Turning up the Lights - Fabrication of Brighter SERRS Nanotags

Laura C. Martin, Iain A. Larmour, Karen Faulds and Duncan Graham*

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Brighter SERRS nanotags ideal for improved SERRS imaging were prepared by the controlled addition of electrolyte producing a dimer enriched solution, which was incubated with a Raman reporter before being stabilised by a polyethylene glycol (PEG) shell.

There are many techniques available to follow cellular processes or identify chemical components *in vivo*. One approach is surface enhanced (resonance) Raman spectroscopy (SE(R)RS) coupled with nanotags,[1-4] which provide improved chemical information compared to fluorescence spectroscopy and are ideal for multiplexing.[5-7] Nanotags, are formed by adsorbing a SE(R)RS active molecule (a Raman reporter), usually a dye molecule, onto the surface of a metallic nanoparticle, which is then encapsulated in a polymer shell. The presence of the polymer shell ensures the nanotags are stable when placed into the high salt environments found *in vivo*, a situation that usually leads to uncontrolled aggregation of the nanoparticles and subsequently increased variability within the recorded signal. Solutions of nanotags have previously been prepared but they consist of functionalised individual nanoparticles which limits the maximum achievable signal.[2] SERRS signals are significantly increased when silver is used rather than gold and when individual nanoparticles, are coupled together to form agggregates.[8-11] However, it is difficult to prepare small clusters, ideally dimers, in solution. Here we show how to produce dimer enriched suspensions of nanotags which have massively increased signal compared to single nanoparticles and are stabilised to harsh chemical and biological conditions through shelling with PEG.

Dimers and small clusters of nanoparticles have been prepared by the use of multi-dentate ligands[12, 13] and DNA hybridisation,[14] but these result in the molecules linking the nanoparticles being present in the 'hot spot' of the dimer. Therefore, the linker must also be the Raman reporter which increases the complexity of the system. A 2-dimensional approach using a meniscus force deposition technique and a nanofabricated template has been reported[15] but there are scalability issues when compared to solution based methods. Etching of single crystal nanoparticle cubes led to dimer formation. However this process was carried out in the presence of poly(vinyl pyrrolidone) (PVP) and thiol molecules were required to displace the PVP and form a Raman active layer, therefore limiting the availability of Raman reporter molecules.[16, 17] Chen *et al.* have reported a method to form dimers with an amphiphilic diblock copolymer coating.[18, 19] However, this method relies on the alteration of the hydrophobicity of the nanoparticle surface and requires reaction at elevated temperatures for hours. This process also resulted in the formation of empty polymer particles which had to be purified by centrifugation.

Here we report a method that creates a solution enriched with silver nanoparticle dimers, which was carried out at standard temperature and pressure.

Electrolyte addition to a colloidal solution causes aggregation and ultimately flocculation. However, careful control of the amount of electrolyte added can lead to a reduction in the Coulombic repulsion barrier (V_{max}) without its complete removal. This leads to a solution that is enriched with dimers and other small clusters. Meyer *et al.* used 20 mM KCl to lower V_{max} into a range of $0 < V_{max} < 15\ k_BT$, creating a solution enriched in small clusters suitable for single molecule detection by SERRS.[20, 21] However, the authors estimated that only one-third of the colloids had aggregated. Therefore, an important aspect of the work reported here is to quantify the partial aggregation which occurred upon electrolyte addition.

Silver colloid prepared by a modified Lee and Meisel preparation[22, 23] was used with a concentration of ~ 0.2 nM and average size of 46 ± 2 nm, as determined by scanning electron microscopy (SEM). To prepare the samples for SEM analysis, a range of samples (Table 1) were placed on 3-aminopropyltrimethoxysilane (APTMS) modified silicon wafers for one minute. This ensured the colloid was attracted to the wafer surface but there was no additional aggregation caused by the surface. This was confirmed by a deposition time series (supplementary information, Fig. S1) where the presence of larger aggregates could be observed from four minutes onwards. Such aggregates were not observed for deposition times of two minutes or less, therefore, a one minute deposition time was conservative but valid.

The partially aggregated nanoparticles were then investigated by SEM and the results are given in Table 1. The results are shown as the nanoparticle weighted percentage populations of each grouping and the control experiment, of colloid only, showed small amounts of dimers, trimers and tetramers. However, upon addition of 20 mM KCl, a significant increase in the amount of dimers along with a slight rise in the amount of trimers and tetramers was observed. There were no significant alterations within the solutions during the first three days after mixing. When the Raman reporter dye was introduced, at a concentration of 200 nM, it did not lead to significant differences in the recorded cluster size populations. There was an insignificant number of larger aggregates (>8 particles) observed within the > 400 clusters/particles that were counted. These results show that approximately a third of the solution existed as either trimers or tetramers, over a third existed as dimers while the final

third remained as monomers.

UV-Vis studies of the colloid after salt addition were carried out and indications of aggregation were absent, i.e. no red-shift or intensity reduction of the plasmon absorption of the native colloid was observed. (Table S1.) An increase in absorption at longer wavelengths with the formation of small aggregates was not observed, however, it has been reported that such a feature may not be observed due to the broad native absorption often observed for metallic colloids.[16]

A stable, dimer enriched, colloid solution was therefore formed and the next stage was the introduction of a reporter molecule. The Raman reporter was chosen to have an absorbance at the same wavelength as the interrogating laser, 632.8 nm in this instance, resulting in further enhancement of the SERS signal by a contribution from the reporter resonance, resulting in SERRS. The reporter molecule chosen was Nile blue, a common biological stain, which has a λ_{max} of 627 nm. A concentration profile was carried out and representative spectra are shown in Figure 1. The intensity of the largest peak, at 591 cm^{-1}, did not increase above a concentration of 400 nM, indicating that a monolayer of reporter had formed on the surface of the nanoparticles, therefore all enhancing spots were populated. As a result of this, a reporter dye concentration of 200 nM, ~2000 dye molecules per nanoparticle was chosen. This sub-monolayer coverage provided space on the colloid surface for the attachment of the encapsulating polymer molecule but still provided a significant SERRS signal from the dimer enriched colloid after 48 hours. The generated signal was found to be stable for weeks (Figure S2),. Table 1 also confirms that this concentration of Raman reporter did not significantly change the cluster percentages recorded, i.e. it did not result in further aggregation of the colloid.

The dimer enriched nanoparticle solution, although stable in a carefully controlled electrolyte solution, was not stable to higher salt environments similar to those found *in vivo*. Further aggregation occurred with the addition of higher salt concentrations. Therefore, it was necessary to protect the partially aggregated nanoparticle solution by forming a protective shell around the small clusters. This was achieved by the addition of a thiol-PEG-5000 solution after the colloid, salt and dye had been mixed for 48 hours, to achieve a stable dimer enriched and therefore SERRS active suspension. The thiol group on the PEG ensured a high affinity of the polymer for the silver nanoparticle surface. The SERRS of control and partially aggregated samples after encapsulation were recorded, Figure 2. It was clear that the partially aggregated sample gave a much improved SERRS response over the control samples, where no aggregation would be expected. This also suggests that the thiol-PEG-5000 did not displace the Raman reporter from the nanoparticles. A slight increase in the SERRS response from the control was observed after PEG addition and washing which was the result of some aggregation during the washing step (Figure S3). However, the SERRS response from the dimer enriched sample was stable during the preparation steps with only small fluctuations immediately after PEG addition and washing, the response returned to that seen before encapsulation (Figure S4).

Figure 3 shows representative SEM images of the control and the dimer enriched nanotag solutions; the encapsulating shell is clearly visible and was measured to be 11.6 ± 3.1 nm. There were no significant alterations in the percentages of nanoparticles involved in each cluster grouping after encapsulating. UV-Vis studies showed that these encapsulated clusters were stable to high salt concentrations of up to 2 M NaCl (Figure S5).

In summary, the solution based preparation of brighter nanotags by the controlled aggregation of silver nanoparticles to create a dimer enriched solution is reported. Incubation of the colloid with a suitable Raman reporter molecule, followed by encapsulation by a thiol-PEG molecule produced a stable, polymer coated nanotag solution. These nanotags offer improved signal intensity and stability and should be ideal for a range of SERRS applications including *in vivo* imaging. Importantly, this approach is applicable to a wide range of Raman reporter molecules and therefore a wide range of nanotags suitable for SERRS multiplexing can be produced.

Notes and references

Centre for Molecular Nanometrology, WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, UK. E-mail: duncan.graham@strath.ac.uk; Tel: +44 141 548 4701

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Sample	Time / Hrs	Monomers	Dimers	Trimers	Tetramers
Colloid + salt	24	21.2	37.4	27.0	14.4
Colloid + salt	48	24.6	36.2	17.0	22.4
Colloid + salt	72	30.5	40.6	18.3	10.8
Colloid + salt + dye	48	37.9	36.4	13.6	12.1
Control	48	70.9	13.7	9.0	6.4

Table 1. Percentage populations of different cluster types of partially aggregated colloid measured at a range of times after mixing. The salt added was 20 mM KCl and the dye was 200 nM final concentration, ~400 particles/clusters were counted from each sample.

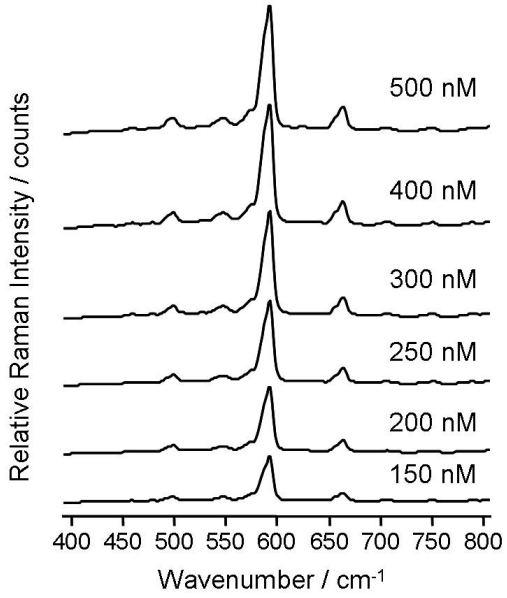

Fig.1. Concentration profile of Nile blue SERRS signal in partially aggregated silver colloid. 20 mM KCl was added to cause partial aggregation, the mixture was left for 48 hours before spectra were accumulated for 60 seconds at 632.8 nm. Spectra have been offset for clarity.

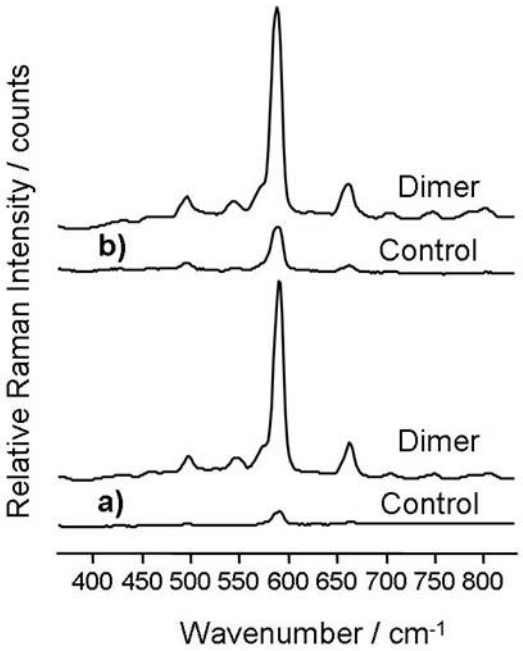

Fig.2. Representative SERRS response of nanotags (a) before and (b) 72 hours after encapsulation. Dimer refers to the partially aggregated colloid mixture and the control was colloid and dye with no added electrolyte. Spectra were recorded for 10 seconds and have been offset for clarity.

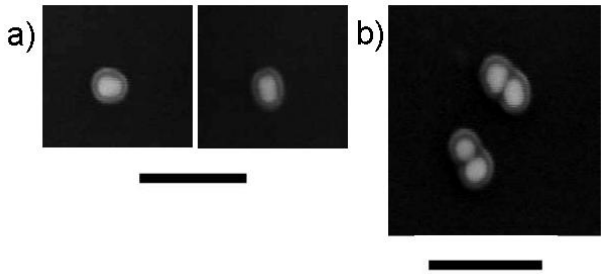

Fig. 3. Representative SEM images of control and dimer enriched nanotags. The scale bars are 200 nm.