JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2004; **47**: 493–500.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.832

Research Article

An investigation into the use of Raman microscopy for the detection of labelled compounds in living human cells

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Summary

We report on the first detection of vibrationally labelled compounds by Raman microspectroscopy in living cells and demonstrate the feasibility of this technique for bioanalysis. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: deuterium; raman microspectroscopy; caco-2 cells

Introduction

Recent developments in bioimaging, using confocal fluorescence microscopy and related fluorescent techniques have made a major impact in molecular biology. These techniques have made possible the direct imaging of the dynamics of molecular, structural and chemical environments at a cellular level. They provide a wealth of information using a spatially and time resolved spectroscopic technique in living cells. These techniques have over the last two decades immensely contributed to our understanding of the structure and function of biological macromolecules.[1,2]

The basic principle underlying fluorescent measurements requires the covalent attachment of a fluorescent probe to the biomolecule under investigation. While for proteins and DNA the fluorescent probe hardly changes the biological activity, structure, physical and chemical properties of

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Contract/grant sponsor: Surrey Foundation Fund
Contract/grant sponsor: The Royal Society
Contract/grant sponsor: AIRC
Contract/grant sponsor: EPSRC

Received 18 September 2003
Revised 3 April 2004
Accepted 15 April 2004

the biomolecule, attachment of a fluorophore to a small molecule, e.g. a drug or a lipid of low molecular weight, changes these parameters considerably. For this reason, investigations using fluorescent labelled small molecules have to be evaluated with great care and some scepticism and require alternative methods for their study.

In this contribution we suggest a complementary technique, namely Raman microscopy or micro-spectroscopy,[3,4] as a promising spectroscopic research tool allowing the time resolved study of the dynamics of molecular, structural and chemical environments at the cellular level. This technique does away with the necessity for a high molecular weight molecular label that changes the fundamental properties of the biologically active molecule under investigation.

Raman spectra of both tissues and single cells have been obtained using micro-spectroscopy. Spectra of animal cells,[5,6] plant cells and bacterial cells have been recorded.[7–9] Both direct techniques[6–14] and SERS techniques[15] have been used. Bacterial cells have been studied for identification purposes as well as for the multidimensional analysis of their chemical composition.[9] To the best of our knowledge there have been no reports on the use of vibrationally labelled compounds in living cells using either Raman or infrared micro-spectroscopy.

As an alternative bioimaging method we would like to introduce the concept of vibrational imaging using vibrationally labelled compounds in conjunction with Raman micro-spectrometry. As labels we chose deuterated compounds as well as isothiocyanates. Both the C-D and N = C = S stretching frequencies are observed at wavenumbers not obscured by water absorption. It should therefore be possible to directly monitor labelled compounds in aqueous biological systems. The C-D stretching frequency at 2200–2100 cm^{-1} and N = C = S at 2110 cm^{-1} offers the additional advantage that they do not interfere with any other bands in biological systems. The use of the deuterium label should allow a general approach to the detection of any chosen compound of biological interest, providing a deuterated version of the compound is accessible using synthetic chemistry and the label does not exchange on the time-scale of the experiment. The physical, chemical and biological properties of the labelled compounds remain largely unaffected by the deuterium label allowing relevant data on the distribution and chemistry of the labelled compound at the cellular level to be obtained.

Results and discussion

Setting the scene: Raman micro-spectroscopy of onion epidermis cells

In order to obtain an insight into the performance of our microspectrometer (Renishaw 2000 Raman microscope with Diode laser at 782 nm or Argon ion laser at 512 nm) we decided to record Raman spectra of onion epidermis cells[16]

prior to the more sensitive human cells. Beside the sensitivity of the human cells the plant cells show a very defined cellular structure under view by the inverted optical microscope coupled to the micro-spectrometer. This feature would also allow us as well to assess the possibility of analysing the chemical composition of individual organelles and compartments of the plant cell. Furthermore very little Raman micro-spectroscopy of living plant cells has been reported in the literature.[7,8]

Carrier material: Different carrier materials on which the cells were to be mounted or grown at a later stage were tested for their performance. The ideal carrier material should be reflective to allow sufficient Raman light back onto the detector, be compatible with the living cells, both biologically and chemically and should possess at least one Raman active resonance that would allow focussing of the cell sample. Ca F_2 was shown to be a superior material by Schuster *et al.*[9] but a solubility of 32 mg/l in H_2O of this material seemed to be unsuitable for growing human cells over a period of several days on this carrier material. We decided to investigate stainless steel, aluminium sheets and polished silicon wafers as the materials of our choice. All materials gave a good signal to noise ratio using the onion epidermis cells (see Figure 1).

Focussing: Although not reported in detail in previous work on Raman micro-spectroscopy we found that focussing the laser spot into the cell proved to be an exceptionally capricious task. The Raman signals from the cellular components within the cell interior are very weak. For this reason we decided

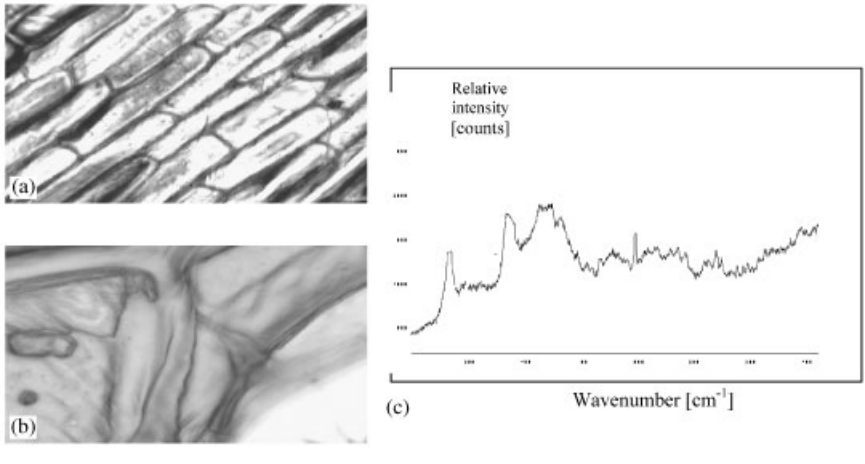


Figure 1. (a) Onion epidermis cells on stainless steel at $\times$200 magnification through inverted microscope; (b) Onion epidermis cells at $\times$500 magnification through inverted microscope; (c) Raman spectrum from centre of b using a 782 nm diode laser and 64 scans acquisition

to use polished silicon wafers as our carrier material of choice. We used silicon wafers of chip manufacture quality that show an even surface and a strong Raman line at 522 cm^{-1}. This line allowed us to adjust the laser focus in each individual experiment by optimizing the intensity of this Raman band. Once the laser focus in the z coordinate was optimized we further assumed that the cell material would be found between 1 and 3 μm above the silicon surface. All experimental spectra acquired did show that this assumption was valid. Polished silicon is suffers from the disadvantage that a first harmonics and third harmonic vibrations as well as Si–O surface vibrations are obscuring the spectrum around 942 and 1900 cm^{-1}.[17] On the positive side these signals could be easily used as internal standards in our measurements.

Time scale of experiment: Since within the technique a high-energy laser is focussed onto a living object the change of temperature of the sample over the period of the spectral acquisition seemed to us to be an important parameter to consider in order to obtain biologically meaningful results. Using a sample of onion epidermis cells covered in 0.5 ml of serum medium or phosphate buffer, respectively (and later Caco-2 cells), we found that at a laser power of 21 mW and a starting temperature of 20°C (as determined by an infrared thermometer measuring the sample surface temperature) a temperature of 40°C was reached within 280–300 s. From these results we concluded that the timeframe, in which acquisition of the spectra should take place should be ideally below 5 min, therefore avoiding thermal damage to the living cells. For future imaging experiments cooled microscope stages would have to be devised to allow longer acquisition times.

Spectra from the cells: Slices of onion epidermis cells have been prepared according to the literature[16] and spectra have been obtained with excitation wavelengths of 514 nm (Argon ion laser) and 782 nm (diode laser). Background fluorescence was, as expected, significantly higher using the 514 nm excitation, however, reasonable quality spectra could nevertheless be obtained.

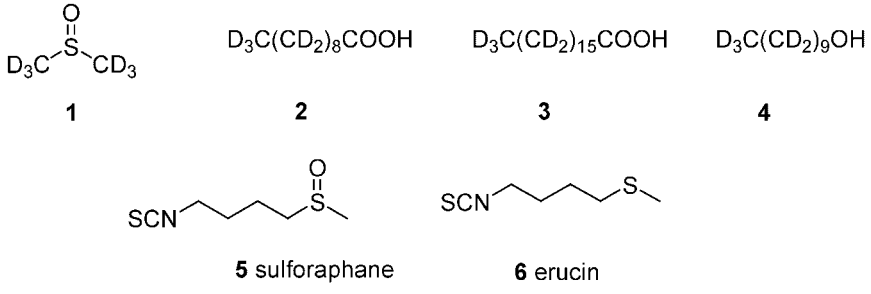
Focussing of the laser onto the cell was achieved using the optimization of intensity of the 522 cm^{-1} Si–Si absorption of the carrier material followed by moving the laser focus up by 3 μm. Both line-scan and depth-scan methods as introduced by Schuster *et al.* were used to demonstrate unambiguously that the laser focus was within the cell and the spectra originated from the cells.[9]

A number of interesting bands appear in the spectra. For tentative assignments have been presented in the literature.[7,9]

Use of vibrationally labelled compounds and their detection in living Caco-2 cells

As our first deuterated molecule of choice we opted for deuterated dimethylsulphoxide (d_6-DMSO **1**). DMSO is known to efficiently penetrate

cell walls and accumulate in cells without being toxic at concentrations of up to 4 M in aqueous solution. Furthermore, work by Friend has shown that DMSO possesses interesting biological activity on human leukemia cells.[18,19] Deuterated DMSO is a standard solvent in NMR spectroscopy and is commercially available at high deuterium incorporation and purity.



Scheme 1.

Caco-2 cells grown on a polished silicon wafer (Caco-2 cell culture conditions: The Caco-2 human carcinoma cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 2 mM glutamine, 1% non-essential amino acids, 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2, 95% O_2 atmosphere. Cells were seeded onto autoclaved silicone wafers in six well cell culture plates at a density of approximately 10^6 cells per ml. Cells were cultured for 14 days post-confluence in order to differentiate the cells. For references see: Ho *et al.*[20]) were treated with a 1 M solution of d_6-DMSO in aqueous cell culture medium for 2 min and thoroughly washed with culture media.

Focussing of the laser was carried out as previously described using the Si–Si band. To improve sensitivity we opted to use the acquisition of static spectra centred around 2100 cm^{-1} with a total acquisition time of 4 min. Figure 2 shows the spectra of d_6-DMSO obtained from within the cell. Since the Caco-2 cells grow confluently line scans cannot provide an answer as to whether the spectra recorded were from within the cell. In this case only a depth scan can unambiguously show the origin of the spectra. As seen in Figure 2 the C-D stretching frequency at 2141 cm^{-1} is only observed in a spectrum recorded at a focus of 2 μm above the silicon surface. A spectrum recorded 10 μm above the Si surface shows no C-D signal, but some background fluorescence, presumably due to the fluorescent components of the culture medium. A spectrum 100 μm above the surface shows neither a C-D band nor significant background fluorescence. It is worth noting that by optical inspection, the

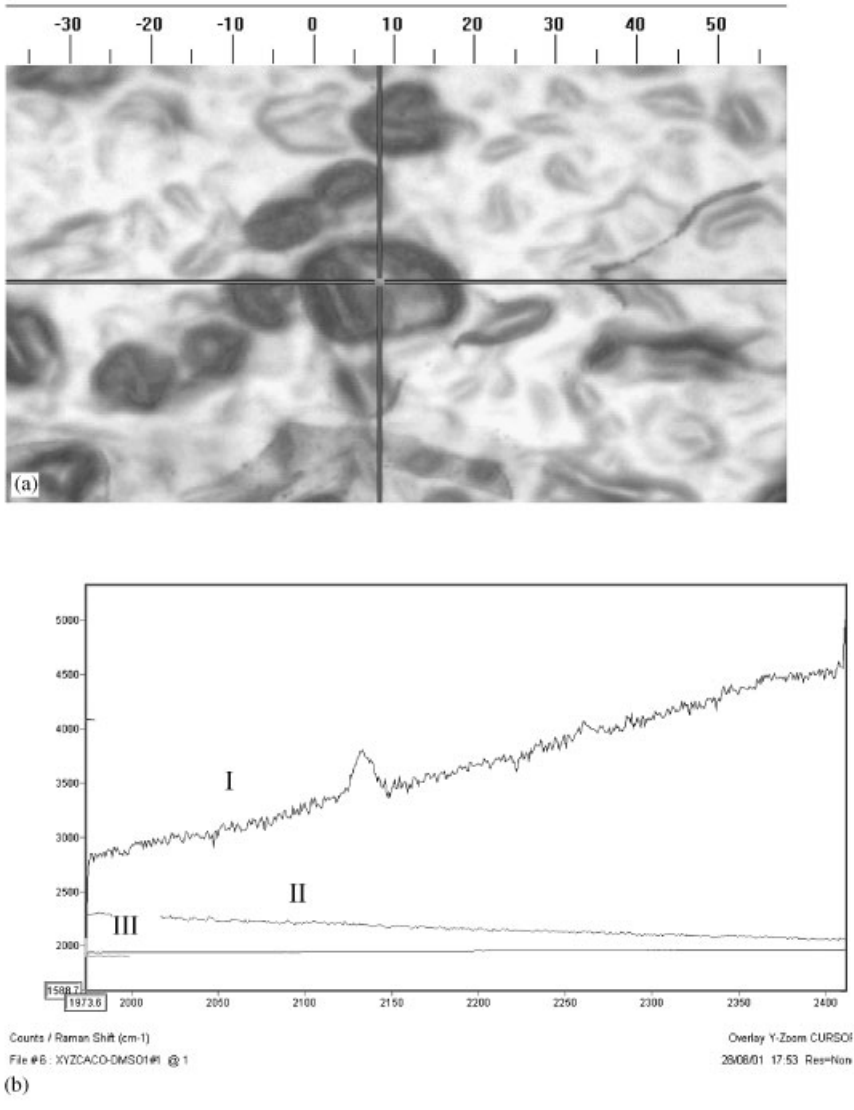


Figure 2. (a) Caco-2 cells using inverted optical microscope $\times$ 500 magnification; (b) Raman spectra (782 nm diode laser, static spectrum centred at 2100 cm^{-1}) 4 min acquisition time of Caco-2 cells incubated with 10% aqueous d_6-DMSO 1; I Spectrum from within cells (2 µm above Si surface); II Spectrum 10 µm above Si surface; III Spectrum 100 µm above surface

Caco-2 cells show no visible damage after treatment with DMSO and subsequent laser irradiation.

To obtain insight into the actual d_6-DMSO concentration within the cell and the sensitivity of the experiment we recorded a calibration curve of d_6-

DMSO in aqueous solution. From the calibration curve it can be estimated that the actual DMSO concentration within the cell as measured by Raman micro-spectroscopy is about 8 ± 1 mM. A x/y line scan over the array of the cells revealed concentration differences of d_6-DMSO of up to 20%. However this experiment needs to be evaluated with great care since increased acquisition times could result in evaporation of culture medium resulting in concentration changes.

After the successful detection of [D-6]-DMSO we also attempted to detect fully deuterated fatty acids **2** and **3**, however without success. Deuterated alcohol **4**, however, could be detected after 2 min incubation of the cells followed by washing as previously, using its C-D stretching frequencies at 2140 cm^{-1}. The chemopreventive isothiocyanates sulphoraphane **5** and erucin **6**[21,22] whose medium strong NCS vibration at 2108 and 2110 cm^{-1} could be easily detected in 0.1 M aqueous solution with our instrument were also incubated with the cells. After incubation, however, no NCS vibration could be detected suggesting that the isothiocyanates were rapidly metabolized.

Conclusion

In conclusion we have shown that Raman micro-spectroscopy is a valuable tool in the analysis of living plant cells. The technique is non-invasive and non-destructive, no chemical reagents are required for staining the cells and the sample preparation is extremely simple.

Most importantly, we have demonstrated the feasibility of using vibrationally labelled compounds, in our case using the C-D label, to detect labelled compounds within living human cells. Although the concentrations of labelled compounds employed and detected here are an order of magnitude in concentration higher than most biologically relevant concentrations the technique is viable. Future research will concentrate on improving the sensitivity and experimental set-up in order to achieve better detection limits. The new technique offers promise as a new bio-imaging technique, avoiding the necessity of a fluorescent label altering the chemical and biological properties of a small bioactive molecule or radiolabelled compound. We hope that the technique will in future be a valuable tool in the study of biological problems.

Acknowledgements

The authors acknowledge financial support for this work from the Surrey Foundation Fund, The Royal Society and the AIRC. EPSRC funding for the Renishaw Raman microscope is acknowledged. We thank Wacker Chemicals (Burghausen, Germany) for a donation of silicon wafers. Furthermore we thank Dr Henryk Herman for helpful discussions and Prof. J. R. Lu (UMIST, Manchester) for a gift of deuterated fatty acids.

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