# General Discussion



**Dr Aggarwal** opened the discussion of Dr Nie's paper: Upon heating, the individual hot spots appear to be brighter. Could you please, explain the observed increase in intensity of a given hot spot with increasing temperature?

**Dr Nie** replied: Some hot particles appear to be brighter upon heating, but there are also particles with decreased SERS intensities. We believe that an adsorbed molecule could move from one spot to another by thermally activated diffusion on the particle surface. The particle could appear brighter or dimmer depending on whether or not the second hot spot is more enhancing than the first one.

**Professor Natan** asked: Is there a way to distinguish between adsorbate motion and (adsorbate–metal atom) motion?

**Dr Nie** replied: An adsorbed molecule could move by itself on the particle surface, or it could move with an underlying metal atom or a cluster of atoms attached. The latter case becomes especially important on silver and gold surfaces because these metal atoms are known to be fairly mobile. As of now, I have not seen any experimental data or designs to differentiate these two modes of surface diffusion. However, I would expect that a molecule moving from one site to another could show spectral frequency fluctuations because the adsorption energy is likely to be different for different sites. On the other hand, if the molecule moves together with an atomic cluster on the particle surface, it is less likely to show significant frequency changes.

**Dr Foulger** asked: Is reduction in "blinking" with silica coating due to adsorption forces (chemical or van der Waals) between adsorbent and silica, this being an additional "bond" to that between Au and the adsorbent?

**Dr Nie** replied: We have found that silica coating reduces blinking, but does not eliminate it. This reduction is most likely caused by a "steric barrier" introduced by silica deposition, but not an "energy barrier" because the vibrational spectra of the adsorbed molecules do not change after silica coating.

**Professor Smith** asked: Do you know how much chloride is on the surface? I speculate that the NaCl causes a reaction with silver oxide to form a silver chloride layer. At higher temperature and bathed in light this will be a photoreactive surface which could create more active sites. This may account for the increased intensity from spots at higher temperatures.

**Dr Nie** responded: We don't know exactly how much chloride ions are on the particle surface, and photo-activation of hot particles at elevated temperatures could indeed be possible. The observed temperature effect should be further examined using colloidal gold nanoparticles, which are much less sensitive to photochemistry. But at this time, I am not aware of any temperature-dependent blinking SERS studies on gold.

**Dr Aggarwal** asked: I wonder if the increase in intensity of a given hot spot with increasing temperature could be due to an increase in the Raman resonance enhancement factor with temperature?

**Dr Nie** answered: This is unlikely because I have not seen any report that resonance Raman enhancement could increase with temperature. In fact, there is evidence that the resonance enhancement factors often reduce with temperature because the absorption spectra becomes broader and less resolved at higher temperatures.

**Professor Käll** stated: In recent publications[1,2] we have developed a theory that combines classical EM descriptions of the Ag nanoparticles and the EM enhancement mechanism with a quantum mechanical description of the light molecule interaction. These theoretical investigations show that:

(1) An EM enhancement of $\sim 10^{10}$ gives a Raman cross-section of the order $10^{-16}$ cm$^2$, in good agreement with single molecule SERRS of R6G.

(2) The calculated spectra shows a strong background of similar shape and relative intensity as in R6G exp. This background is due to surface-enhanced fluorescence, which is not quenched efficiently if the EM-enhancement is very high ($\sim 10^{10}$).

(3) The anti-Stokes/Stokes ratio is significantly enhanced for relatively low laser irradiance levels ($\sim 1$ mW μm$^{-2}$). This vibrational pumping is due to the continued Raman and fluorescence optical transitions in the molecule.

1 H. Xu, X.-H. Wang, M. P. Persson, H. Q. Xu, M. Käll, and P. Johansson, *Phys. Rev. Lett.*, 2004, **93**, 243002.
2 P. Johansson, H. Xu and M. Käll, *Phys. Rev. B*, 2005, **72**, 035427.

**Dr Nie** responded: Schatz and coworkers have also shown that under certain geometric conditions, the electromagnetic effect could lead to SERS enhancement factors on the order of $10^{10}$. Regarding your second point, however, I think that surface-enhanced electronic Raman scattering (in the form of radiative decay of excited electrons and likely mediated by surface-absorbed molecules) also contributes to the background emission. It cannot be due to unquenched fluorescence alone because similar background signals are also observed with simple molecules such as bipyridine that do not fluoresce in the visible spectrum. Regarding your third point, our experimentally measured anti-Stokes/Stokes ratios indicate vibrational pumping in the ground electronic state or an underlying resonance effect, which is broadly consistent with your results.

**Dr Pettinger** asked: If the background must be attributed to a fluorescence of the same molecule which contributes to SERS, then the experiment tells us the following: The enhanced fluorescence and enhanced Raman signals differ only by about one order of magnitude. That means also their cross-sections differ only by this amount. ($\sigma_{\mathrm{SERRS}} \sim 10^{-16}$ cm$^2$, $\sigma$ enhancement l–10 $\times$ $10^{-16}$ cm$^2$.) The enhancement of fluorescence competes apparently with quenching at the metal surface in such a way that there is not much net gain in fluorescence. But there is a spectacular change of the shape of the background, it is nearly flat. Is this then signalling a reduced lifetime?

**Professor Käll** replied: Yes, the molecular cross-sections for Raman and fluorescence processes could well be of the same order, if the fluorophore is situated at a site of ultra-high field-enhancement (see our recent calculations in Xu *et al.*[1] and Johansson *et al.*[2]

Many SERRS experiments on fluorophores, such as Rhodamine 6G, show a broad but peaked background, very similar to our calculations. The detailed shape and width of this fluorescence background will be sensitive to the photophysical properties of the molecule (or molecule–ion complex) and the various enhancement and decay phenomena at play. However, it seems difficult to explain a completely flat background as the result of a fluorescence process, which requires a finite life-time (and thus a finite peak width) to be meaningful.

1 H. Xu, X.-H. Wang, M. P. Persson, H. Q. Xu, M. Käll, and P. Johansson, *Phys. Rev. Lett.*, 2004, **93**, 243002
2 P. Johansson, H. Xu, and M. Käll, *Phys. Rev. B*, 2005, **72**, 035427

**Professor Tian** asked: You have attributed the new peak(s) as surface carbon. I am wondering where this carbon came from? It may due to laser-induced decomposition of probed molecules or impurities from the solution.

**Dr Nie** replied: There is strong evidence that the new peaks come from amorphous or graphitic carbon on the particle surface (see Fig. 4 of our paper), which is believed to come from laser-induced decomposition of surface-adsorbed species such as organic dyes and impurities.

**Professor Kneipp** asked: I made a comment on a possible weak fluorescence signal that sometimes appears along with a single molecule Raman signal. This fluorescence is shifted from the R6G fluorescence in solution by a few nm.

 

Is this surface fluorescence from R6G or from other surface species?

**Dr Nie** replied: This weak fluorescence signal is likely to arise from unquenched R6G fluorescence when excited at visible wavelengths.

**Dr Glembocki** asked: How well do the 'amorphous' carbon peaks that you talk about compare with real amorphous carbon Raman in relative intensities and positions?

**Dr Nie** replied: The peaks are similar in both intensities and frequencies, but are not identical. The work by Moyer and coworkers (see ref. 7 of our paper) shows that the surface-enhanced carbon spectra depends on the size of carbon clusters on the particle surface.

**Dr Cohen** commented: With R6G and 514 nm the unlikely underlying resonance will give you an increase in apparent temperature.

Agreed, unless you take the underlying resonance into account the anti-Stokes/Stokes ratio is meaningless and will not reflect the underlying temperature.[1]

1 R. C. Maher, L. F. Cohen, J. C. Gallop, E. C. Le Ru and P. G. Etchegoin, 2005, http://www.arxiv.org/abs/physics/0511074

**Professor Kneipp** asked: Has the Pas/Ps ratio been found to be the same for all Raman modes?

**Dr Nie** replied: We have only selected the most intense peaks for calculations, and have not systematically examined other peaks.

**Dr Cohen** stated: We have published[1] the technique to use the anti-Stokes/Stokes ratio to map out the underlying resonance for R6G and silver and the resonance that dominates the anti-Stokes/Stokes ratio is independent of geometry—it is related to R6G and silver and it is at a maximum between 514 nm and 633 nm (and it is related to the modified optical properties of R6G) in close proximity to the surface of silver.

1 R. C. Maher, L. F. Cohen, E. Le Ru and P. Etchegoin, *J. Chem. Phys.*, 2005, **123**(8), 084702.

**Dr Nie** responded: In our anti-Stokes/Stokes temperature calculations, we did not take into account the possibility of an underlying resonance effect. Our calculated temperatures are in the range 500–600 K, which do not appear to reflect the true surface temperature of nanoparticles in water solution. This result is in fact consistent with a resonance effect, pumping of the excited vibrational levels, or both.

**Professor Natan** opened the discussion of Professor Graham's paper: Is there a benefit to the off–on beacon which appears to be operative (as opposed to on–off) in some of your experiments?

**Professor Graham** replied: Yes! There most certainly is a benefit in this particular set of experiments.

**Dr Nie** asked: The SERRS beacons may not work as proposed due to the complex kinetic, thermodynamics and adsorption configuration problems associated with oligos on metal particle surfaces.[1] Have you studied and understood these fundamental issues?

1 D. J. Maxwell, J. R. Taylor and S. M. Nie, *J. Am. Chem. Soc.*, 2002, **124**(32), 9606–9612.

**Professor Graham** replied: We have not studied these issues with regard to our SERRS beacons.

**Professor Cass** asked: Weihong Tan has used fluorescent molecular beacons for imaging mRNA in live cells. Do you think that the SERRS beacons will also be applicable to live cell imaging?

**Professor Graham** replied: We believe that the SERRS beacons will be applicable to live cell imaging, however, there are obviously some issues involved including the stability of the SERRS beacon probe toward nucleases and also the viability of using silver nanoparticles within a living cell. We think that by passivating the surface by coating them in the beacon molecules we will protect the cells from the silver particles, however, until we try it we do not know.

**Dr Stone** asked: The most useful aspect of this work will be its application for medical diagnostics which is likely to require multiple DNA targets. Can you comment on the likely method of multiplexing the technique of SERRS beacons? Will it be spectral signal or spatially separated?

**Professor Graham** replied: Yes you can multiplex with the SERRS beacons. One way would be to spatially separate and also to combine a combination of the labels to give you multiplexing within that spatial separation. For instance, if one particular fluorophore was used with benzotriazole dye A and another fluorophore was used with the same benzotriazole dye then you would be able to multiplex due to the difference in the SERRS signals from the different fluorescent dyes. Essentially all we have to do is change the combination of the labels to allow us to multiplex. There will be a limit on the multiplexing due to the separation and deconvolution of the signals, however, what that limit is, is uncertain at present. We can look at SERRS beacons in solution to give us a multiplexing target in solution or we can use a surface species in an array format which will allow us to do spatial separation as well.

**Professor Natan** opened the discussion of Professor Green's paper: Is there any concern with respect to diffusion into the toroid voids? How is the spot-to-spot and substrate-to-substrate reproducibility?

**Professor Green** replied: There is no diffusion problem. I would estimate that the reproducibility is within a factor of two.

**Professor Kosower** asked: Does the benzotriazole group dissociate when combining with the $Ag^0$ surface (normally covered with oxide)?

Is there any chemistry (*i.e.* radical formation, $D^- \rightarrow D^{\bullet}$—no longer strongly bound?) that interferes with performance? Is chloride used to generate torus?

**Professor Graham** responded: We think the benzotriazole group combines with silver(I) on the surface of the nanoparticles. We do not have any evidence for dissociation of the benzotriazole group as when nanoparticles functionalised with these molecules are centrifuged and the supernatant removed we can no longer find any of the benzotriazole dye in the supernatant. We have not observed any chemistry which interferes with the performance of these dyes.

**Professor Green** replied: Chloride ions are not involved in torus formation.

**Professor Natan** asked: Can both speakers comment on what the goals are in terms of sensitivity with comparison to other existing bioanalytical methods?

**Professor Graham** responded: The goal of our research is to be as sensitive as possible using routinely available equipment. In order to compare our sensitivity to that of another existing bioanalytical method, we have compared the sensitivity of standard routinely available fluorescence equipment with a standard Raman spectrometer using fluorescently labelled oligonucleotides which will give both a fluorescence signal and a SERRS signal. This work was published[1] and indicated that SERRS was at least three orders of magnitude more sensitive than fluorescence.

1 Karen Faulds, Romina P. Barbagallo, Jacquie T. Keer, W. Ewen Smith and Duncan Graham, *Analyst*, 2004, **129**, 567–568

**Professor Green** replied: We need to be clear about how we define sensitivity. If we mean the detection limit for DNA on the surface, then the method will readily detect a surface loading of 2 ×

 

$10^{13}$ DNA molecules $cm^{-2}$ over the beam area, namely $1 \times 10^{-7}$ $cm^{-2}$. This corresponds to $2 \times 10^{6}$ molecules. An array spot size of 30–50 microns might be anticipated which would correspond to a detection limit of *ca.* 0.5–2 $\times 10^{8}$ molecules. In practical terms it is more useful to define detection limit in terms of the solution concentration that can be distinguished from zero (with a given degree of confidence). This will be very dependent on the length of the probe molecule, the solution conditions (ionic strength, pH) and the complexity of the DNA being detected. This makes it difficult to compare methods unless comparable samples are used.

**Dr Corish** asked: Is the shift in A : C ratios, used as the metric for successful hybridisation, sequence order dependent? For example, if the bacterial sequence used in the paper is randomised, keeping the same base composition, do you get the same ratio shift?

**Professor Green** replied: For the relatively short chain lengths used (18 and 45 mer) we have found no significant dependence on the sequence.

**Dr Abdelsalam** asked: Have you tried to use your method of making silver torus to make any other metals like gold?

**Professor Green** answered: We have not tried.

**Professor Kneipp** asked: Can you quantify how many mismatches you have in your strand?

**Professor Green** replied: We have only tried one base mismatch.

**Dr Russell** asked: Would you please comment on the long term stability of your structures? (*i.e.* do you use the samples immediately or can you leave them "on the shelf for days, weeks?")

**Professor Green** replied: A freshly prepared substrate is stable for a week if left in ethanol or water of pH $\sim$ 11.

The act of functionalization with a thiol anchor is the protection that our substrates require for long life.

**Dr Zoorob** asked: Due to the 0.36 fill factor, does the Si peak from Raman of wafer flood the SERS signal of the DNA. The Si peak at 520 $cm^{-1}$ is not shown, is it much larger than the SERS signal? Does the $SiO_2$ Raman background dominate the spectra seen between 600 $cm^{-1}$ and 1600 $cm^{-1}$.

Are these two points a problem for the experiment considering the SERS signal is quite weak?

**Professor Green** replied: The Si peak is not shown because we display the spectrum from 600 wavenumbers onwards. The Si peak is comparable (not larger) with our main peaks. The amorphous-$SiO_2$ features are very weak. Neither matter raised is of any significance.

**Dr Pearson** asked: Can you detect RNA hybridisation? What would be the limits of detection for RNA or expression analyses?

**Professor Green** replied: We have not tried RNA. But in principle it should behave as DNA.

**Dr Alexander** asked: Professor Green, your paper calls to mind a series of papers published by Georgiadis and co-workers[1–3] whereby they were able to follow the adsorption onto gold surfaces of thiolated ssDNA and the subsequent hybridisation of the probe ssDNA with target ssDNA, all by SPR. They further reported that the hybridisation DNA may be denatured leaving only the initial ssDNA probe attached to the surface, which can then be reused for further hybridisation and that this may be followed by SPR. Have you tried a similar approach as it would be interesting to see what happens to the probe during this hybridisation–denaturisation cycle or to see if it is possible to re-use the Ag-substrates presented in the paper?

1 A. W. Peterson, L. K. Wolf and R. M. Georgiadis, *J. Am. Chem. Soc.*, 2002, **124**, 14601.
2 K. A. Peterlinz, R. M. Georgiadis, T. M. Herne and M. J. Tarlov, *J. Am. Chem. Soc.*, 1997, **119**, 3401.
3 R. J. Heaton, A. W. Peterson and R. M. Georgiadis, *PNAS*, 2001, **98**, 3701.



**Professor Green** replied: We have not tried the Georgiadis procedure for removing the analyte from the attached probes. Thank you for your suggestion, we will certainly try it.

**Professor Kneipp** stated: Denaturation and renaturation of DNA can be monitored in SERS[1,2]

1 K. Kneipp and J. Flemming, *J. Mol. Struct.*, 1986, **145**, 173.
2 J. Flemming and K. Kneipp, *Stud. Biophys.*, 1989, **130**, 45.

**Dr Alexander** replied: Indeed, while the work referred to by Professor Kneipp[1,2] reported the detection of SERS spectra of pre-denaturated and pre-hybridised DNA, silver colloid was used as a substrate, and as such is less than re-useable. In light of the work presented in the current paper, it would be interesting to monitor the changes attendant upon hybridisation and denaturisation *in situ*, or to validate the re-usability of the substrates in a manner similar to that of the SPR studies.

1 Professor Kneipp *et al.*, *J. Mol. Struct.*, 1991, **244**, 183
2 *J. Mol. Struct.*, 1986, **145**, 173

**Dr Stone** opened the discussion of Professor Goodacre's paper: You have demonstrated the use of a generic SERS enhancer (silver colloid) coupled to multivariate analysis to distinguish between related species of bacteria. There is a great variability likely to be found in the signal as you map across the sample. Do you have a likely methodology for improving the signal reproducibility? Furthermore, do you have a handle on the impact of this signal variability on the analysis, *i.e.* have you attempted to keep hot spot data *etc.* to understand any likely problems?

**Professor Goodacre** replied:
(1) As suggested the key issue is to 'fire the laser' directly at the bacteria with metal particles to obtain SERS. The ideal scenario would be to have a uniform close packed array of metal particles annealed to a surface. Then, upon locating a bacterial cell one could be certain of obtaining a SERS response. The difficulty is in developing such a surface for this application and to make it reproducible which also provides significant signal enhancement for a broad range of analytes.
(2) We overcome the problem of signal variability by using a low magnification objective lens which naturally interrogates a larger area, thus providing a more average and representative SERS signal across the sample. In addition, as reported[1], our spectral pre-processing methodologies and multivariate analysis overcome much of the difficulties associated with the analysis of SERS data.

1 Roger M. Jarvis and Royston Goodacre, *Anal. Chem.*, 2004, **76**, 40–47.

**Professor Kosower** asked: Does $Ag^+$ present around $Ag^0$ particles cause disruption of cell membranes?

**Professor Goodacre** replied: If you are suggesting is $Ag^+$ toxic to bacterial cells then indeed it is! It is a well documented bactericidal metal and hence why it is used in water filters to stop bacterial fouling.

**Professor Natan** commented: An important factor for clinical utility is robustness of instrumentation, and of the 30+ companies that currently manufacture instruments, only one has paid any attention to this.

**Professor Goodacre** replied: This is of course an extremely important consideration; it may be up to the SERS and indeed Raman bio-community to collaborate in experiments to determine the

 

degree of reproducibility across instruments. Indeed, it would be useful to coordinate this and perform a Round Robin Test.

**Dr Pearson** asked: What are the impacts of sample processing in the spectra?

**Professor Goodacre** replied: The major desired impact for spectral processing prior to chemometrics is that we correct for background differences in the spectra. In broader terms, the impact of spectral pre-processing is that for robust applications these steps need to be standardised so that the procedures become reproducible across laboratories.

**Dr Le Ru** asked: Have you observed whether the cells were modified upon laser irradiation? Colloid-induced breaking of the cell wall (through heating) has been observed even at relatively low power, and was even shown to be a possible route to killing cancer cells.

**Professor Goodacre** replied: In a previous study[1] we obtained extensive SEM images of the sample matrix, following collection of numerous SERS spectra. We did not observe any degradation of the bacterial cells. The Gram-positive organisms studied for this article have an extremely tough outer membrane composed of peptidoglycan, so it is possible that they were robust enough to avoid photochemical damage. However, we have not attempted to culture after exposure to irradiation or indeed potentially toxic $Ag^{+}$.

1 Roger M. Jarvis, Alan Brooker and Royston Goodacre, *Anal. Chem*., 2004, **76**, 5198–5202.

**Dr Corish** asked: What levels of cell surface components are discernible by SERS? Could you experimentally test by exogenously expressing cell surface proteins and determining the SERS response?

**Professor Goodacre** replied: It is certainly true that vibrational modes of chemical species associated with proteins are being detected by this method, and we have previously shown that outer cell surface components were being probed. The idea of expressing a specific outer membrane protein and measuring this by SERS is an interesting idea.

**Professor Cass** asked: Can you use functionalisation of the particles (antibodies, aptamers, small ligands) to target the nanoparticles to particular cell surface markers to improve specificity?

**Professor Goodacre** answered: Yes, and such work has already been performed by other groups. However, in order to target a particular structure such as a protein expressed on a cell surface in this way one needs to have full antigenic information about the cell structure being targeted. By contrast, we are more interested in using SERS to probe non-selectively the surface of bacterial cells.

**Dr Roy** asked: The cells/bacteria have an inhomogenous coverage by Ag nanoparticles. The Raman signal is obtained from a local area while the Ag particles are present. How does it matter in real life applications?

**Professor Goodacre** responded: Massively! and that is why, as with many in the SERS community we must focus on improving the properties of our experimental design with respect to the localisation of a target analyte within the enhancement volume of the substrate. Hence, it is why improving surface-based SERS would help here.

**Mr Donaldson** commented: I wanted to confirm that the comments in this discussion imply that we can functionalise SERS particles to give them specificity for certain membrane proteins. A SERS spectrum of a membrane protein would be a significant achievement in the field because of their low abundance. Membrane proteins are notoriously hard to study. SERS/SERRS sensitivity to low abundance membrane proteins would generate much excitement in molecular biology.

**Professor Goodacre** replied: The difficulty with such an application is that this is a chicken and egg scenario, where in order to develop a functionalised SERS active particle, the chemistry of the target

structure, or protein has to be understood. If an antibody exists and it is accessible to the SERS or SERRS reagent, then there is cause for optimism.

**Dr Cant** asked: Your current method of sample preparation requires pre-mixing of the bacteria and colloid. Have you considered deposition of the bacteria directly onto a SERS active surface and do you foresee any effect to the spectra obtained?

**Professor Goodacre** replied: Yes, please refer to our previous answer.

**Professor Graham** opened the discussion of Dr Pal's paper: The scheme is misleading. The talk is about a carboxy group and the scheme shows an ester. EDC will react with phosphates very nicely so I am not convinced the CFV is coupled to the ester/carboxyl group.

**Dr Pal** replied: The procedure followed here is the same as that for coupling to a carboxyl group of the carboxyl modified oligonucleotide with the amino compound mentioned in the ''User guide to DNA modification'' by Glen Research. The phosphate group still remains protected under the conditions described. It is true that Carboxy-dT as its structure shows in Scheme 1 (of our paper) contains an ester group. But during the coupling reaction with CFV in the presence of EDAC it is hydrolyzed to the carboxyl group and reacts. This might be due to the fact that because of the presence of the hydrochloride salt of EDAC, *in situ* hydrolysis of the ester group takes place prior to the coupling process. Therefore, the scheme is basically a coupling between an amino group and a carboxyl group although the starting material is an ester.

**Professor Graham** asked: What are the detection limits?

**Dr Pal** replied: The limit of detection (LOD) is $\sim$1.25 ng $\mu l^{-1}$ and the absolute LOD is $\sim$12.5 pg. This is discussed in ''Results and discussion'' under ''SERS analysis''.

**Dr Cohen** asked: General sensitivity limits for different techniques. Can we quantify?

**Dr Pal** responded: Among the various methods for gene identification, technologies using radioactive labels, luminescence labels and SERS labels are very sensitive. Sensitivity, however, depends on the substrate, analyte and conditions imposed. Quantification is also possible as is done for BRCA1 using molecular beacon probe and a miniature biochip.[1]

1 M. Culha, D. L. Stokes, G. D. Griffin and T. Vo-Dinh, *Biosens. Bioelectron.*, 2004, **19**, 1007–1012.

**Professor Goodacre** replied: It is actually unfair to say the DNA is label free as it has a phosphate backbone. One can put PNA down on the surface and then bind DNA to that. Secondary ionisation MS can be used to measure phosphate.

I'm in agreement with Rick (Professor Van Duyne) that the community needs to benchmark these with other analytical approaches.

**Professor Cass** answered: When expressing sensitivity we need to distinguish between concentration limit of detection rather than amount detection. The ability to manipulate very small volumes through microfluidics means that amount detection can be made very small with quite modest concentration detection limits.

**Professor Graham** responded: The point made about reporting solution concentrations is probably correct but not always as simple as concentrating molecules on a surface.

**Dr Corish** replied: SERS can certainly deliver in principle the types of sensitivity and multiplex required for real applications but reproducibility is a key issue especially when moving into 'dirty' biological samples.

**Professor Natan** commented: SPR is a surface chemical phenomenon that is (a) commercial and (b) has figured out the ''units'' issue (*i.e.* concentration *vs.* mass sensitivity.

 

**Professor Cass** opened the discussion of Dr McCabe's paper: How does the SERRS bead multiplexing compare with, for example, the Luminex fluorescence bead arrays?

**Dr Corish** replied: The Luminex system is an encoded substrate where 5.6 μm latex beads are mixed with differing ratios of two fluorescent dyes. The resulting palette of 100 beads can be used to identify a particular analyte of interest in a multiplexed system. However, the final measurement of analyte concentration is determined by a third fluorescent dye, thereby limiting the sensitivity of this approach. Additionally, Luminex beads are read by flow cytometry, a feature that is not required for the SERRS beads. However, the commercial applications for both platforms are largely identical.

**Professor Baumberg** asked: How small can you make beads? (*e.g.*, dimers with thin encapsulation). Also, there is a very large variation in cluster size—if you look at SERS intensities from individual beads do you see a huge variation? Does this cause large problems for multicomponent beads?

**Dr McCabe** replied: We are able to make very small beads with small aggregates. We have not tried to determine the minimum bead size possible. Yes, we do see a large intensity variation between beads with different silver cluster sizes. Even if this was not the case it does not need to cause a significant problem for the multi-component analysis of these beads, as the assay format is based on measurement of a number of beads at one time, which gives us averaging of the signal. With multi-component beads, the loading of the dyes can be balanced to accommodate dyes with different scattering cross sections.

**Mrs Cintra** asked: How stable are the beads? What shelf life do they have?

**Dr McCabe** replied: The beads are stable in most buffers and some solvent systems for sufficient time to carry out a range of analysis. Solid samples are still active months after preparation, no longer term testing has been done. However, we believe they will be stable in solid form for years based on similar work that was published on polymer films containing silver and dye.[1]

1 A. McCabe, W. E. Smith, G. Thomson, D. Batchelder, R. Lacey, G. Ashcroft and B. F. Foulger, *Appl. Spectrosc.*, 2002, **56**(7) , 820–826.

**Dr Le Ru** asked: How photostable are these beads? Do you see any sign of photodegradation?

**Dr McCabe** replied: We have not seen any evidence of photodegradation under ambient light conditions. However, if the beads are exposed to a high power density by focussing on one bead using a microscope and laser irradiation, it appears that above a threshold level a change in the SERRS scattering can be induced in some beads. This is not a problem in the envisaged analysis procedure, which will use lower laser powers and fast integration times.

**Dr Vlčková** asked:
(1) What was the reason for selection of the dye concentration specific for each dye and how is the concentration related to coverage of Ag nanoparticles by the dye molecules? (monolayer, submonolayer?)
(2) Did Ag nanoparticles aggregate after the addition of the dye, or in the course of the polymerization procedure?

**Dr McCabe** answered:
(1) We believe we are working at close to monolayer coverage based on studies of addition of the dye in suspension. With multiple labelled systems the concentration of the dye within the monolayer was adjusted to accommodate different Raman cross sections.
(2) The addition of dye at high concentrations has been shown to cause aggregation of the silver particles. However, we have electronic spectra that show that the largest amount of aggregation is a result of the centrifugation process. We have also seen some evidence that further aggregation can occur as a result of polymerisation.

**Dr Glembocki** asked: How thick is the shell around the aggregate and how does this affect the SERS sensitivity?

**Dr Cormack** replied: The individual silver nanoparticles that are aggregated to form the cores of the beads are typically 25–35 nm in diameter. In the specific example shown, the average diameter of the beads lies in the range 1–1.5 μm. Methods to precisely control the size and aggregation state of the silver nanoparticles in the cores of the beads are currently under development. Furthermore, when using precipitation polymerisation as the means for bead production it is possible to tune the average bead diameter in the range of 100 nm to 10 μm. Alternative polymerisation strategies allow access into SERRS active beads that lie out-with this size range.

**Professor Kosower** asked: How many different dyes can be multiplexed? (competition with Illumina).

**Dr McCabe** replied: We have yet to determine the maximum potential for multiplexing but it is considerable. We have shown in polymer samples that multiplexing with 4 dyes can give between 50 and 100 codes with simple data analysis procedures.

However, currently we have 50 dyes available.

**Professor Natan** commented: There are plenty of encoded bead technologies; the real benefit is increased sensitivity.

**Dr McCabe** responded: We believe the combination of sensitivity and multiplexing gives this technology a real advantage.

**Dr Nie** commented: This is an excellent ''research'' project, but I don't see how it can compete with dye or QD-encoded beads. For direct biolabeling, I don't see how the aggregated beads could compete with the core-shell encapsulated gold nanoparticles, as published by Professor Natan[1] and Dr Nie[2]

1 S. P. Mulvaney, M. D. Musick, C. D. Keating and M. J. Natan, *Langmuir*, 2003, **19**(11), 4784–4790.
2 W. E. Doering and S. M. Nie, *Anal. Chem*, 2003, **75**(22), 6171–6176.

**Dr McCabe** replied; The current beads were intended for *in vitro* molecular biological analysis. As already stated, the advantages are in sensitivity and multiplexing.

As a further comment to Dr Nie's question SERRS gives an approximately 100–1000 fold increase in sensitivity compared to fluorescence (whether organic or inorganic) while at the same time avoiding the spectral overlap inherent to fluorescence that prevents extensive multiplexing. Even when 3–4 fluorescent dyes are mixed within a bead, an additional fluorescent marker is required to quantify. This situation does not apply to these SERRS beads. The current beads also take advantage of the aggregation enhancement that has been central to much of this week's discussion. The particles of Professor Natan and Dr Nie referred to are single nanoparticles in structure and therefore are limited in their enhancement capability.

**Dr Roy** asked: For biological applications, the smaller the biosize the better mobility will be. However, it will be more difficult to detect. So what should the size range be where both mobility and detection will be feasible?

**Dr McCabe** responded: Two possible fields of use are in analysis for molecular biology (DNA analysis, *etc*.) and *ex vivo*/*in vivo* analysis for molecular biology. The current synthesis route for these beads does result in structures that are larger than ideal for the full range of molecular biology applications. However, no work has yet been carried out to reduce the final bead size, and there is nothing inherently problematic in producing a bead that would be 100–200 nm in diameter. This would be suitable for *in vitro* applications, but a further reduction in size is necessary for *in vivo* uses.

**Dr Vlčková** asked: This question is related to Fig. 1 (p. 3) of your paper:

 

What types of surface functionalities are planned to modify the surface of the beads for the purposes of their specific applications? What procedure will be used for introduction of the functionalities?

**Dr Cormack** replied: Typically, the beads are synthesised by conventional free radical polymerisation methods under precipitation polymerisation conditions. One of the many advantages of free radical polymerisation chemistry is that it is highly tolerant of a diverse range of functional groups being present during the polymerisation. Thus, functional groups that can be readily incorporated into the polymer beads, either by homo-polymerisation or copolymerisation, including carboxylic acids, amines, alcohols, active esters, aryl groups, alkenes, alkyl halides, *etc*.

**Dr Corish** commented: Fluorescent-encoded beads are limited in their multiplex capability and even if mixed (*e.g*. 3–4 dyes per bead at different levels) still require a fluorescent marker to quantify. Single nanoparticle beads (such as Emory and Dr Nie's) may not benefit from the aggregation enhancements already spoken about in the rest of this meeting.

**Professor Tian** asked: I agree with the last comment that the SERRS labelled beads may have an advantage in terms of spectral resolution in comparison with the semiconductor quantum dot labelled beads. The question is how to further increase the signal intensity and improve the stability. I am wondering if it is possible to use a confocal laser (Raman) microscope to manipulate the silver or gold nanoparticle aggregates to try to further increase the SERRS intensity? Since the beads' diameter is around several micrometers, it could be possible to focus the laser spot just at the beads' center without damaging the shell materials.

**Dr McCabe** replied: This may very well be possible. We have made no attempt to do this, although we recognise that control of the silver aggregation is key to not only the beads SERRS signal intensity but also its variability.

**Dr Roy** asked: What would be the optimum size (refers to the previous question) considering real life biological applications?

**Dr McCabe** replied: This will depend on how many single nanoparticles we can make active and on the specific application. A reasonable goal with the current technology would be 100 nm containing approximately 10 silver particles.

**Dr Cohen** asked: Surely smaller can be better?

**Dr McCabe** replied: 1–2 μm beads are a good balance of silver cluster size and bead size for molecular biology applications. The larger silver clusters ensure that almost all beads are active. Clearly, smaller beads will be needed for other biological applications.