chemical synthesis of coupled particles or through milling structures with a focused ion beam (FIB).19

This paper focuses on correlative scanning electron microscopy (SEM)-SERS analysis of plasmonic gold nanoparticles and particularly on particle dimers with a spontaneously formed gap size which result from sparse aggregates deposited on a surface. The orientation of the dimer particle with respect to the linear polarization of the incoming laser field is also investigated. The goal of the investigation is to

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Experimental methods

Near field simulations

The electric near field enhancement of plasmonic structures depends strongly on the structure shape, size, composition, and the excitation wavelength. The field enhancement of a dimer compared to a monomer is presented in Fig. 1. Fig. 1A shows the near field distribution of a gold monomer nanoparticle, Fig. 1B shows the near field for a dimer with the excitation light along the dimer axis, Fig. 1C shows the same dimer situation with the excitation light orthogonal to the dimer axis. The simulations are used to obtain the near field enhancement distribution on the nanostructure surface. Using the power 4 approximation the surface averaged EF is obtained from the simulation and reported in the figure caption. As can be observed, the EF is a strong function of the position on the particle. From the calculation, the maximum EF, which coincides with the dimer hot spot, and also the average EF over the nanostructure surface are obtained. The structure averaged EF together with the number of adsorbed molecules determines the measured SERS signal intensity. The gold particles used in this research are 60 nm in diameter, the Raman excitation wavelength is 783 nm, and the gold refractive index is obtained from ref. 23. A substrate has been included in the simulations, and to make the simulations comparable with the experimental situation, approximate values for the refractive index of the steel substrate are used at 785 nm wavelength $n = 2.9 + 3.3i$.

Simulations of the electric near field distribution of experimentally observed structures are performed. As a reporter molecule for the field enhancement, the organic dye molecule rhodamine 6g (R6g) is adsorbed on the gold surface. The EF is averaged over the structure surface and follows a fourth power law of the local electric field.

$$\text{EF} = \frac{1}{S} \left( \frac{|E(\omega_{\text{laser}})|^4}{|E_0(\omega_{\text{laser}})|} \right) \text{dS}$$

(1)

The change in the dielectric constant at the frequency of the field at the Raman shift was not taken into account. The calculated structure averaged EF is obtained to compare the simulated EF with the measured SERS intensity. The simulations are performed with the metallic nanoparticle boundary element method (MNPBEM) toolbox. The program functions as a toolbox for MATLAB, which is useful for direct presentation of the data. MNPBEM solves Maxwell’s equations for a dielectric environment where bodies are separated by sharp interfaces. This approximation holds true for the experiments performed in this project, the gold nanoparticles are described with their dielectric function and are placed in a dielectric environment such as vacuum, air or water. The potential-based BEM approach performs calculations based on structures defined by surface elements, instead of volume elements. This property decreases computation time which is important when many structures are modelled. A comparison of MNPBEM based calculations with MIE-theory and discrete dipole approximation (DDA) based simulations has been reported.

The purpose of the simulations is to calculate the field and enhancement of observed dimer structures. The two parameters of interest are the gap size and orientation of the dimer to the excitation polarization. Results of these simulations are shown in Fig. 2. Fig. 2A shows the EF averaged over the dimer structure in a logarithmic color representation. The enhancement factor has been calculated for different gap sizes (horizontal axis) and excitation polarization (vertical axis), ranging from 0.5 nm to 15 nm and 0 to $\pi/2$ radians respectively. Fig. 2B shows the polarization kept at 0 radians and the gap size influence is shown logarithmically. Fig. 2C shows the polarization influence with the gap size at 1 nm. The calculations show the
**Microscopy and data analysis**

A confocal Raman microscope integrated with a dual-beam FIB-SEM is utilized for correlative Raman-electron microscopy. The Raman microscope ($\lambda_{sca} = 785$ nm) is bolted onto a port of the vacuum chamber of the dual-beam system. No additional foot space was needed for the hybrid microscope system, the optical objective (NA = 0.65) was specially designed for application in a vacuum and the optical materials of the objective were selected for negligible background interference and therefore an optimal signal-to-noise ratio of Raman scattered light. The objective is positioned adjacent and parallel to the electron microscope column. Correlative imaging is performed by moving the sample between the SEM and Raman position. This calibrated movement is performed with the FIB-SEM stage. The coordinates of any chosen region of interest (ROI) are thus preserved. The integration of the Raman microscope with the FIB-SEM system causes no limitations to the operation of the FIB-SEM. This has been extensively tested on multiple different samples.\(^{20}\) The integrated Raman-FIB-SEM system enables *in situ*, high resolution imaging of plasmonic structures combined with SERS to probe local field enhancements.

Sparse distributions of self-assembled particle aggregates were deposited on a substrate from a suitably diluted solution of 60 nm gold nanoparticles. The particle density of the sample is verified by electron microscopy. A large number of dimer aggregates were spontaneously formed and sufficiently well-separated on the substrate to enable Raman amplitude measurements of individual objects, in agreement with the resolution of the far-field Raman microscope. In order to acquire data from a sufficiently large number of dimer structures, Raman microscopy images were prepared from an area of more than 2000 $\mu$m$^2$ in an array of $\sim$25 000 spectra. Both SEM and SERS images reveal the locations of particle clusters. Dimer particles can only be selected with certainty from the SEM image. The dimer particles were subsequently measured by high resolution electron microscopy to measure the gap size with the highest precision. Application of SEM after the Raman measurements avoids contamination of the nanoparticle clusters by electron beam induced deposition\(^{26}\) and prevents the potential destruction of Raman reporter molecules adhered to the gold nanoparticles. The electron microscope is operated with 15 kV electron energy in immersion imaging mode, in which the through-the-lens (TTL) detector is used for secondary electron (SE) detection. The SEM resolution for this detection mode is specified at 1.1 nm. The Raman experiments are performed with 500 $\mu$W excitation power, an integration time per pixel of 1 second and an image step size of 300 nm in $x$- and $y$-direction. The Raman microscope has a lateral resolution of 850 nm, and the excitation polarization is oriented along the $y$-axis as indicated in Fig. 3.

The correlative analysis of the ROI is shown in Fig. 3. Fig. 3A shows an overlay image of Raman and SEM data. The SEM image was obtained with a magnification of 1500×. The Raman data is represented with a logarithmic colour scale, in which the signal intensity corresponds to the fitting parameter of a linear least squares fit of the recorded spectra with the known R6g SERS spectrum. The scale of this printed image is too coarse to observe the nanoparticles. A part of the image, indicated in the white rectangle, is therefore enlarged to show the position of the nanoparticles in the SEM recording (Fig. 3B) in the blue colour scale for increased contrast. The selected dimer is imaged with SEM at a higher magnification (Fig. 3B) in the blue colour scale for increased contrast. The selected dimer is imaged with SEM at a higher magnification of 250 000×, as shown in Fig. 3C. From the high resolution SEM image the dimer orientation, particle diameters, and gap size are obtained. For the dimer in Fig. 3C the angle, with respect to the light polarization is 40°, the diameters of the nanoparticles are both 60 nm and the gap size is $6 \pm 1.1$ nm. The measurement of the gap size between the two particles is taken from a line cross section along the dimer axis, as indicated in the SEM image (Fig. 3C). The cross section intensity is presented in Fig. 3D.

The measured hyperspectral Raman data sets were background corrected and filtered by singular value decomposition (SVD).\(^{27}\) The spectra contain the SERS signal and some fluorescence, which originates from the fluorescence enhanced signal from the R6g molecules and from the gold nanoparticles.\(^{28}\) The SERS signal was isolated using a filter with a smoother function specifically designed to separate background fluorescence and Raman spectra.\(^{29}\) A pretreated
Raman spectrum, averaged over 9 pixels, selected in Fig. 3B, is presented in Fig. 3E. Fig. 3 exemplifies the large range in scale during data acquisition. Fig. 3A covers an image range of $50 \times 45 \mu m^2$, whereas Fig. 3C demonstrates nanometer resolution analysis of one of the objects in Fig. 3A.

**Materials and methods**

Gold nanoparticles with a diameter of 60 nm prepared by the citrate reduction method were obtained from Nanopartz™ at 37.7 pM concentration. Rhodamine 6g dye was obtained from Sigma Aldrich (batch: 252433-250MG) in solid form. SERS labelling of nanoparticles was performed by dissolving R6g in ethanol with a concentration of 8 μM. The mixing ratio of gold nanoparticle solution and R6G solution was 7 : 1. The dye and nanoparticle concentrations were verified by UV-Visible absorption spectroscopy before and after mixing of the compounds, and the absorption spectra are presented in the ESI (Fig. 1†). The R6G covered nanoparticles were deposited and dried on a substrate directly before placing them in the correlative light-electron microscope. Raman silent and conducting μ-Rim slides (BioTools Inc.) were used as substrates. The electron microscope is a dual-beam NOV A600 NanoLab from FEI, and the Raman microscope, fabricated by HybriScan Technologies B.V., employs a 785 nm excitation laser.

The laser power at the surface is 0.5 mW. The stability of the SERS signal from nps under laser irradiation was verified by recording the Raman signal of dimer structures for 20 seconds under constant illumination. A constant R6g SERS spectrum is observed for all spectra during the entire measurement time, as shown in the ESI (Fig. 2†). The R6g SERS spectrum is spectrally very similar to the spontaneous Raman R6g spectrum indicating little chemical interaction between the gold nanoparticle and the dye molecules, as shown in the ESI (Fig. 3†). It has been observed that larger plasmonic structures, consisting of more than 10 particles, become damaged during the Raman experiment. This is observed as a sudden change in the SERS signal of the large clusters during Raman microscopy, something which can also be seen in Fig. 3A.

**Quantitative SERS**

The combination of in situ, correlated SEM imaging, Raman spectroscopy, and near field simulations enables a systematic
approach to investigate the EF, but a more quantitative interpretation based directly on the Raman measurement is desirable. For analysis of the structure averaged EF a thorough understanding of the sample and microscope is required. The unenhanced Raman signal is determined from the R6g Raman scattering cross section and the number of adsorbed molecules on the plasmonic structure. Further a calibration of the microscope signal collection efficiency is performed to calculate the SERS signal intensity from the measured signal. With the knowledge of these contributing factors the average SERS EF is calculated as the ratio of the SERS enhanced intensity over the calculated unenhanced Raman intensity.

$$\text{EF} = \frac{I_{\text{SERS}}}{I_{\text{RS}} - I_{\text{NRS}}}$$

This reveals the EF averaged over all reporter molecules present on the plasmonic structure. This enhancement can then be compared with the surface average EF calculated with near field simulations.

The R6g Raman scatter cross section is measured using an internal reference standard method. A solution of R6g in ethanol was prepared and 5 volume percent toluene was added as an internal reference. The concentration of R6g in solution is measured by UV-Vis absorption spectroscopy. The Raman cross section for the Raman band at 998 cm⁻¹ of toluene is known, namely, \(\sigma_g = 3.53 \times 10^{-30} \text{ cm}^2 \text{ sr}^{-1}\) at 632.8 nm excitation. The cross section has been corrected for the excitation/scattering wavelength dependence and the dispersion of the polarizability for a Raman excitation wavelength of 785 nm to \(\sigma_R = 1.62 \times 10^{-30} \text{ cm}^2 \text{ sr}^{-1}\). The measured Raman scattering cross section of prominent R6g Raman bands is presented in ESI Table 1. The Raman cross sections of R6g in ethanol have been corrected for the internal field factor term to compare with the situation in a vacuum. The internal field factor is unrelated to the SERS electromagnetic contribution, but takes into account the effect of the field concentration in the medium. The formulae are provided in the ESI†. The change of the internal field factor from ethanol to vacuum results in a change of the Raman scattering cross section change with a factor of 0.36.

The number of R6g adsorbed molecules has been directly measured by fluorescence spectroscopy in the following way. An R6g solution was made with a known concentration, and measured by UV-Vis absorption spectroscopy. Gold nanoparticles were added and the concentration measured by absorption spectroscopy. The solution was subsequently centrifuged at 10g for 120 minutes to obtain a supernatant without nanoparticles, absorption spectroscopy of the supernatant is applied to verify complete removal of the gold nanoparticles from the solution. The fluorescence intensity was measured from the original R6g solution (before addition of nanoparticles) and the supernatant of the Au-R6g suspension (after addition and centrifugation of nps). The adding and centrifugation of nanoparticles result in a removal of R6g molecules that have adsorbed to the nanoparticle surface. The intensity ratio of the fluorescence amplitude of R6g from the two solutions determines the number of adsorbed R6g molecules according to eqn (3).

$$c_{\text{ad}} = c_{\text{Au-R6g}} - c_{\text{R6g}} \left( \frac{I_{\text{Au-R6g}}}{I_{\text{R6g}}} \right)$$

where \(c_{\text{R6g}}\) is the concentration of dye in solution before Au addition with fluorescence intensity \(I_{\text{R6g}}\), \(c_{\text{Au-R6g}}\) is the concentration of the dye in solution before centrifugation, this concentration is slightly different from \(c_{\text{R6g}}\) due to the small volume of Au-nps suspension added to the solution. \(I_{\text{Au-R6g}}\) is the fluorescence intensity of the supernatant after centrifugation. This procedure results in \(3.3 \times 10^4\) R6G dye molecules per nanoparticle, or 2.9 molecules per nm². Earlier values have been reported of 0.9 and 1.3 molecules per nm² for gold particles and 4.7 molecules per nm² for silver particles.

The spectral conversion factor from detected counts to scattered photons underneath the objective has been performed by using sub-diffraction sized particles of polystyrene and with thin layers of polysulfone, with a known Raman scattering cross section. The sub-diffraction size of the samples was chosen to be comparable to the size of the plasmonic structures. Analysis of the plasmonic systems reveals the SERS EF, averaged over the entire observed dimer structure. It is therefore not possible to directly probe the enhancement factor in the dimer hot spot, although this can be estimated from the SEM observed gap size and that the SERS signal predominantly originates from the hot spot. Accurate analysis of the enhancement from observed structures is achieved. The measured enhancements are in good agreement with near field simulations.

**Results and discussion**

The SERS signal was measured for many dimers in conjunction with high resolution SEM measurements of the nanoparticle size, orientation and gap size of the dimer structures. Correlative analysis of several structures is demonstrated in Fig. 4. High resolution electron microscopy images in Fig. 4 (A, D and G) provide the structure morphology parameters. Measured gaps in the dimers are 4 nm, 6 nm and 6 nm respectively. The gap size was determined from the dimers by a procedure presented in the ESI†. Fig. 4 further shows the SERS spectra recorded from the same structures (Fig. 4B, E and H) and the corresponding simulations of the near field enhancement (Fig. 4C, F and I).

The experimental and theoretical SEM–Raman analysis confirms the general notion of the electromagnetic enhancement in Raman light scattering. One observation made by electron microscopy is the faceted shape of the nanocrystals. The presence of facets increases the field enhancement with respect to the calculated enhancement for perfectly spherical particles. The random presence of ridges, the sharpness of ridges and the orientation of ridges with respect to the dimer axis all modify the actual enhancement factor. This may
explain the difference in signal enhancement observed for the three dimers presented in Fig. 4. The effect of the gap shape has been investigated in other work, where the dark field spectroscopy is applied for the analysis of gaps with a parallel or anti-parallel alignment of the facets from the individual nanospheres. For dimers with the same gap distance but different orientation of facets a higher local field enhancement is shown for gaps without parallel alignment of facets, however a larger gap volume is achieved for parallel alignment increasing the number of molecules in the hot spot.

A comparison of the amplitude of the SERS signals with the simulated EF from all observed dimers was made. The simulation predicts a prominent decrease in signal when the gap size increases. The gap size is a well-known property of np aggregates that has a dominant influence on the signal strength. A comparison between the measured and simulated SERS enhancement as a function of gap size is shown in Fig. 5. The presentation of the enhancement factor as a function of the gap size requires a correction of the signal for the variation in dimer orientation with respect to the polarization direction of the incident field. The amplitude of the SERS enhancements in Fig. 5 corresponds to a dimer orientation parallel to the light polarization for np's with a diameter of 60 nm. The original data, without the correction for the orientation, is presented in Table 2 in the ESI.

A comparison of the simulated and measured enhancement factor, shown in Fig. 5, reveals only an approximate agreement between the gap size and the measured signal strength. Although the general trend matches reasonably well a precise agreement is absent. The error bars in Fig. 5 indicate an uncertainty in the measurement of the gap size, originating from the finite SEM resolution. The decrease in penetration of the electron beam in small gaps in between particles that compose a dimer places an uncertainty on the accuracy of the gap size measurements, especially for gap sizes of 3 nm and smaller. The strong influence on the local field enhancement by the gap size therefore results in an uncertainty in the expected enhancement factor. The signal enhancement varies strongly with nanometer small gap sizes and even small errors in the measured gap will strongly affect the simulated EF, which is reflected in Fig. 5.

It can be noted in Fig. 5 that the experimentally measured EF is on average lower compared to the EF determined from near field calculations, the logarithmic average for all measured structures equals a ratio of 1.5. Such a difference in observed enhancement can be attributed to morphology variations of the nanocrystals (vide supra), but potentially also to a chemical contribution to the SERS enhancement. The precise knowledge of the structure could help in future research of the interpretation of the chemical and electromagnetic contribution to the SERS EF. We conclude that the electro-magnetic contribution to the EF can be simulated for an ideal particle dimer and the comparison with the experimental results provides an approximation for the chemical EF, the approximation can be improved by inclusion of a more detailed description of the morphology of the nanoparticles that together compose the dimer particle.

Conclusions
An integrated correlative Raman-scanning electron microscope has been applied to the analysis of plasmonic structures. The
integrated microscopy system is used to quantify the Raman signal strength of molecules adhered to plasmonic systems. This is demonstrated by correlative analysis of spontaneously formed dimers, but can be applied to any synthetic plasmonic structure. Analysis of the dimer gap resonances is performed by observation of the structure with SEM and probing of the field enhancement with SERS of the adsorbed reporter molecules. Experimental results are combined with simulations of the field enhancement based on observations of the structure with SEM. Electron microscopy correlated with SERS analysis enables the fast identification of dimers, observation of the particle size, dimer gap size, and dimer orientation with respect to the polarization angle.

Quantitative analysis of the SERS enhancement factor is performed with an integrated commercial Raman microscope (HybriScan Technologies B.V.). This enables an accurate analysis of the enhancement factor, which is compared to the EF calculated from near field enhancement simulations. However, also regularly a mismatch with simulations is revealed. This is attributed to roughness in the particle shape, and by the accuracy with which the gap size is observed. Experiments reveal the structure averaged enhancement, which is naturally smaller than the enhancement factor in the dimer hot spot. The observed enhancement ranges from a factor of ~200 to ~20 000 for dimers with a gap size ranging from 2 to 9 nm, and for all observed dimer orientations. This observed enhancement is lower than most reported observations of dimer structures, but it is an expected result based on the excitation wavelength, the use of the structure averaged EF, and the use of spontaneously formed dimers. The project has focused on the analysis of dimer structures specifically, but can be applied for any plasmonic structure. We conclude that correlation SEM Raman analysis enables a new method for quantitative analysis of plasmonic structures providing opportunities for the analysis of plasmonic sensors, and SERS labels suitable for both SEM and SERS microscopy.

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