Biopharma Imaging and Analysis

Advancing Towards a More Detailed Picture of Chemistry

By Dr A Knowles, Raman Division Manager and Dr S FitzGerald, Raman and XRF Micro-Analysis Specialist at Horiba JobinYvon

Dr Adrian Knowles has been a member of the Raman division of Horiba Jobin Yvon for over nine years. He has been involved in promoting and developing the technique within ever broadening application fields. A background in Raman and vibrational spectroscopy was established at university, where his PhD research involved Raman and FTIR analysis, microscopy in inorganic complexes and solution chemistry. He now holds a role in the business development of such techniques in key application areas such as pharmaceutical analysis, HTS screening and the life sciences.

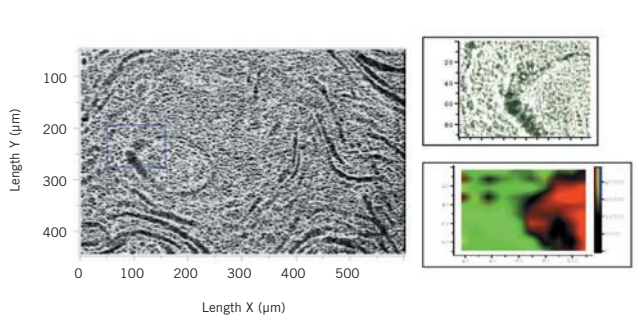
Dr Simon FitzGerald is the Raman and XRF microscopy specialist for Horiba Jobin Yvon in the UK. His interest and experience in spectroscopy began at Durham University, where he read Chemistry and was involved in time resolved spectroscopic analysis of luminescent materials for industrial and medical applications. Since joining Horiba, he has been working in close partnership with international researchers to establish the use of new spectroscopic methods for sample analysis and imaging.

There is a wealth of analytical and imaging techniques available for the measurement of biopharma samples. The samples themselves can be wide-ranging and take numerous forms: single cells, tissues, crystals or blended formulations. To study and develop such samples many techniques, including fluorescence microscopy, NIR imaging, AFM and SEM, have been used.

So why add two more techniques to this list? What these and many other techniques do not do is to characterise the chemistry and composition of the sample under examination. Raman and x-ray fluorescence (XRF) microanalysis offer information in these further dimensions; Raman is particularly good at probing the biochemistry, whilst micro-XRF provides information on elemental composition. With recent advances in technology, both of these techniques allow samples to be analysed on the micron scale.

Figure 1: Raman of Histological Sample of an Aorta

White light image of extended area (left), magnification of image (top right), and Raman chemical image of the section (bottom right)



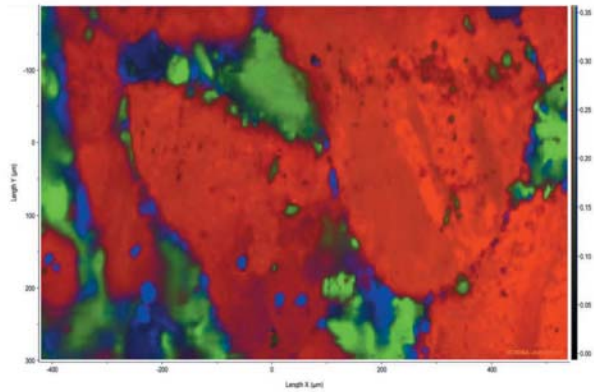
RAMAN SPECTROSCOPY

Raman spectroscopy is not a new technique; it was first described by CV Raman in 1928, but it is only in recent years that it has become a useful routine tool in the analysis and study of biological and pharmaceutical samples. The technique uses light scattered from a laser to build up a chemical fingerprint characteristic of the sample. The single colour laser light is scattered from the sample at different wavelengths and these various wavelengths of light provide the molecular and structural information. It is extremely sensitive to subtle changes in the chemistry of a sample and since it is a technique using light irradiation, it can be adapted for use on a microscope. In the last few

years, the potential of the confocal Raman microscope has become apparent, coupling chemical information with microscopic scale. The advantage of the new generation of fully confocal Raman microscopes is that they have the ability to very precisely discriminate the area of the sample being analysed, thus determining the chemistry of individual particles or cells. With advances in laser technology such as narrow beam NIR laser diodes, and advances in CCD detector technology (open electrode, deep depletion and back illuminated chip formats) the sensitivity of the latest Raman microscopes enables analysis and images to be obtained far more quickly than previously possible. This means that samples delicate or sensitive to environmental conditions can now be analysed with greater ease and with less risk of degradation.

Figure 2: Raman Mapped Image of a Tablet Formulation

The different colours result from the various components present within the tablet



X-RAY FLUORESCENCE (XRF)

Energy dispersive XRF has long been a useful analytical tool for macro sampling, providing fast, high sensitivity elemental characterisation. Typical instruments incorporate an x-ray generator or source which is used to irradiate the sample – interaction of the incident x-ray beam with the sample results in fluorescent x-rays being emitted, which characterise the elemental composition of the sample. An energy dispersive x-ray detector is then used to capture the whole XRF spectrum in one shot.

The initial forays into micro-XRF analysis were exciting but not overly practical – the intensities of obtainable narrow x-ray beams were extremely low, thus requiring unacceptably long acquisition times to produce usable data. It was only on specialised set-ups such as synchrotron sources that this intensity issue could be countered (1). Perhaps more importantly, for sensitivity to the lighter elements (for example, sodium, magnesium, aluminium and silicon), which are of great importance in bio-pharma applications, samples had to be subjected to high vacuum conditions – not the best environment for samples which are often delicate, powdered or water containing.

Recent developments which have seen the successful integration of mono-capillary x-ray optics into such XRF instruments represent an important advance. These groundbreaking capillaries allow highly efficient coupling to the x-ray generator/source without degradation in beam diameter. As a consequence, samples can now be analysed at atmospheric pressure at speeds up to 50 times faster than previously possible, and with spatial resolutions down to just 10 μm. The era of micro-XRF is truly born.

NEW DIMENSIONS FOR IMAGING

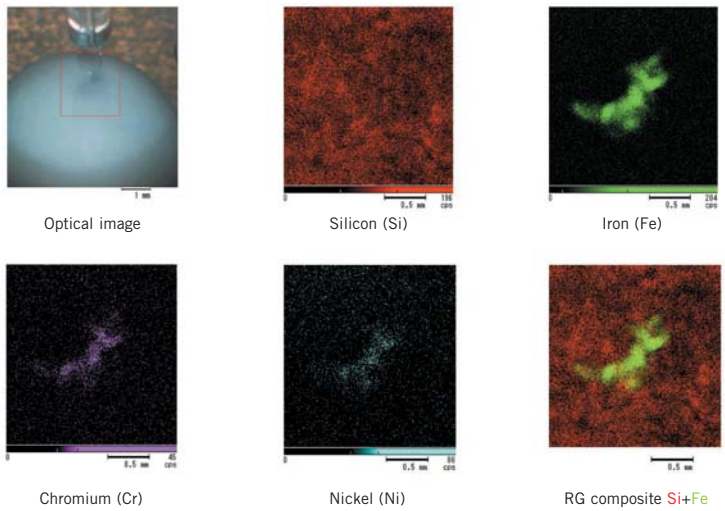
Fluorescence microscopy in its various forms has become an established technique for imaging cells and tissues. Fluorophores are used as tags which label biomolecules and are a means of detecting diseased states, DNA faults and biological mechanisms.

However, what fluorescence microscopy does not reveal is precisely what chemistry may be involved or discreet changes in composition or molecular structure. For example, Raman has been used to produce chemical images of cells and biological tissues where diseased states may be detected. An example of this has been in the analysis of cancerous tissues. The pathological state of tissue showing advanced malignancy is easy to determine through conventional microscopy of an histological sample, but for cases where the cancer is far less advanced or in its earliest stages, the visual changes in the cell become more ambiguous. However, even at this early stage the chemistry has begun to change and Raman has recently been shown to be capable of detecting this subtle change (2). It can also be used to image the localisation of further diseased states or active drug therapies within cells, elucidating where a drug should be targeted or where it may migrate. A technique such as Raman has the potential to detect and image even the subtlest changes in chemistry or structure.

The confocal Raman microscope can even be employed in the study of single cells. Researchers at the Centre for Ecology and Hydrology (CEH) in Oxford have recently demonstrated that the biochemical fingerprint generated by Raman microanalysis of single bacteria cells can be used to distinguish genus and growth stage, and to identify and quantify uptake of isotope labelled nutrients. That the majority of bacteria cannot be cultured in a lab makes the ability to analyse real populations at the single cell level extremely important (3).

The many advantages of Raman mapped imaging – such as high information content, high spatial resolution and non-destructive nature – can all be used to good advantage for imaging tablet formulations (4), so that the correct polymorph, good sample distribution (discrete particles rather than large agglomerates) and contaminants can all be determined.

Figure 3: XRF Mapped Elemental Images of a Tablet Formulation



The wealth of information provided by Raman allows the effects of mixing and compaction to be investigated, can identify subtle chemical polymorphs of active components, provides clarification and evidence for patenting, and provides far more information than just an image.

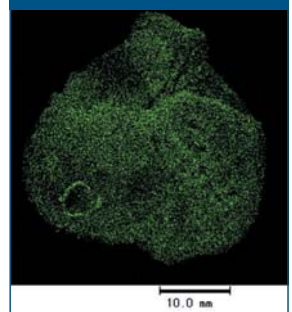
Such applications within the biopharma industry can also gain from the elemental information provided by micro-XRF.

This technique is ideally suited to foreign particle analysis, where it can provide fast identification of a contaminant. The penetration of the incident x-ray beam depends upon the matrix, and can range from millimetres (light element/organic matrix) to microns (heavy element matrix) – so, for pharmaceutical tablet formulations it is possible to benefit from penetration well beyond the visible surface, and as shown in the example, it allows foreign particles even deep within a tablet to be located and identified.

The elemental mapped imaging highlights the relatively uniform distribution of silicon containing stearate species, together with a small steel fragment (identified through primary composition of iron with smaller quantities of chromium and nickel). It is important to note that this major contaminant was not visible with standard optical techniques, and other methods to identify the particle would have required its removal and subsequent chemistry. In comparison, the speed and simplicity of micro-XRF is clear.

Targeted drug discovery and development depends on understanding of drug action, and this can be aided through XRF mapping of tissue sections following drug treatment.

Figure 4: XRF Image of Rat Stomach



This image illustrates the concentration of zinc; the gastric ulcer is clearly visible (bottom left)

For example, zinc components are believed to contribute to the healing of gastric ulcers, and so micro-XRF has been used as a fast but informative technique to probe this. A zinc containing medicine was orally administered to a rat suffering from a gastric ulcer. A subsequent tissue section was taken and soaked in formalin. Analysis of the tissue clearly identified increased zinc concentration in the immediate vicinity of the ulcer.

CONCLUSION

Whilst there are many fast optical imaging techniques used within biological and pharmaceutical research, these often provide only limited information. Emerging micro techniques such as Raman and XRF give access to a whole new dimension for imaging, combining spatial and spectroscopic information. Their non-destructive nature, with no requirement for complex sample preparation or pre-treatment ensures that natural composition is in no way perturbed.

By investigating real biochemistry and element distribution on the micron scale, it is possible to enhance drug design and production, unlock further secrets of drug-tissue interactions, and continue to gain insight into the complexities of the natural world. ◆

The authors can be contacted at info@jobinyvon.co.uk

References

1. **Kemner KM, Kelly SD, Lai B, Maser J, O'Loughlin EJ, Sholto-Douglas D, Cai Z, Schneegurt MA, Kulpa CF and Nealson KH, Elemental and Redox Analysis of Single Bacterial Cells by X-ray Microbeam Analysis, *Science* 306, p686, October 2004**

2. **Beljebbar A, Sockalingum GD, Morjani H and Manfait M, Raman and SERS microspectroscopy on living cells: a promising tool towards cellular-drug response and medical diagnosis, *Biomedical Applications of Raman Spectroscopy*, Michael D Morris, Abraham Katzir, Eds, SPIE, 3608, pp175-184, 1999**

3. **Huang WE, Griffiths RI, Thompson IP, Bailey MJ and Whiteley AS, Raman Microscopic Analysis of Single Microbial Cells, Analytical Chemistry, 76, pp4,452-4458, 2004**

4. **Horiba Jobin Yvon, technical application notes on microanalysis, http://www.jobinyvon.co.uk/ukdivisions/raman/application_notes.htm**