Intracellular Analysis by SERRS and Nanoparticles

R. Stevenson[*], L. Senior[*], J. Alexander, M. Geboers[*], K. Faulds[*] and D. Graham[*]

[*] Centre for Molecular Nanometrology, Department of Pure and Applied Chemistry, WestCHEM, University of Strathclyde, Thomas Graham Building, 295 Cathedral Street, Glasgow, United Kingdom, G1 1XL

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, John Arbuthnott Building, 27 Taylor Street, Glasgow, United Kingdom G4 0NR

ross.stevenson@strath.ac.uk

ABSTRACT

We report the use of 'bare' nanoparticles as both a cellular delivery vehicle and a spectroscopic detection platform to enable surface enhanced resonance Raman scattering (SERRS) detection within cells. We have shown how this approach can be used to observe the conversion of a specific Raman active substrate whose enzyme is naturally occurring within that cell line. The technique has been used to localize unique Raman signals at sites within a cell, suggesting a novel approach for enzyme localization and representing a major advancement upon both current SERRS and fluorescence based research.

Keywords: SERRS, SERS, nanoparticles, intracellular detection.

1 INTRODUCTION

Raman spectroscopy (RS) provides information on discreet modes of molecular vibrations from an analyte, requiring the collection of light inelastically scattered from the target molecule. It is an inherently weak process with only an estimated 1 in every 10^6 photons scattered inelastically, meaning sensitivity can be an issue. In 1974, Fleischman and co-workers found it possible to significantly enhance the Raman output by introduction of a strongly scattering neighbouring metal surface.[1] This development led to surface enhanced Raman scattering (SERS) and early work studied analytes adsorbed electrochemically to roughened silver electrodes that provide an excellent scattering surface. Most coinage metal surfaces can be applied to SERS and since the initial discovery, the majority of researchers have used either nano-engineered roughened metallic surfaces or colloidal metal nanoparticles to facilitate SERS. Gold and silver nanoparticles are generally favoured for most applications owing to their large surface area, low cost and ease of synthesis. It's estimated that more than 1000 SERS research articles are published annually, making SERS studies one of the largest growing spectroscopic research areas.[2] In some cases, further signal enhancement can be achieved by careful consideration of the wavelength of the incident light source. If a laser wavelength can be selected that is in resonance with the absorbance maxima of the nanoparticle and the analyte, surface enhanced resonance Raman scattering (SERRS) can be achieved. This is ideally illustrated where an additional enhancement is obtained through molecular resonance by using an analyte containing a chromophore with an electronic transition close in frequency to the excitation frequency of the laser used. It has been demonstrated that enhancements of up to 10^{14} are possible with SERRS.[3] SERS and SERRS have both been used to detect sub nM levels of oligonucleotides, small molecules and proteins.[4; 5] The techniques also offer the opportunity to resolve multi component mixtures in a non-intrusive manner.[6; 7] Raman spectra contain fingerprint regions where multiple sharp vibrational peaks are observed across a relatively small bandwidth, around two orders of magnitude narrower than fluorescence profiles. The ratios of peak heights and their exact placement are indicative of unique species making it possible to observe a number of molecules with similar spectra, indeed it has been shown possible to distinguish 3 Raman tags uniquely from one another across a spectral region of ~ 6 nm.[8] This makes the technique stand out above the fluorescence alternative where broad spectral overlap limit the multiplexing potential.

The ability to detect and quantify specific proteins and enzymes within a cell would be a major advancement to the field of proteomics. Any tool capable of detecting the variation of cellular protein levels could provide key insights to cell cycle control and also how cells deal with extracellular stimulation. The control of cellular machinery and the factors that cause the up- and down-regulation of genes within a cellular environment is a research area still in its infancy. Current techniques to observe and quantify cellular proteins rely largely on the destruction of cells by lysis, and the separation (generally electrophoretical methods) and quantification (typically by Western Blot) of

the proteins contained within. The ability to quantify proteins/enzymes *in vitro* and further the ability to localize multiple targets to specific areas within the cells remains elusive. Fluorescence approaches can be used to detect specific targets within a cell. The most common approaches involve either the introduction of antibodies covalently attached to fluorophores/quantum dots, or the use of recombinant technology to fuse green fluorescent protein (GFP) or one of its derivatives to the antigen of interest or to a relevant protein.[9; 10] There are suggestions that attaching proteins or antibodies to large unwieldy quantum dots or large proteins (i.e. GFP) will prevent many proteins from acting in a wild-type manner within the cell.[11] For this reason the attachment of small molecules to an antibody may be the preferential approach to the detection and localization of specific proteins in a true *in vitro* manner. Unlike SERRS, fluorescence techniques also suffer from photobleaching which can lead to major problems.[12] Here we report the measurement of enzyme activity within cells using SERRS and nanoparticles.

2 EXPERIMENTAL

2.1. Optimum SERRS Conditions

To ensure that optimal Raman signals were observed in the *in vitro* analysis, plate based solution experiments were first carried out with a range metallic nanoparticle solutions and differing wavelengths of excitation. The earliest analysis looked to ensure that a change in the Raman signal was achieved when the enzyme acted upon substrate (Figure 1(A)). Next it was necessary to ensure that surface enhanced Raman and not just Raman spectroscopy was being observed. The substrate was added to a well containing reaction buffer, enzyme and either phosphate buffered saline (PBS) (control), gold colloid (20 nm) or silver colloid (40 nm). By observing the difference in Raman spectra between the control and the nanoparticle solutions, it was clear to see that the introduction of a strongly scattering surface led to an enhancement in the Raman signal, thus it was reasonable to conclude that SERS was being observed (data not shown).

Further it was possible to use different wavelengths of excitation to observe the best Raman spectra. Each condition, gold, silver or PBS, was observed with excitation at 514.5 nm, 632.8 nm and 785 nm. The data collected, showed clearly that gold colloid and excitation at 632.8 nm had the optimal SERRS response. By showing that a single wavelength excitation source elicited a greater response than either of the alternatives, it shows that SERRS must be the underlying mechanism for the greater enhancement. Gold colloid has an absorbance maximum around 520 nm and the product has an absorbance with a maximum peak at 652 nm. By coupling the two with excitation at 632.8 nm, best Raman results were observed.

A time course study was set up to analyse the emergence of the Raman signal over a defined period in the presence or absence of gold nanoparticles. The study was carried out in triplicate over 30 minutes with sample data collected every 5 minutes. Results obtained show that as the enzyme acts on the substrate, the increase in Raman signal can be monitored by measuring the change in peak height at 600 cm^{-1} (shown in Figure 1 (B, C)). Almost a 10 fold greater response for the time analysis was achieved with nanoparticles present than without. Further our analysis showed that the Raman signal can be collected in a dose dependant manner (data not shown).

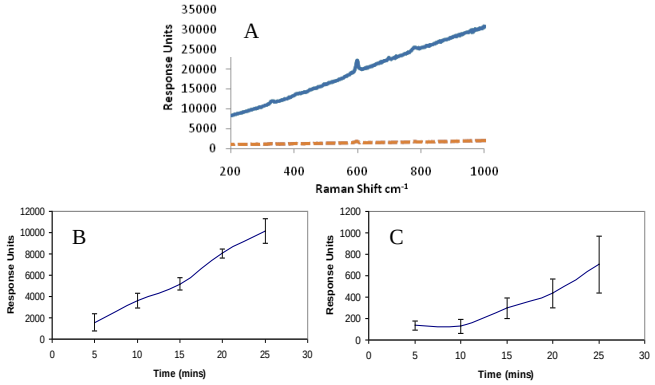

Figure 1. Raman spectra and time course analysis for the emergence of Raman signal. The Raman spectra (A) for substrate with enzyme (blue line) and without (red dashed line). The emergence of a peak at 600 cm^{-1} was observed over a time course in the presence (B) and absence (C) of gold nanoparticles. Analysis was carried out at 632.8 nm, in triplicate.

2.2 Cellular Studies

After optimization of SERRS conditions, nanoparticles and substrate were added to cells to test if it were possible to analyse the production of a Raman active product within a cellular environment. Macrophage cells were used in the analysis as not only do they naturally express the wild-type enzyme, they are also naturally attuned to uptake foreign materials by either endo- or phago-cytosis. Macrophage cells have previously been used to internalize nanoparticle and evidence collected to date suggests they easily uptake metallic colloidal solutions.[13] In our approach, we first looked to analyse the cytotoxicity of gold nanoparticles in the cellular environment. Cells had gold nanoparticles (100 µl, varying concentration) added, and the cell viability was checked periodically over 120 hours (Table 1). Over the first 3 days there appeared to be no deleterious effect of the presence of nanoparticles. After 5 days there appeared to be evidence of cytotoxicity, however around the same period the control cells also were deteriorating suggesting that the 5 day result should be disregarded.

		Cell Viability (%) ± Standard Deviation		
Nanoparticle	**Concentration (pM)**	**Day 2**	**Day 3**	**Day 5**
Silver	**0**	100 ± 0	100 ± 0	76.1 ± 14
	10	100 ± 0	100 ± 0	48.55 ± 3.6
	100	100 ± 0	100 ± 0	57.9 ± 25.6
Gold	**0**	100 ± 0	100 ± 0	90.25 ± 5.4
	10	100 ± 0	100 ± 0	92.85 ± 1.1
	100	100 ± 0	100 ± 0	87.15 ± 11.1

Table 1. Nanoparticle cell viability assay. Gold nanoparticles (20 nm, 1 nM) were added to cells and the cell viability was analysed periodically over 5 days.

To analyse the SERRS signal *in vitro*, nanoparticles and substrate were added to cells grown on glass cover slides and were left to incubate for 48 hours, giving the foreign material time to act within the cells. Upon completion of the time course, coverslips were washed with DMEM buffer, fixed with 4% glutaraldehyde and attached to a glass slide using DPX mountant. Fixed cells were then point scanned using a Renishaw Raman spectrometer and a 632.8 nm excitation source. Spectra were observed from inside (B) and outside the cell (C), as shown in Figure 2. Accumulation times of 2s were sufficient to observe the substrates Raman signal intracellularly and further the low background noise from the extracellular environment allowed easy comparison of Raman active areas.

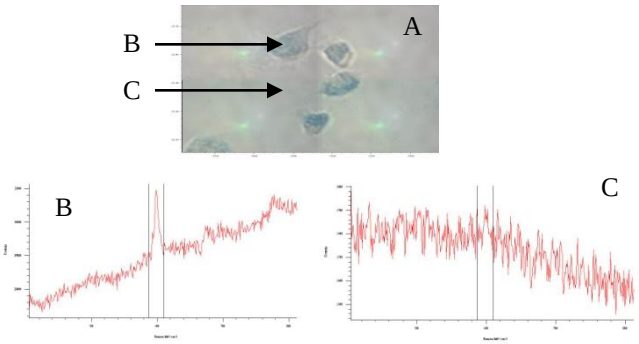

Figure 2. **Cellular Raman detection**. Raman spectra observed intra- and extracellularly, (B, C respectively). The region for the Raman peak is highlighted in both, around the 600 cm^{-1} region. The bright field image (A) shows the points from where both spectra were recorded. Four cells can be seen in figure A.

3 DISCUSSION

SERRS has been used to detect intracellular enzymes working in an *in vitro* environment. This work differs from those technologies already available as it allows the non-destructive analysis of a cellular component by Raman spectroscopy. The use of SERRS allows multiplexing potential that far outweighs any fluorescent alternative and the absence of photobleaching from SERRS gives the technique a further edge. For *in vitro* analysis of enzyme activity, a solution based assay was first prepared to observe the substrate acted upon by the enzyme in idealized conditions. By finding the optimum nanoparticle and wavelength combination, it was possible to move to cells with the approach. In the earliest cell experiments, nanoparticles were added to cells in a controlled manner, allowing observation of the toxicity of 20 nm gold nanoparticles to macrophage cells. In line with previously published results, our data suggests that nanoparticles have no deleterious effects on the viability of cells.

Nanoparticles and enzyme substrate were then added to cells to allow the analysis of the SERRS signal intracellularly. Observation of the unique Raman peak at 600 cm^{-1} clearly showed that it was possible to observe the turnover of substrate by enzymes contained within the cellular matrix. Further, the low background signal from the extracellular environment allowed easy comparison of areas of positive Raman signal. Although this work requires further investigation, the data collected suggested that there were discreet areas within the cellular environment where larger Raman spectra were observable. This may indicate that either larger numbers of nanoparticles or turned over substrate were localized at specific regions within the cell. Although this is still preliminary research, results collected to date suggest that SERRS could be used as a tool to probe intracellular compartments, and localize cellular machinery to nm resolution.

4 CONCLUSIONS

This report shows the feasibility of the use of SERRS to detect Raman fingerprints from within a cellular environment. Our results have shown that by careful consideration of nanoparticle substrate and the laser excitation source, optimal Raman signals can be observed. In this report a Raman active substrate was used and its conversion by enzymes in a solution based, then by wild-type enzymes in an *in vitro* environment were monitored using Raman spectroscopy. Our results show it possible to observe the substrates conversion within the cell, and further it may be possible to pin-point the signal to specific locations at nm resolution. The development of this technology may lead to real time detection of multiple proteins or antigens within a cell by SERRS, providing new opportunities in proteomics research.

REFERENCES

[1]M. Fleischmann, P.J. Hendra, A.J. McQuillan. Chemical Physics Letters 26 (1974) 163-166.

[2]D. Graham, R. Goodacre. Chemical Society Reviews 37 (2008) 883-884.

[3]X. Zhang, J. Zhao, A.V. Whitney, J.W. Elam, R.P. Van Duyne. J Am Chem Soc 128 (2006) 10304-10309.

[4]K. Faulds, L. Fruk, D.C. Robson, D.G. Thompson, A. Enright, W.E. Smith, D. Graham. Faraday Discuss 132 (2006) 261-268; discussion 309-219.

[5]S. Nie, S.R. Emory. Science 275 (1997) 1102-1106.

[6]K. Faulds, R. Jarvis, W.E. Smith, D. Graham, R. Goodacre. Analyst 133 (2008) 1505-1512.

[7]K. Faulds, F. McKenzie, W.E. Smith, D. Graham. Angew Chem Int Ed Engl 46 (2007) 1829-1831.

[8]G.v. Maltzahn, A. Centrone, J.-H. Park, R. Ramanathan, M.J. Sailor, T.A. Hatton, S.N. Bhatia. Advanced Materials 21 (2009) 3175-3180.

[9]W. Margolin. Methods 20 (2000) 62-72.

[10]A.P. Alivisatos, W. Gu, C. Larabell. Annu Rev Biomed Eng 7 (2005) 55-76.

[11]B. Ludin, A. Matus. Trends in Cell Biology 8 (1998) 72-77.

[12]M.D. Porter, R.J. Lipert, L.M. Siperko, G. Wang, R. Narayanan. Chemical Society Reviews 37 (2008) 1001-1011.

[13]J. Kneipp, H. Kneipp, M. McLaughlin, D. Brown, K. Kneipp. Nano Letters 6 (2006) 2225-2231.