Faraday Discussions

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DISCUSSIONS

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SERS in biology/biomedical SERS: general discussion

Jeremy Baumberg, Steven Bell, Alois Bonifacio, Rohit Chikkaraddy, Malama Chisanga, Stella Corsetti, Ines Delfino, Olga Eremina, Claudia Fasolato, Karen Faulds, Holly Fleming, Roy Goodacre, Duncan Graham, Mike Hardy, Lauren Jamieson, Tia Keyes, Agata Królikowska, Christian Kuttner, Judith Langer, Carin Lightner, Sumeet Mahajan, Jean-Francois Masson, Howbeer Muhamadali, Michael Natan, Fay Nicolson, Evelina Nikelshparg, Konstantinos Plakas, Jürgen Popp, Marc Porter, Daniel Prezgot, Nathalie Pytlik, Sebastian Schlücker, Alessandro Silvestri, Nick Stone, Zhong-Qun Tian, Ashish Tripathi, Marjorie Willner and Pieter Wuytens

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Jürgen Popp opened a general discussion of the paper by Roy Goodacre: What is the advantage of using SERS instead of ordinary Raman spectroscopy?

Roy Goodacre replied: Raman and SERS in the context of using stable isotopically labeled substrates to investigate metabolism within the environment are complementary, and we have indeed used Raman and FT-IR in very similar systems.[1] Raman will measure the whole cell and give information on, for example, protein labeling inside the bacteria as seen in shifts in the ring vibration in phenylalanine. By contrast, SERS generally provides highly selective and specific information on the cell surface,[2] which is of course the part of the cell in contact with the environment and is therefore sensitive to changes within the immediate area surrounding the cell. Some bacteria are able to reduce metal ions inside the cell[3] and this can be used to probe within the cell as well.

1 H. Muhamadali, M. Chisanga, A. Subaihi and R. Goodacre, Combining Raman and FT-IR spectroscopy with quantitative isotopic labelling for differentiation of *E. coli* cells at community and single cell levels, *Anal. Chem.*, 2015, **87**, 4578–4586.

2 R. M. Jarvis and R. Goodacre, Rapid discrimination of bacteria using surface enhanced Raman spectroscopy, *Anal. Chem.*, 2004, **76**, 40–47.

3 R. M. Jarvis, N. Law, I. T. Shadi, P. O'Brien, J. R. Lloyd and R. Goodacre, Surface-Enhanced Raman Scattering from Intracellular and Extracellular Bacterial Locations, *Anal. Chem.*, 2008, **80**, 6741–6746.

Ashish Tripathi commented: My comment is regarding the question raised about why we perform SERS of bacteria at all when normal Raman (NR) provides spectral information already?

SERS provides cell wall information, while NR provides a complete cellular spectra.

As an example, consider the case of methicillin-resistant *S. aureus* (MRSA). The MRSA cell wall is functionally different than that of *S. aureus*.[1] Certainly, SERS can be a valuable tool in diagnostically determining these differences.

1 L. I. Llarrull, J. F. Fisher and S. Mobashery, *Antimicrob. Agents Chemother.*, 2009, **53**, 4051–4063.

Fay Nicolson asked: Have you carried this out using a sample from the environment? How do you think the low environmental pH will affect the Raman shifts? Do you expect to quantify this to the same extent?

Roy Goodacre replied: For SERS and Raman, not yet. We have used FT-IR to probe complex communities of bacteria from activated sludge which can digest phenol.[1] We would like to try the same system with SERS and Raman but have not yet had the opportunity.

With respect to pH shifts in the environment, we have not investigated this yet. Acidic conditions may cause molecules to be ionised differently (according to the different pKa values in the molecule substructures) and we would expect this to result in shifts in the Raman bands. If pH is considered then we would expect to be able to quantify to the same extent.

We note that environmental samples are not directly mixed with NPs. There will be a washing step to remove any variability from the environment, as the variations within the metabolic fingerprint of the microorganisms are of main interest. This step should remove any pH variations.

1 E. S. Wharfe, R. M. Jarvis, C. L. Winder, A. S. Whiteley and R. Goodacre, Fourier transform infrared spectroscopy as a metabolite fingerprinting tool for monitoring the phenotypic changes in complex bacterial communities capable of degrading phenol, *Environ. Microbiol.*, 2010, **12**, 3253–3263.

Steven Bell said: In this work you use *in situ* borohydride reduction to generate your particles rather than adding particles that had been prepared separately. Does this approach have any advantages over just adding conventional particles and drying the sample before analysis?

Roy Goodacre replied: That's a very good point and our very first SERS paper for bacterial characterisation used the mixing of bacteria and colloids approach.[1] You get SERS with this but you need to have colloids and bacteria together otherwise you measure just the Raman spectra of the bacteria. The main advantage with the *in situ* synthesis that we have used in the current work is that the nanoparticle synthesis is localised around the cells, which results in better reproducibility.

1 R. M. Jarvis and R. Goodacre, Rapid discrimination of bacteria using surface enhanced Raman spectroscopy, *Anal. Chem.*, 2004, **76**, 40–47.

Steven Bell asked: Since your particles are located on the surface of the cells it is very reasonable to attribute the signals to compounds found in the cell wall, but is there any chance that some intracellular material may have found its way onto the surface of the particles and is contributing to the signals?

Roy Goodacre answered: That is likely to be true. In the work previously described,[1] we could identify some of the SERS peaks as being due to surface materials (polysaccharides from the cell wall NAG and NAM and extracellular L-cysteine rich polypeptide toxins), so in the present study we expect most of the signals to be from the surface as electron microscopy shows that the Ag nanoparticles are surface associated. If the preparation method is changed then we can observe signals from inside the cell.

1 R. M. Jarvis and R. Goodacre, Rapid discrimination of bacteria using surface enhanced Raman spectroscopy, *Anal. Chem.*, 2004, **76**, 40–47.

Jürgen Popp asked: How does the type of bacteria influence the outcome? Is there any difference between Gram-positive and Gram-negative? If the bacteria have flagella, does this influence the result?

Roy Goodacre answered: Different bacteria will have different SERS spectra. We have unpublished work to suggest this and have analysed both Gram-negative and Gram-positive bacteria. The main difference is that due to the chemical process in Gram-negative bacteria the borohydride will associate with lipopolysaccharides, while for Gram-positive bacteria the borohydride is likely to interact more with teichoic and/or teichuronic acid. We have not looked at the influence of flagella on this system, so we cannot say at this stage whether there would be an additional signal from these.

Alois Bonifacio asked: In your paper,[1] the spectra in Fig. 6a are rather different from those in Fig. 3a, especially in the region around 1300 cm^{-1}. There is an intense, broad band in Fig. 3a in this region, which is apparently missing in Fig. 6a. Could you comment on the reason behind this spectral difference?

1 M. Chisanga, H. Muhamadali, R. Kimber and R. Goodacre, *Faraday Discuss.*, 2017, DOI: 10.1039/c7fd00150a.

Roy Goodacre responded: These spectra are different because in Fig. 3a the bacteria are analysed directly using Raman/SERS. By contrast in Fig. 6a, in order to obtain a mixture of differentially isotope labeled bacteria, we washed the cells and diluted them and this may have removed some chemical species that are associated with the surface. We tentatively identify the band at 1300 cm^{-1} to be from DNA and adenine (Table 1 in the paper) so it is possible that adenine, as it is water soluble, has been removed.

Alois Bonifacio commented: PCA has been used to reduce the data dimension, and the first 20 PCs were retained to generate the PC-DFA model. In my experience, this number of PCs is unusually high: loadings of PCs after 15, even for SERS spectra, often have quite a lot of noise, which might be carried into the DFA

model. What was the rationale or criteria used to decide the number of PCs to use?

Roy Goodacre responded: This is a good point and you are correct that we do not want to over-fit the data. However in this case PC-DFA is being used in a semi-supervised way. The PC-DFA scores plot is shown in Fig. 4 in the paper[1] and the number of *a priori* groups used by the DFA algorithm was set to 26 (*i.e.* this grouping reflects the replicates only). DFA tries to minimise within group variance and maximise between group variance, and so in our approach it is not given the level of ^{13}C-^{12}C or ^{15}N-^{14}N. In Fig. 4 you can see linkage in the clustering, where in DF1 the clustering reflects the increasing incorporation of ^{13}C into cells and in DF2 the level of ^{15}N in bacteria. This is natural and not artificial so the algorithm has been used correctly.

When we use PLSR, which is now used to predict the incorporation of ^{13}C or ^{15}N into bacteria, we do use this as a supervised learning method. To validate this supervised learning approach you will note that in Fig. 5 some of the data are used to train/calibrate PLSR (these are samples 0, 10, 20 … 90, 100) and some are used for an independent hold out test set (samples 5, 15, 25, … 85, 95). Both the training and test set are predicted very accurately with PLSR which means that the SERS spectra do contain quantitative information on the level of ^{13}C and ^{15}N incorporation during bacterial growth.

1 M. Chisanga, H. Muhamadali, R. Kimber and R. Goodacre, *Faraday Discuss.*, 2017, DOI: 10.1039/c7fd00150a.

Tia Keyes questioned: Is there a time dependence to your SERS signal or are you constrained to collecting your spectra within a particular time window after growth of the nanoparticles within the medium? In other words, does the SERS signal evolve over time *e.g.* due to biochemical changes at the bacterium interface or because of uptake of the particles by the bacteria over time?

Roy Goodacre responded: We do not see time dependencies as all of the samples are collected in the early stationary phase (*ca.* 15 h of growth) in this study to ensure that the cells have used (incorporated) the maximum amount of carbon and nitrogen supply available, which would facilitate their detection by SERS.

Marjorie Willner asked: Were the bacteria measurements done in bulk? Did you interrogate individual bugs to see if there was cell-to-cell variation?

Roy Goodacre answered: We did both of these. The cluster analysis (PC-DFA; Fig. 4) and quantitative predictions (PLSR; Fig. 5) are done on bulk. The spectra and SERS images shown in Fig. 6 are from single bacteria. We do observe some cell-to-cell variation but the dominant variable is the level of ^{13}C or ^{15}N incorporation.

Marjorie Willner said: For real world applications, do you foresee disrupting the biofilms in order to collect data?

Roy Goodacre replied: We have not tried the nanoparticle production method on biofilms yet. At this stage, yes we would disrupt a biofilm into single cells. After one identifies the important microorganisms, *i.e.* those that are using the primary carbon source that has been ^{13}C labelled, one can then isolate these bacteria and analyse their DNA (in particular the 16S rDNA) in order to identify them. We have done this previously using Raman activated cell ejection.[1]

1 Y. Wang, Y. Ji, E. S. Wharfe, R. S. Meadows, P. March, R. Goodacre, J. Xu and W. E. Huang, Rapid Raman activated cell ejection for isolation of single cells, *Anal. Chem.*, 2013, **85**, 10697–10701.

Ines Delfino remarked: What is the size of the data matrix? More precisely, how many spectra do you use in your analysis and what are the number of points per spectrum? Do you think that a data analysis method based on the use of a specific spectral window rather than on the whole spectrum (such as interval-PLS) would help?

Roy Goodacre responded: The SERS spectra contain 1015 bins between 489–4909 cm^{-1} and for the PLSR modelling we used the whole spectrum. For the PLSR training set we used 90 spectra (from samples with 0, 10, 20 … 90, 100% ^{13}C (or ^{15}N) incorporation) and for the test set we used the samples from the 5% intervals (75 spectra).

At this stage the predictions look really nice (see Fig. 5 in the paper), and so we would not perform any variable selection. If the accuracy in predictions in the future starts to degrade then we would use iPLS or VIP (variable importance in projection) scores from PLS as well as other variable selection methods.

Evelina Nikelshparg commented: We investigate quite a similar system, of mitochondria placed on nanostructured films to achieve long-distance enhancement and noninvasively investigate cytochromes inside living mitochondria. What is the distance of the enhancement of particles produced by bacteria? Have you tried to use nanostructured films to investigate deeper regions of bacteria?

Roy Goodacre responded: We have not looked at the distance/range of the enhancement effect from this synthesis so we cannot say at this stage. We do suspect it is localised to the surface for the reasons already discussed in terms of the types of chemistry we are probing.

Konstantinos Plakas asked: If you want to use this in an environmental context, is there any long-term toxicity associated with the gold nanoparticles?

Roy Goodacre replied: As there may be toxicity from silver nanoparticles, rather than gold, all of these analyses would involve sampling an environment. The last thing we want to do is (*e.g.*) to kill the primary microorganisms that are removing toxins from contaminated ecosystems.

Karen Faulds said: Would you expect to see differences between different strains of bacteria that would allow you to differentiate them within a population?

Roy Goodacre answered: Yes we would and we have done this previously with nanoparticles mixed with bacteria for SERS,[1] as well as *in situ* nanoparticle synthesis.[2] The nice thing here is that we might be able to ascertain which is the primary ^{13}C or ^{15}N consumer and also identify it without recourse to DNA sequencing.

1 H. Muhamadali, D. Weaver, A. Subaihi, N. AlMasoud, D. K. Trivedi, D. I. Ellis, D. Linton and R. Goodacre, Chicken, beams, and *Campylobacter*: rapid differentiation of foodborne bacteria *via* vibrational spectroscopy and MALDI-mass spectrometry, *Analyst*, 2016, **141**, 111–122.
2 R. M. Jarvis, A. Brooker and R. Goodacre, Surface-enhanced Raman scattering for bacterial characterisation using a scanning electron microscope with a Raman spectroscopy interface, *Anal. Chem.*, 2004, **76**, 5198–5202.

Lauren Jamieson enquired: Have you investigated using this technique to monitor changes to the metabolism of bacteria as a result of, for example, antibiotic action or genetic modification? Also, have you tried following the kinetics of incorporation of the isotopically labelled glucose and ammonium chloride into the bacteria over time?

Roy Goodacre answered: This is a really good question. SERS measures the metabolic output of a bacterial system, *i.e.* it measures the bacterial phenotype which is equal to its genes plus the environment. In fact bacteria are just like us and our phenotype changes if we eat, say, chicken tikka masala. We have not done this with this system as bacteria don't like curry (!) but there are many papers that have used SERS, Raman and FT-IR spectroscopy to investigate phenotypic adaptations. Bacteria are highly dynamic so these methods have been used to measure growth-related changes as well as challenges with antibiotics. As far as the kinetics of the incorporation of stable isotopes over time, we have not done this in this study. In previous work using Raman spectroscopy we investigated substrate utilisation and cross feeding, monitoring both the changes in ^{13}C loss (we were using reversed labelling) and ^{2}H incorporation.[1] The use of D_2O is a nice method to measure general metabolic activity.

1 Y. Wang, Y. Song, Y. Tao, H. Muhamadali, R. Goodacre, N.-Y. Zhou, G. M. Preston, J. Xu and W. E. Huang, Reverse and multiple stable isotope probing to study bacterial metabolism and interactions at the single cell level, *Anal. Chem.*, 2016, **88**, 9443–9450.

Zhong-Qun Tian addressed Roy Goodacre: This approach is important but challenging because many of the biosystems of interest are alive and much more complicated than materials and chemical sciences. I would like to ask your opinion and advice on what you think are the most exciting targets for bio-SERS in order to make some real contributions to life and medical sciences in the future? I also wonder how strong is the interference of SERS-active nanoparticles (with sizes between 20–80 nm) inside living cells and when in aggregated forms? To what extent will life science scientists be happy to accept complicated situations? For reducing the interference, several groups have introduced the nanoparticles outside living cells and probed membrane structures. It's evident that the membrane structure is dynamically changeable, leading to very complex SERS spectra, fluctuated with time. It seems to be important to find a method, such as the use of linkers, to fix the nanoparticles on the cross-membrane proteins. This

could be a solution because the time resolved SERS from these proteins may provide useful information. I would like to know your opinion on these comments.

Roy Goodacre answered: I think SERS has great potential for bioanalysis. For medicine and life sciences the ability to target certain molecules with molecular specificity is allowing people to measure drugs and metabolites in human biofluids and to do so with absolute quantification. For environmental microbiology SERS may help us understand the system a lot better so that it may be manipulated to, for example, enhance the decontamination of polluted environments or produce bacteria that are able to perform bespoke functions for synthetic biology/metabolic engineering.

With respect to probing bacterial surface structures as well as the biochemical composition inside cells, Malama Chisanga might like to comment on this.

Malama Chisanga commented: The method we have used in this paper gives highly reproducible SERS signals, and works very well with both Gram-negative and Gram-positive bacterial species. Interestingly, when bacterial cells are firstly soaked in a strong reducing agent followed by silver nitrate, the nanoparticles are coated onto the biological components on the cell surfaces. When the reagents are interchanged, so that cells are soaked in silver nitrate prior to re-suspension in a strong reducing agent, the SERS spectral features look very different which is probably because the nanoparticles become attached to the metabolites and biopolymers inside the cells instead.

Christian Kuttner communicated: You explained that SERS is a valuable tool to study microbial communities and their temporal expression of phenotypes. Could you comment on the experimental efforts required to follow the step from genotype to phenotype?

Roy Goodacre answered: In order to go from genotype to phenotype or *vice versa* as SERS measures the phenotype, we would have to incorporate some genomic information. Incorporation of metabolomics and/or genomics approaches using SERS fingerprints would, we believe, assist in assessing labeled structures in DNA (sequencing techniques) followed by metabolite profiling of metabolites (GC-MS). Combined, these would provide information about genetic changes in a well-controlled environment. This approach has been performed by others for the incorporation of genotypic information from FISH with Raman of stable isotopes.[1]

1 W. E. Huang, K. Stoecker, R. Griffiths, L. Newbold, H. Daims, A. S. Whiteley and M. Wagner, Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence *in situ* hybridization for the single cell analysis of identity and function, *Environ. Microbiol.*, 2007, **9**, 1878–1189.

Holly Fleming communicated: Does the use of silver nanoparticles have any antimicrobial effect, and does that affect the SERS spectra of the cells?

Roy Goodacre answered: In our experiments we do not observe any antimicrobial effects from the silver nanoparticles. When the NPs are synthesized

outside the cells, the samples are spotted onto CaF_2 disks and fixed so that the metabolic fingerprint of the cells remains unchanged. In addition, all of the samples are randomised before being analysed, to make sure that samples from each of the growth conditions are exposed to the NPs for random durations of time. The clustering patterns on the PC-DFA scores plot (Fig. 4 in the paper) confirm that there are no clear trends and significant effects from exposure to the NPs.

Duncan Graham opened a general discussion of the paper by Pieter Wuytens: An enzyme will break bonds but also make bonds if the free energy change is favourable in that direction. Have you seen any evidence of bond formation rather than hydrolysis in your system?

Pieter Wuytens replied: No, we have not observed this. We believe bond formation is unlikely for the proteases used here (trypsin and endoproteinase Glu-C).

Mike Hardy said: In the experiments, the GNF sequence Raman signal decreases by around 50% after tryptic cleavage ($I_{1003}/I_{829–860}$). It is postulated that the process is impeded by steric hindrance. You claim that there is fair control of the inter-dome spacing (12 ± 2 nm). Thus, could you increase the gap size slightly (*i.e.* whilst maintaining sufficient signal strength) to check this idea? Thank you.

Pieter Wuytens replied: This is an interesting suggestion. It is possible to a certain extent. We can increase the gap size by approximately 5 nm to 15–20 nm. Although the SERS signal will decrease, it is expected to still be sufficient for this measurement.

I wish to add that, apart from the narrow gap, there are other possible causes for steric hindrance. These include a dense packing of the peptide substrate and 90 degree corners at the bottom of the gold nanodomes.

Alessandro Silvestri asked: Did you compare the kinetic rate of the enzymatic reaction in solution and in the nano-gap? Is it faster or slower? Did you observed some positive effects due to the compartmentalization of the substrates?

Pieter Wuytens answered: We have not done a complete study on the kinetics of the enzyme reaction. The current data suggests that cleavage occurs within the first minutes after adding the protease. However, more experiments are necessary to quantify the reaction rate.

Jean-Francois Masson remarked: Following on from the previous discussion, the diffusion of molecules in very small gaps is slowed if the molecules have a diameter on the order of 1/2 to 1/3 of the diameter of the pore, which is the case here. Hence, the diffusion of proteases in the gap will be much slower that in the bulk. However, once a molecule is in the gap, the local concentration of the substrate is high and thus the protease should appear more catalytically active due to the short diffusion distance to travel. The system of an enzyme in a gap is thus much more complex than in the bulk. It would therefore be interesting to compare the catalytic activity of the protease in the gap to free reaction, to see

which factors dominate and whether the reaction is faster or slower than in the bulk.

Karen Faulds commented: Do you see differences in the overall intensity of the spectra when you use different enzyme concentrations? Would it be expected to observe different rates for different enzyme concentrations?

Pieter Wuytens replied: Yes, we expect to see a slower reaction rate when lower enzyme concentrations are used. We are working on increasing the time resolution of the experiment (which is currently about one minute per measurement) before carrying out quantification of the experiment. However, the kinetics are more complex than in a bulk solution, as just discussed. Note that we expect the remaining SERS intensity after the reaction to be the same, as this is determined by the amount of sterically inaccessible substrates.

Lauren Jamieson asked: How do you envisage transferring this platform to an *in vitro* environment? Inside a cell is there any chance you will get a SERS signal from any other biomolecules attaching to the substrate or other biomolecules interfering with the action of the SERS platform?

Pieter Wuytens answered: Under the assumption that the peptide monolayer is sufficiently stable, we believe that interfering SERS signals will not be a major hurdle. Because the gold is functionalized with a peptide layer with a thickness of a few nanometres, this will provide the dominant contribution to the SERS signal.

However, there may be other issues when transferring our approach from a reference environment to an *in vitro* measurement. Possible problems could be unspecific cleavage or a reduced accessibility of the peptides. Further experiments should clarify these issues.

Christian Kuttner asked: The binding of peptides to gold is mediated by terminal thiols. Could you comment on the stability of this binding and possible changes to the design of the gold-bound peptide architecture to make it more robust?

Pieter Wuytens responded: The CALNN pentapeptide was designed by Levy *et al.*[1] to form a stable SAM. The idea is that the gold–sulfur bond is isolated by a hydrophobic layer (here, amino acids A and L), followed by a hydrophilic shell to achieve a wetable surface (here amino acids N, N). We experimentally observed that this layer has an improved stability under a 1–10 mM concentration of dithiothreitol when compared to a normal cysteine-based bond. Nevertheless, improvements are possible. For example, a long alkane-chain with a PEG linker is expected to form a more stable self-assembled monolayer.[2] This could possibly be combined with dithiol binding, as was suggested in the discussion.

1 R. Lévy, N. T. K. Thanh, R. C. Doty, I. Hussain, R. J. Nichols, D. J. Schiffrin, M. Brust and D. G. Fernig, Rational and combinatorial design of peptide capping ligands for gold nanoparticles, *J. Am. Chem. Soc.*, 2004, **126**, 10076–10084.
2 R. Derda, D. J. Wherritt and L. L. Kiessling, Solid-phase synthesis of alkanethiols for the preparation of self-assembled monolayers, *Langmuir*, 2007, **23**, 11164–11167.

Marc Porter said: Can you comment on whether you observe an exchange process that is dependent on the size (*i.e.* length) of the previously adsorbed species?

Pieter Wuytens answered: We made a highly stable monolayer under up to 10 mM dithiothreitol concentrations when using a long alkanethiol chain (undecanethiol) linked to a peptide chain, synthesized as previously described.[1] We have not done a systematic study on the influence of the chain length. The work of Nayak *et al.*[2] is interesting in that regard.

1 R. Derda, D. J. Wherritt and L. L. Kiessling, Solid-phase synthesis of alkanethiols for the preparation of self-assembled monolayers, *Langmuir*, 2007, **23**, 11164–11167.
2 S. Nayak, W.-S. Yeo and M. Mrksich, Determination of Kinetic Parameters for Interfacial Enzymatic Reactions on Self-Assembled Monolayers, *Langmuir*, 2007, **23**, 5578–5583.

Christian Kuttner commented: One possibility to increase robustness would be to switch from one terminal binding site to a dithiol. Bidentate anchoring of ligands to gold has been shown to provide augmented robustness.[1]

1 F. N. Gür, F. W. Schwarz, J. Ye, S. Diez, and T. L. Schmidt, *ACS Nano*, 2016, **10**, 5374–5382.

Karen Faulds asked: Have you tried to multiplex different enzymes? Is it challenging to design different substrates to achieve sufficient differences in the spectra to allow identification of multiple enzymes simultaneously?

Pieter Wuytens responded: Multiplexing experiments are currently ongoing. The peptide substrates proposed here rely on natural aromatic amino acids for providing a dominant, distinguishable contribution to the SERS signal. As there are only three of those (phenylalanine, tyrosine and tryptophan), multiplexing will require the use of alternative molecules, such as non-natural aromatic amino acids.

Duncan Graham opened a general discussion of the paper by Judith Langer: Can you say a little more about the valency effects of the galectin you're using? Have you looked at any sizes of the clusters you've formed? I presume this is under diffusion/kinetic control so does this mechanism give rise to clusters that reach a certain size and then stops? Have you varied the density of the glycan on the particle?

Judith Langer replied: Our SERS platform based on aggregation by linking nanoparticles was designed to sense divalent or multivalent galectins, and the glycan ligand was chosen to selectively bind galactose in galectin proteins. Galectin-9 is a divalent, tandem-like galectin having two identical carbohydrate recognition domains which are recognized by the glycan ligand linked to the nanoparticle surface. Monomeric galectin-3 with a single carbohydrate recognition domain can be also bound by the glycan ligand but as no aggregation takes place, no SERS signal was detected.

We looked at several cluster sizes by different techniques. The composition of these clusters was studied by gel electrophoresis and the presence of galectin-9 within the aggregates could be clearly identified. The time-dependent DLS

measurements indicate that for intermediate and low galectin concentrations, clusters reach a certain size and then stop to grow. As an example, we measured a 2.8 nM sample which showed steady cluster growth to approx. 300 nm, whereas the 1.6 nM sample showed immediate formation of the NP dimers without further growth. These findings were supported using TEM. We therefore conclude that the slowly rising SERS signal in the 1.6 nM sample originated from the steady formation of new NP dimers without further cluster growth (within the observed time window). Yes, we varied the glycan but also the Raman reporter concentration. The density of both components was optimized in order to avoid pre-aggregation effects (*e.g.* induced by the protein buffer solution) and to maximize the SERS output. This implies that the density of Gal-9 recognizing the glycan ligand which at same time acts as a nanoparticle stabilizing agent as well as the SERS reporter must be properly adjusted. Once the glycan ligand/reporter ratio (here 5 : 1) and its concentration (1.25 μM) were optimized, we synthesized a larger batch with the same constitution and performed the concentration-dependent and aggregation dynamics studies.

Jeremy Baumberg said: To use this for sensing, you need signals that are linear in the analyte. However one of the things that changes with concentration is how the aggregation itself works. You go from diffusion-limited to reaction-limited colloidal aggregation, leading to changes in the aggregate size/shape (spindly/compact). How does this affect your results?

The threshold you see is peculiar, with no signal seen until a critical concentration. Why is this? It suggests there is some sort of charge issues in solution, so have you changed the buffer/pH? If you want to use it as a detection technique, you want it to be quite robust.

Judith Langer answered: We did not study how exactly the shape of the aggregates affects the SERS response. Furthermore, we found for high and intermediate galectin concentrations relatively large cluster distributions which contributed to the SERS signal. Only the low concentration of 1.6 nM results exclusively in NP dimers. In general, the average cluster size and the distribution correlates with the galectin concentration leading to a linear SERS response. Why we do not see a SERS signal until a critical concentration is related to the time window of the observation. We focused here on real-time measurements between 0–30 min but we found that for 0.8 nM of galectin, an incubation time of 2 days was needed to detect a SERS signal. This was too long to include the result for sensing applications within the mentioned time window. The pH and buffer concentrations were carefully kept constant for all of the measurements.

Tia Keyes commented: Is it possible to inhibit binding or disaggregate your particles by adding free galactose or lactose to your solution?

Judith Langer replied: As the immobilised galactose linkers and free galactose are selectively binding the same type of protein and thus compete with each other, the nanoparticle aggregation surely is inhibited. This will strongly depend on the concentration of the free galactose added. Whether or not the free molecules can dissociate clusters already formed, I cannot say at this moment. It depends on whether the value of the dissociation constant for the reaction with free galactose

is (a bit) smaller than for the reaction with Au-linked glycan or not. Thank you for this idea.

Christian Kuttner asked: The formation of hot spots is achieved by induced particle aggregation. The decrease in absorbance at 400 nm is an indication that the concentration of Au^0 in dispersion decreases about 20%. Is this process reversible or irreversible, and to what degree are particles lost by aggregation?

Judith Langer responded: Indeed, the samples with a high galectin-9 concentration such as 3.6 nM aggregated so fast that a significant amount of clusters precipitated leading to an irreversible loss of "material" after a few minutes. Through sonication, some parts could be redispersed but not completely. This also depended on the reaction time elapsed. The specific cluster losses were not quantified, but as a general trend we observed an increased redispersion with decreasing cluster sizes. On the other hand, the low concentration (1.6 nM) sample containing only dimers showed a steady SERS signal increase over a few days and no hint of irreversible precipitation.

Marjorie Willner commented: Do you plan to test the specificity and performance of these sensors in more complex media before undertaking multiplexing experiments? Have you considered that the conformation of the free galectin could differ from that on the cells and thus a different calibration curve would be needed?

Judith Langer replied: We have not yet tested the performance and specificity in more complex media but it will definitely be needed before going to galectin-9 sensing directly in cell cultures. We have shown that the sensor is stable in buffer solution but the behavior in complex cell medium which contains additional proteins can be totally different. The aggregation dynamics will also be changed in cell medium. For such experiments, we need a new calibration curve. The same holds for other varying parameters such as the conformational change of the analyte which could affect the SERS activity. The efficiency must be checked, analysed and the sensor must be calibrated for all new parameters.

Agata Królikowska remarked: Moving to the real samples – you have shown a plot where there are two different responses of the proposed sensor, depending on the ratio of nanoparticle and galectin concentration (Fig. 3b in your paper[1]).

In the real sample, you are not able to control this ratio. How will you solve this problem?

1 J. Langer, I. Garcia and L. M. Liz-Marzán, *Faraday Discuss.*, 2017, DOI: 10.1039/c7fd00123a.

Judith Langer answered: In Fig. 3B we show that the mixing volume ratio of both reactants affects the SERS response. We have to notice that in all of the experiments performed, the nanoparticle concentration in the final mixture was kept constant. Consequently, for a given mixing ratio, the initial volumes of the nanoparticle sensor and galectin-9 solution as well as nanoparticle concentration are fixed. The "real" sample of unknown galectin-9 concentration could be probed as a function of the mixing volume ratio and compared to the respective

calibration curves in order to determine the galectin-9 amount. In the inset of Fig. 1 displayed here, we show a linear dependence of the SERS signal on the $V_{(galectin)}/V_{(nanoparticle)}$ ratio (measured at t = 450 s) for a constant final galectin-9 concentration of 1.6 nM. A more complicated issue in real samples is the change in the SERS response caused by the presence of non-specifically binding proteins. In this regard we found that, for example, the amount of 20% of human serum albumin in a 1.6 nM galectin-9 resulted in a 30% lower SERS signal. Upon increasing the amount of human serum albumin to 30%, the SERS signal dropped down to 32%. For real applications, it will be necessary to study the interference of further proteins in more detail.

Karen Faulds asked: Your nanoparticles are really nice and homogeneous with very low background signals. Do you have any advice on functionalising the nanoparticles to reduce aggregation and keep the background low?

Judith Langer responded: The aggregation of nanoparticles depends strongly on their size, the stabilizing agent and the ion strength/pH. The background is often related to the absorption and luminescence of the nanoparticles and

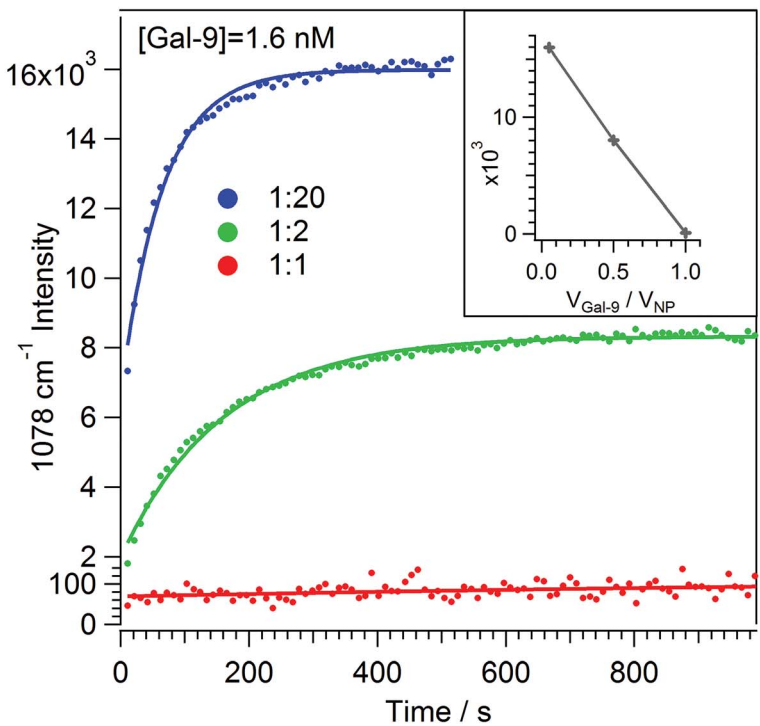


Fig. 1 Time-dependent evolution of the 1078 cm^{-1} vibration of 4-MBA for different galectin-9-nanoparticle mixing volume ratios keeping constant final protein and nanoparticle concentrations. Inset: linear dependence of SERS signal intensity measured at 450 s on mixing volume ratio.

molecules attached to the surface. For aggregates composed of spherical nanoparticles covered with small aromatic thiols (not resonant) and excited at 785 nm, the background is normally low. Higher stability is observed for smaller nanospheres than larger spheres. The compromise for SERS applications is to find the balance between nanosphere size (approx. 30–50 nm), optimal (high) coverage of the Raman reporter molecule (ideally charged) and a sufficient enough density of the stabilizing agent in order to prevent non-desired aggregation.

Claudia Fasolato opened a general discussion of the paper by Sebastian Schlücker: As you have demonstrated, optimization of the acquisition parameters in imaging experiments can lead to excellent results in the analysis of single cells. In your paper, there are some suggestions for a route to making such results quantitative, for example by estimating the amount of specific receptors per single cell with imaging experiments. Can you comment on the variability of your SERS signal from cell to cell, and against control cell populations?

Sebastian Schlücker answered: Our data set is probably not large enough to draw any statistically relevant conclusions, however, we certainly performed negative control experiments. Correlation of the Raman intensities in iSERS experiments with concentrations of the target protein requires *ex vivo* concentration series such as immuno sandwich assays. However, we have not yet performed such experiments in this context.

Nick Stone asked: You identify a set of experimental parameters to provide a stable set of results for your instrument. Have you considered that these signals are likely to be related to the plasmonic heating of cells and NPs, and therefore there is probably a more universal parameter of maximum temperature achieved in the cells? Have you considered looking at the Stokes/anti-Stokes ratio to observe the temperature threshold, and then stay below this?

Sebastian Schlücker answered: I completely agree that local heating is an important aspect limiting the maximum applicable laser power density. However, we have not considered Stokes/anti-Stokes measurements for technical reasons since our commercial Raman microscope is not equipped with a notch filter but only with a long pass filter for measurements on the Stokes side. The dominant marker band of the Raman reporter is around 1340 cm^{-1} which, according to the Boltzmann factor even at elevated temperatures, would be difficult to be detected at the anti-Stokes side.

Michael Natan remarked: Highly multiplexed detection of protein targets in thin tissue sections is a very important and timely problem. In addition to the challenges you've already listed, I would add "gaining a thorough understanding of commercial workflows in wide use today (using immuno-fluorescence)". To demonstrate the potential of SERS, this is an absolute baseline in performance that has to be met.

Duncan Graham commented: Just to back up the SERS *versus* fluorescence debate, we have data from tissue section imaging using 4 different SERS nano tags with antibodies to 4 different targets. The background is very low and all 4 can be

clearly seen using SERS. The fluorescence imaging of the same 4 targets is much harder and can't multiplex in the same way as the SERS. Our ultimate aim from here is to go *in vivo* where the SERS will perform much more favourably than the fluorescence due to the low background and depth penetration of Raman/SORS *versus* fluorescence.

Marc Porter said: Given the complications associated with antibody cross-reactivity, can you speculate on the extent of multiplexing you may be able to achieve?

Can you describe how the gold nanoparticle dimers were prepared? Is it a Janus particle approach?

Sebastian Schlücker responded: This is a very important aspect which needs to be tested empirically for a particular marker panel. I can only speculate that five antibodies should be no problem but when we approach 10–15 and more this becomes the limiting factor.

Dimers were prepared using a solid phase-supported approach by depositing the first particle on a glass surface, adding a dithiol linker molecule and then adding the second particle. The corresponding paper will appear soon.

Zhong-Qun Tian commented: Your study is very interesting. I wonder if you could reduce the number of nanoparticles to few and use different linkers and Raman markers to fix them into different membrane crossing proteins, in order to observe their kinetic behavior and to see if these different proteins are correlated? Moreover, can you promote the study into the higher level – act as a receiver and *in situ* monitor some species released from the cell through the crossing proteins when the cells are initiated by the change in local pH or through the injection of chemicals, *etc.*?

Sebastian Schlücker answered: These are very interesting and challenging questions. Reducing the number of nanoparticles is possible: we are able to detect the SERS labels/nanotags at the single-particle level within short acquisition times (<1 s). In principle they could also be used to target membrane crossing proteins. The static iSERS studies on single cells or tissue biopsies require washing steps for removing any free/unbound antibody-particle conjugates since we observe SERS from the particle irrespective of whether binding to the target has taken place or not. Dynamic studies would probably require very fast washing steps. In cases where the nanoparticle should be permanently conjugated to the membrane crossing protein, the question arises to what extent the large size and weight of the particles modifies the intrinsic dynamics of the system. The idea of using the particles as a receiver instead of just a label would probably require reporter molecules which are specific to the corresponding target proteins. However, synthetic chemical receptors currently lack the required specificity.

Carin Lightner said: Did you image the nanostars after exposing them to high powers? Do you expect the shape change of the nanostars to have a significant impact on the enhancement?

Sebastian Schlücker responded: We repeated iSERS experiments on the same single cell with gold nanostars conjugated to antibodies. However, we did not perform experiments in which we extracted the gold nanostar-antibody conjugates from the cells after the iSERS experiments and analyzed them by TEM or SEM. In cases where some significant morphological changes such as "degradation" of the tip occur after optical illumination, I would expect that most likely the SERS intensity decreases. Since we observe reproducible SERS spectra with relatively constant Raman intensities, we conclude that the nanostars do not undergo significant morphological changes which would lead to a reduced plasmonic enhancement of the nanostars.

Christian Kuttner asked: Different approaches have been presented for the controlled fabrication of nanostars[1] and nanoclusters[2–4] with well-defined structural and optical properties. While nanostars present their hot spots at the tip ends, the hot spots of nanoclusters are formed in the gaps, to some extend hidden inside the assemblies. Could you comment on the possible advantages and disadvantages of these morphologies for SERS applications?

1 W. Niu, Y. A. A. Chua, W. Zhang, H. Huang and X. Lu, *J. Am. Chem. Soc.*, 2015, **137**, 10460–10463.
2 M. Gellner, D. Steinigeweg, S. Ichilmann, M. Salehi, M. Schütz, K. Kömpe, M. Haase, and S. Schlücker, *Small*, 2011, **7**, 3445–3451.
3 J. H. Yoon, J. Lim and S. Yoon, *ACS Nano*, 2012, **6**, 7199–7208.
4 R. P. M. Höller, M. Dulle, S. Thomä, M. Mayer, A. M. Steiner, S. Förster, A. Fery, C. Kuttner and M. Chanana, *ACS Nano*, 2016, **10**, 5740–5750.

Sebastian Schlücker replied: Gold nanostars are probably better suited for label-free SERS applications since the analytes can more easily approach the tips compared to the small gap regions of clusters including particle assemblies.

This may be especially critical for larger (bio)macromolecules. As for SERS labels/nanotags, we have had very good experiences with very bright core/satellite assemblies in which relatively small aromatic Raman reporter molecules are chemisorbed onto the surface of the core particles. Overall, the choice of a particular particle geometry certainly depends on the specific application.

Holly Fleming opened a general discussion of the paper by Jean-Francois Masson: How long do the neurons "live" after extraction? How do you see D-SERS translating to *in vivo*?

Jean-Francois Masson answered: The neurons are stable at room temperature for a few hours under constant buffer perfusion. However, they are extracted about 10 days before the experiments, following a standardized procedure in neurochemistry.

This is a long-term objective of this project. We foresee that sensors based on fiber optics could be implemented in living animals using procedures very analogous to the implementation of microelectrodes in the field of electrochemical neurochemistry. Electrodes of a similar size have been successfully implemented in the brains of living rats for about a decade now and continuously monitor the level of dopamine in the brains of freely moving rats. We envision

that SERS experiments could be performed using similar techniques in about a decade.

Evelina Nikelshparg asked: Can differences in the binding kinetics of different molecules have an impact on the quantitative analysis? How could we deal with the problem of irreversible binding of molecules to a SERS substrate?

Jean-Francois Masson responded: The affinity of molecules to the surface will have an impact on the frequency of measured events. At identical concentrations, molecules with a greater affinity to the surface of the nanoparticles will be observed more frequently. This was seen in our binary experiment with ATP and dopamine. ATP had a larger frequency of detected events than dopamine.

Irreversible binding could be an issue. In our current conditions, we did not see irreversible binding as the medium did not contain molecules that were susceptible to strong binding with the nanoparticles. However, this could be a concern in more complex media. In that case, protective surface chemistry with some sort of microdialysis technique may be needed.

Roy Goodacre commented: That's very nice work. I am thinking of additional experiments and application areas, so I wonder if you can differentiate between ATP, ADP and AMP? The ability to measure these *in situ* for cancer biology would be very neat. Currently these measurements using enzyme assays linked to luminescence are notoriously difficult for absolute quantification.

Jean-Francois Masson answered: This is indeed an excellent suggestion. We have started another project that was presented as a poster on the detection of ATP near cells. We have not yet done the controls with ADP and AMP, but this is planned for the near future. You are correct about luminescence being difficult and I would add non-optimal as luminescence is a bulk measurement not suited for ATP measurements in close proximity to cells.

Daniel Prezgot commented: In your control experiments while testing individual neurotransmitters you observe a significant amount of false positives (around 20%) for ACh and GABA. However your experiments on neurons seem to produce proportionately less of these events (perhaps <10%). How were you able to improve your selectivity for these experiments despite the more complex sample matrix?

Jean-Francois Masson responded: A few factors can explain these differences. We first further optimized the code for the neuron experiments. We also dropped the power of the laser for the neuron experiments, resulting in a slightly lower SERS intensity, which can reduce the number of false positive detection if the signal no longer exceeds the threshold imposed in the data processing algorithm. Lastly, the difference may be partly explained by the variance of the results.

Steven Bell asked: Could you clarify how you decide which frequencies to include in your barcodes and possibly indicate how sensitive the results are to the number of frequency values included?

Jean-Francois Masson replied: In the first step, we extracted all the frequencies of higher intensity than a threshold for a series ($\geq$1000 spectra) of SERS spectra of standards. The threshold was set as three times the median value of the entire spectrum. We then performed a combinatorial computational analysis of the number of frequencies to include in the final barcodes for every standard. We verified the sensitivity and selectivity for each set of parameters. Note that the frequencies to be considered in the barcode are solely based on intensity. Only the most intense Raman peaks are used in the barcoding process.

There is a potential impact on the results with the different number of frequencies of the barcode. A greater number of frequencies in the barcodes usually leads to a higher number of event detected (more sensitive) up to a certain number of frequencies. Then, barcodes containing too many bars are too restrictive and a lower sensitivity is observed. However, a higher number of frequencies will improve the selectivity of the chemometric data analysis. We therefore performed a selectivity assay *e.g.* to count the number of events of every neurotransmitter for a solution containing a single neurotransmitter, and optimized the barcode algorithm to maximize the sensitivity and selectivity.

Olga Eremina communicated: It is known that such neurodegenerative diseases as Alzheimer's and Parkinson's are characterized by a loss of dopaminergic activity. As a rule, the level of catecholamine concentrations can go down to nanomolar or picomolar levels. What limits of detection can possibly allow the proposed technique to be used for the early diagnosis of dementia?

Jean-Francois Masson answered: We are in the process of calibrating thoroughly the D-SERS nanosensor for different neurotransmitters. However, we have measured pure solutions of dopamine and observed D-SERS spectra for this molecule for concentrations in the low nanomolar ($\leq$10 nM) range. We anticipate that the required limits of detection could be reached with an optimized D-SERS nanosensor and proper calibration.

Olga Eremina communicated: Could you comment on the possibility of this approach being selective across L-DOPA (dopamine precursor) and epinephrine, norepinephrine, metanephrine, *etc.* (dopamine metabolites)?

Jean-Francois Masson responded: In principle, we should be able to detect metabolites of dopamine. While we have not done this experiment at this time, we were able to detect pyruvate and lactate with the same method in a recent article we published.[1] These two molecules are structurally very similar, suggesting that structurally-related metabolites could be measured.

1 F. Lussier, T. Brulé, M. Vishwakarma, T. Das, J. P. Spatz and J.-F. Masson, *Nano Lett.*, 2016, **16**, 3866–3871.

Olga Eremina communicated: Have you investigated the toxicity of the applied Au nanoparticles? Have you observed the influence of the developed D-SERS nanosensor on a living neuron?

Jean-Francois Masson responded: We have not investigated the toxicity towards a living neuron, but we do not expect any impact of the nanosensor on the neurons. The nanosensor is located about 10 microns from the living neuron, such that there is no direct contact of the Au NP with the neurons. The Au NP are also immobilized on the glass nanocapillary, hence should not be available to neurons. The fluorescence images show no changes in the morphology of the neurons following the D-SERS experiments. Hence, we are confident that the experiments have no impact on neurons.

Holly Fleming opened a general discussion of the paper by Sumeet Mahajan: Your paper only discusses 60 nm gold particles, have you looked at any other sizes or materials of particles? Would you expect anything different to be seen – are there particle size limits to what you observe in your paper?

Sumeet Mahajan responded: In this paper we have used 60 nm gold nanoparticles. In some of our previous work we have successfully used 40 nm gold nanoparticles.[1,2] The limits to nanoparticle size may arise due to the dependence of the uptake mechanism, and hence uptake, on the size/shape of the nanoparticles. We have discussed this in a recent review.[3]

1 A. Huefner, W.-L. Kuan, K. H. Müller, J. N. Skepper, R. A. Barker and S. Mahajan, *ACS Nano*, 2016, **10**, 307–316.
2 A. Huefner, W.-L. Kuan, R. A. Barker and S. Mahajan, *Nano Lett.*, 2013, **13**, 2463–2470.
3 J. Taylor, A. Huefner, L. Li, J. Wingfield and S. Mahajan, *Analyst*, 2016, **141**, 5037–5055.

Jürgen Popp asked: How did you ensure that the particles were really inside the cell, not just sticking outside?

Sumeet Mahajan answered: We relied on the natural process of endocytosis for nanoparticle uptake. Therefore, in order to visualise that indeed the nanoparticles were inside the cell we tested all treatment conditions with confocal microscopy. We took *z*-stacks for 3D visualisation and used a cytoplasmic dye (CellTracker) to see the cells and their boundaries, and used the Rayleigh scattering signal to image the nanoparticles. In this way we were able to confirm that the majority of nanoparticles were indeed inside the cells under all of the treatment/uptake conditions in our experiments.

Stella Corsetti addressed Sumeet Mahajan and Jack Taylor:

(1) How did you produce the mean spectra shown in Fig. 3 and 7 in your paper?[1] It would be useful to add a brief explanation.

(2) What is the standard deviation associated with the mean spectra shown in Fig. 3 and 7 and the standard deviation associated with the difference spectrum shown in Fig. 7? Usually if different regions of a cell containing label free nanoparticles are probed with a focused beam different SERS spectra are obtained. It would be useful to visualise the standard deviation associated with the mean spectra and error bars associated with the difference spectrum.

(3) In Fig. 4 in your paper,[1] it is shown that the first principal component is the one that allows cells that are uptaking different concentrations of nanoparticles to be discriminated. What does the first principal component loading look like? It

would be interesting to observe what are the vibrational bands associated with the components that allow the discrimination.

1 J. Taylor, J. Milton, M. Willet, J. Wingfield and S. Mahajan, *Faraday Discuss.*, 2017, DOI: 10.1039/c7fd00156h.

Sumeet Mahajan replied:

(1) The mean spectra were produced by recording 16 static scans in a point map format across an area exhibiting high scattering from NPs inside a given cell (endosomal compartments are most likely to contain such a concentration of entrapped NPs). This process was repeated for 6 cells in each treatment condition across two repeats (culture wells). The mean spectra were produced by merging all of the collected spectra for each class in iRootLab, processing by feature selection (350–1650 cm^{-1}), polynomial baseline subtraction (order 5), wavelet denoising and vector normalisation. These steps are performed by the software upon every individual spectrum within the merged dataset for each class. Thus, the mean spectrum presented is the average of each processed spectrum for each treatment class (total of $16 \times 6 = 96$ spectra). These mean spectra have been offset for clarity in the plots shown in Fig. 3 and Fig. 7 in the paper.[1]

(2) It is correct that the spectra can differ a lot if taken from different locations. However, as noted in the methods section, we used pre-selected areas for acquiring the spectra. The selection was based on the strong low-frequency plasmon-phonon band visible in the SERS spectrum or Rayleigh scatter. It is likely that these areas in cells therefore were those where clustering of nanoparticles had occurred, which is most likely to happen in the *endo*-lysosomes and this could account for the relative spectral similarity within treatments. A representative example (250 μM, 1% FBS, Fig. 2 displayed here) of the standard deviation in the spectra from Fig. 4 in the paper[1] is presented below. The mean spectra in Fig. 7 in the paper[1] are the same data, presented against other cell treatment spectra. The standard deviation associated with the difference spectrum is presented in Fig. 2 shown here.

From the difference spectrum it is clear that spectral variation is more abundant in high wavenumber regions such as the amide I region than in the lower sections of the fingerprint region, which possibly arises from the limited sample sizes. However, wavenumbers where the mean intensity of one cell treatment class falls outside of the standard deviation range of the other, as evidenced at

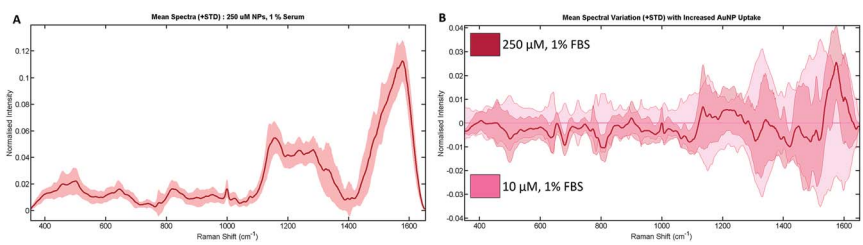


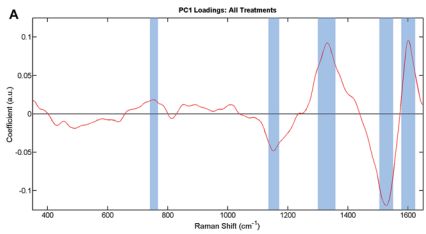
Fig. 2 Mean spectra with the standard deviation (A, left) of cells treated with 250 μM AuNPs in 1% serum-media and (B, right) its spectral variations relative to a 10 μM AuNP dose, indicating NP concentration-induced changes to the cellular environment

637, 680, 798–812, 983, 1063, 1090, 1418, and 1560–1580 cm^{-1}, allow identification of significant NP-induced spectral changes.

(3) Fig. 3 displayed here shows the PC1 loadings of the PCA performed on the whole dataset (5 classes), corresponding to the scores plot provided in Fig. 4 in the manuscript,[1] along with assignment of the 5 vibrational peaks providing the largest contributions to clustering.

The loadings have been obtained after processing the spectra, vector normalisation and mean-centring the data. The table shows the tentative assignments of the most significant vibrational frequencies that lead to segregation by PCA.

1 J. Taylor, J. Milton, M. Willet, J. Wingfield and S. Mahajan, *Faraday Discuss.*, 2017, DOI: 10.1039/c7fd00156h.



Raman Shift/cm^{-1}	Assignment
1590-1610	C=C bend/C-C str, Phe, Tyr, proteins
1510-1540	C=C conjugated in-plane bend
1310-1350	CH_3CH_2 wagging, proteins/nucleic acids
1150-1170	C-C/C-N str, proteins/lipids
755	C-C breathing, Trp, proteins

Fig. 3 The PC1 loadings reveal the wavenumbers which contribute most greatly to the PCA classification of all of the SERS spectra acquired from all five NP treatment classes. The peaks contributing most to the classification are assigned in the accompanying table as potential markers of NP-induced cell stress.

Roy Goodacre said: In your experiments I understand that these nanoparticles are taken up by endocytosis and so are internalised within a membrane. This may explain why the spectra are very similar. Do you know how long it is before the NPs are released into the cytoplasm?

Sumeet Mahajan responded: In our experiments we did not focus on *endo*-lysosomal escape into the cytoplasm. However, other work in literature documents show through pulse-chase experiments and time-lapse imaging that nanoparticles can escape into the cytoplasm in times ranging from minutes to several (>10) hours. It has also been shown that this escape or release is dependent on the size, shape and the chemical nature (corona) of the nanoparticles.

Lauren Jamieson remarked: As you say, nanoparticles incubated with cells in the presence of fetal bovine serum (FBS) are thought to become coated in a protein corona. This protein corona is therefore the closest thing to the nanoparticle surface, and as a result it is expected that the greatest contribution to the SERS signal would originate from this. By lowering the FBS component of the medium from 10% to 1%, could it not therefore be expected that the major difference between the SERS signals will simply originate from a lesser contribution from the FBS signal? How do you account for this variation *versus* other intracellular variations? The characteristics of this coating could also affect how

the nanoparticles are taken up by cells and where they end up inside the cells – can you comment on this also?

Finally, I think this study highlights an important aspect of intracellular SERS measurements and the need to better understand how nanoparticles themselves can alter the biochemistry of cells, without necessarily killing them. How much do you think we need to assess the effect of nanoparticles on the biochemistry of cells before we can be sure that the processes we are measuring using the nanoparticles are real biological changes, and not the result of nanoparticle application?

Sumeet Mahajan answered: You are correct that we do see a change in the spectrum from SERS of nanoparticles outside cells depending on whether they have been incubated with 1% or 10% serum containing media. This data is shown in the supporting information of our paper.[1] So indeed we see mostly the corona in both cases but, as you can compare, the intracellular SERS spectra are more complex in both cases of serum treatment. However, most importantly, we recognise the variations by considering changes between different concentrations of nanoparticles with each treatment. Within each serum treatment (either 1% or 10%) the corona is the same and yet we see spectral variation. Therefore that must come from the cells. Moreover, we use PCA to pick up the subtle differences and also use the difference spectra to understand the differences. I am glad that you concur that indeed it is important to understand how the application of SERS nanosensors (nanoprobes) could alter the biochemistry of cells and that was the key message of the paper. One of the takeaways from our work would be to use a healthy serum content (5% or above is recommended) and then to not just do a viability test but also a cell stress assay to the minimum before proceeding to do further investigations.

1 J. Taylor, J. Milton, M. Willet, J. Wingfield and S. Mahajan, *Faraday Discuss.*, 2017, DOI: 10.1039/c7fd00156h.

Alessandro Silvestri asked: Which superficial functionalization of the nanoparticles is employed in the study? The superficial chemistry of the nano-system can have a tremendous impact on the protein corona formation and intracellular behavior of the NPs. Different functionalization can induce different levels of stress. Considering this factor could improve the results of the present study.

Sumeet Mahajan replied: You are correct that the surface chemistry can determine the corona formation and hence, the intracellular behaviour of the nanoparticles. In this study the nanoparticles were used unmodified, however, they would have a citrate capping layer (from their synthesis/storage) which would determine the initial protein corona formation when the nanoparticles are suspended in cell media. The aim of this study was to investigate whether nanoparticle uptake itself can affect cells and hence the SERS spectra. For this aim further modification of nanoparticles was not necessary. However, this is an interesting aspect and could be investigated further to see the effect of surface functionalisation on uptake and the SERS spectra.

Alessandro Silvestri asked: Which techniques have been employed to characterize the protein corona?

Sumeet Mahajan replied: As mentioned earlier, the aim of this study was to investigate and establish whether nanoparticle uptake can itself affect cells and hence SERS spectra. This did not require us to characterise the protein corona fully. We have recorded the SERS spectra from the corona itself (shown in the supporting information of the paper) which as expected show the characteristic peaks of proteins. However, for other studies it may be essential to characterise the corona fully. Dynamic light scattering (DLS), static light scattering/LSRP measurements, TEM and others techniques including mass spectrometry could potentially be used.

Howbeer Muhamadali remarked: According to Fig. 2b in your paper[1] it is of course clear that upon increasing the concentration of your nanoparticles, the ROS levels are also increasing, suggesting an increase in the stress levels. However, your cell viability results (Fig. 2a) don't show any clear patterns linked with such stress, which also suggests that the cells are doing well in dealing with this stress. Do you have any future plans to investigate further the metabolic response and changes in the cells due to exposure to these NPs? This may assist in understanding the detected changes in the spectral fingerprints of these cells.

1 J. Taylor, J. Milton, M. Willet, J. Wingfield and S. Mahajan, *Faraday Discuss.*, 2017, DOI: 10.1039/c7fd00156h.

Sumeet Mahajan responded: This is correct that cell stress induced by nanoparticles, such as through an increase in ROS levels, does not correlate with viability, which is one of the key messages of the paper. However, you are correct in pointing out the possibility that the cells could be maintaining viability through internal compensatory mechanisms. This is interesting since on page 16 of our paper we discuss one of the peak assignments which relates to NADH and observe an increase at higher concentrations of nanoparticles. The NADH/NADPH redox-couple is known as one of the mechanisms for cells to counter an increase in ROS, so exploring the metabolic response and changes in greater detail through a systematic approach would undoubtedly aid our understanding and indeed will be followed up.

Jeremy Baumberg asked: Have you tried different sorts of corona? Very dense DNA coronas for example (as Mirkin uses). This would be very interesting.

Sumeet Mahajan answered: I agree this would be different and interesting to see what effect they cause. Coronas around nanoparticles will determine the interactions the nanoparticles will have with cells and reactions they will trigger inside. It will likely give another handle to control interactions and study them since with DNA one could control the density, sequence/nature and perhaps other oligonucleotides as well by immobilising them on nanoparticles. DNA *vs.* protein corona comparison would itself be interesting and give useful insights.

Nathalie Pytlik said: You mention in your paper that you have made tentative spectral assignments of the SERS spectra. What kind of reference spectra have you used for it (Raman spectra, SERS spectra, databases)?

Sumeet Mahajan responded: Our tentative spectral assignments are all based on literature, published papers mainly. These have been indicated in the text.

Nathalie Pytlik asked: You write in your paper (page 11, lines 20–22) that the two regions of clustering may indicate different cellular locations. Could you differentiate between two different regions using 3D microscopy?

Sumeet Mahajan responded: We indeed write that the two clusters could indicate two cellular locations. These could be the nanoparticles within *endo*-lysosomal vesicles and those free in the cytoplasm, which is again well known that this happens although the relative distribution can be different under different conditions. In the 3D microscopy images shown in the paper these two locations would not be differentiated. However, potentially an experiment could be done to verify this by use of appropriate stains and with further verification by TEM.

Nathalie Pytlik asked: You have shown in your paper that the corona influences the uptake of nanoparticles inside the cell. This could be caused by different interactions between the corona and the cell or cell wall. However, the cell is less damaged when the concentration of the serum is higher at a stable concentration of nanoparticles, so could the level of damage of the cell also influence the nanoparticle uptake? Meaning that less damaged cells have better protection against nanoparticles and less nanoparticles can, therefore, enter the cell? What do you think has a higher influence on the nanoparticle uptake: the corona or the level of cell damage?

Sumeet Mahajan replied: This is a very important point. This study does not allow us to disentangle the effect of the corona from the amount of serum content in the cell growth media. To obtain a less dense corona we need to have less serum in the cell media, and less serum makes the cells susceptible to more stress. It is entirely possible that the cells under stressful conditions at low media serum content made it easier for us to see the additional stress caused by the nanoparticles. Further from our study we cannot conclude anything about the ability to uptake with respect to the stress conditions being experienced by cells and the corona around nanoparticles. All we can conclude is that there is a correlation between the serum conditions (which affects the protein corona around the nanoparticles) and uptake. It is well known that the corona influences the uptake of nanoparticles (see ref. 26 in our paper and, among many others, a recent paper published in *ACS Appl. Mater. Interfaces*.[1])

1 X. Cheng, X. Tian, A. Wu, J. Li, J. Tian, Y. Chong, Z. Chai, Y. Zhao, C. Chen, and C. Ge, *ACS Appl. Mater. Interfaces*, 2015, 7, 20568–20575.

Karen Faulds commented: It is really important that you have tried to quantify the number of nanoparticles inside the cells when discussing the effect that the nanoparticles have on the cells. There is a lot of conflicting literature on the effect of nanoparticles on cells and the effect of different types (size, shape, surface functionalisation) but very few of these papers quantify the number of nanoparticles within the cell so it is not always clear whether toxicity (or lack of) is due to the number of nanoparticles or the type of nanoparticle.

Sumeet Mahajan responded: I agree that it is extremely important to quantify the number of nanoparticles that are actually inside cells. Thank you.

Christian Kuttner communicated: The density of proteins in the protein corona of nanoparticles is expected to influence their biocompatibility[1] and cellular internalisation. By what means can the density of proteins (*e.g.* sparse *vs.* dense) be quantified? Recently, we applied SERS to investigate the well-defined protein shell of BSA-coated nanoparticles.[1,2] Maybe this could be extended to follow changes in the protein shell composition and density and serve as a model system for intracellular SERS. Have you considered using nanoparticles with a defined protein corona?

1 M. Tebbe, C. Kuttner, M. Männel, A. Fery and M. Chanana, Colloidally Stable and Surfactant-Free Protein-Coated Gold Nanorods in Biological Media, *ACS Appl. Mater. Interfaces*, 2015, **7**, 5984–5991.
2 R. P. M. Höller, M. Dulle, S. Thomä, M. Mayer, A. M. Steiner, S. Förster, A. Fery, C. Kuttner and M. Chanana, *ACS Nano*, 2016, **10**, 5740–5750.

Sumeet Mahajan responded: Indeed, to have nanoparticles that are well characterised and have defined corona would be ideal for intracellular SERS experiments. To study the effect of different types of coronas with controlled (known) composition would be very interesting. Please also refer to the previous discussion with Jeremy Baumberg.

Malama Chisanga asked: I see that you have used gold nanoparticles in this work which are working well. Have you also tried the same experiment with silver nanoparticles, which might actually provide a larger enhancement effect?

Sumeet Mahajan replied: In all our work so far we have used gold nanoparticles due to their relative inertness and biocompatibility compared to silver nanoparticles.

Marjorie Willner addressed Sumeet Mahajan, Jean-Francois Masson and Sebastian Schlücker: During the discussion there was a comment about respecting the complexity of biology. In your individual experiments, do you think you've looked at enough cells to get the whole picture with regard to cellular behavior in your systems? As a community are we performing experiments in a way that give us results that are generalizable? Should we pay closer attention to things such as cell passage number?

Sumeet Mahajan answered: This is indeed a supremely important point. In our individual experiments we examined 6–8 cells per treatment class. We didn't find much intra-class variation and hence, it was sufficient for us to make the conclusions we have made. Nevertheless, that we as a community still examine a relatively fewer number of cells (and other biological materials) than in a typical biological study is also related to the fact that intracellular SERS or SERS mapping experiments are still time-consuming and tedious to perform. The nature of the question being addressed also determines the extent to which the number of cells, repeats and replicates are necessary. With regards to intracellular SERS measurements the questions we are asking are still in the domain of methodology

development, to have a better understanding of how to carry out intracellular SERS experiments appropriately and to establish better procedures and protocols. We should definitely pay attention to all of the biological variables that could affect the results and their interpretation. These include the type of cells, the environmental conditions (temperature, pH, gases), the passage number for cell lines, the treatment and the media composition. A good step would be to at least state these clearly in our experimental sections.

Jean-Francois Masson replied: The number of cells/neurons necessary to establish strong biological data can change significantly depending on the type of cells/neurons. In our case, our neuroscientist collaborators typically look at 5–10 neuron samples under a set of conditions to provide biologically-relevant data. We have looked at 5 neuron cultures in each condition in the current study. Hence, we are on the low end and cannot draw any conclusions at this time. Special care must be taken to make sure experiments are biologically sound and that the changes observed are from the biological samples and not from the experiments. Hence, controls are essential to draw conclusions.

I believe that all of the conclusions we draw from experiments with biological samples must first be validated with established techniques to confirm their validity. We must pay attention to a lot of the details beyond the cell passage number.

Sebastian Schlücker responded: I consider the present work to be a proof of concept study for highlighting the importance of obtaining reproducible results in repeated iSERS microscopic experiments. The number of cells investigated in the iSERS experiments presented here is certainly not statistically relevant.

Lauren Jamieson addressed Sebastian Schlücker, Sumeet Mahajan and Jean-Francois Masson: In the community, SERS is proving its advantages in terms of detection limits, multiplexing capability *etc.* but it is still struggling to replace biological and clinical analysis techniques in current use. What do you think we need to do/show to convince biologists and clinicians to adopt these methods?

Jean-Francois Masson replied: One of the main criteria for adoption, in my opinion, is to develop techniques that use skills or methods biologists and clinicians are used to working with. The learning curve must be rapid for ensuring translation to these fields. This is one consideration we have taken in our project, for example using SERS on a patch clamp, a technique they are used to working with. We also make sure that our workflow is as closely related to the ones they are used to. Lastly, we need to work in close collaboration with biologists and clinicians to ensure compatibility of the methods we develop and to facilitate technology transfer.

Sumeet Mahajan responded: There are multiple reasons for why SERS is finding it hard to replace biological and clinical techniques. Some of the reasons are related to workflow, discipline-specific scientific culture *etc.*, and therefore exist despite SERS having numerous advantages. SERS has still not entered the workflow of a typical biology or medical lab, it is still seen as a very much physical technique which works in the hands of physicists/chemists. The nature of the

questions and problems biologists and clinicians address does not allow them to delve into the intricacies of the techniques they use. Therefore, till we come up with standard protocols and, in general, standardise SERS experiments and have instruments to go with that, it will be hard to convince biologists and clinicians to try SERS on their own. However, what we can do is that when we try to solve biological/medical problems besides playing to the strengths of SERS, we must not take the biology for granted. I agree that having a good collaborator on board and involved can help avoid mistakes obvious from a biological/medical perspective and makes the studies robust to be acceptable to bio- and medical-research community. Another issue is where we publish - biologists/clinicians do not necessarily read the journals chemists and physicists like to publish in; our choice of keywords, which might appear trivial, can be important if we want our work to be read by a different research community. All of the above can go a long way to help translate techniques for use by biologists and clinicians.

Sebastian Schlücker responded: I think we need to clearly demonstrate the unique advantages of SERS by benchmarking it against existing approaches such as immunohistochemistry and immunofluorescence in pathology. Good arguments are, for example, saving time and money, better data quality as well as making better/more reliable clinically relevant decisions, ideally not only with diagnostic but also therapeutic relevance.

Rohit Chikkaraddy addressed Sumeet Mahajan and Sebastian Schlücker: Can you please comment on the strong and sharp SERS line $\sim$1000 cm^{-1}, which is evident in most of the SERS spectra of nucleotide, neurotransmitter and cell work? Why is this bond so stable compared to all other lines around $\sim$1600 cm^{-1} which are broad and fluctuate over time?

Sumeet Mahajan responded: The 1000 cm^{-1} peak is one of the most recognisable peaks in spectra from biological material, cells and tissue. It is attributed to the ring breathing mode of the aromatic phenyl ring structure found in phenylalanine. Phenylalanine is an essential amino acid and is taken up by all cells for protein synthesis among other things. Phenylalanine is also a precursor to a number of neurotransmitters. Therefore it is always observed from cells/tissue. I am not sure it should be observed in the spectra of pure nucleotide bases. Phosphated nucleotides also give a peak around 1050 cm^{-1} and this is different (and often broad) from the phenylalanine peak. The broadness of the peaks in the 1600 cm^{-1} region can be attributed to both spectral congestion (as a number of biochemicals have peaks in that region) and also inter-molecular and hydrogen-bonding interactions. For instance, the amide I band at 1600 cm^{-1} can be affected by such interactions. The lack of hydrogen-bonding interactions of the phenyl structure (which is essentially hydrophobic) in phenylalanine accounts for the relative sharpness of the peak at 1000 cm^{-1}.

Sebastian Schlücker answered: All of the Raman peaks observed in the iSERS approach are SERS signals from the Raman reporter molecules which are present as a self-assembled monolayer on the surface of the gold nanostars, *i.e.* the signals are not conventional Raman scattering from the cell itself. Both peaks can be assigned to phenyl rings modes. The peak at $\sim$1600 cm^{-1} in the non-reproducible

case is assigned to the Raman reporter and one of its photoproducts due to photodegradation induced by too-high laser power densities.

Pieter Wuytens commented: Can gold nanoparticles escape the lipid vesicle during the *endo*-lysosomal pathway? How likely is this to happen?

Sumeet Mahajan replied: Yes, indeed nanoparticles can escape during the *endo*-lysosomal pathway. The escape depends a lot on the size, shape, surface charge and the corona of the nanoparticle and of course on the types of cells themselves. In my understanding for spherical nanoparticles incubated in FBS containing media, that is, the nanoparticles have a well formed protein corona, the escape likely occurs at the lysosomal stage when the proteins (and hence much of the protein corona) are degraded, which allows the nanoparticles to disrupt the vesicular membrane and escape. However, this needs testing or some evidence in literature might already exist.

