SYNCHROTRON INFRARED SPECTROSCOPY IN BIOLOGY AND BIOCHEMISTRY

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Introduction
The absorption of near infrared light by materials provides information about the chemistry and structure of those materials by probing their molecular vibrations. The infrared spectra of biological macromolecules have distinct absorbances that allow Fourier Transform Infrared (FTIR) spectroscopy to monitor macromolecular changes. FTIR can be carried out in aqueous solution, organic solvents, thin films, deposits, flow cells and membrane environments using μg quantities. Moreover, protein secondary structure can be determined, individual bonds in an enzyme can be monitored and processed on time scales of μs or a few ns with rapid scan- or step scan-based instruments. Lipid polymorph structure and lipid phase transitions can be studied along with the physical states and conformational transitions of nucleic acids. Carbohydrates and glyco-conjugates and the interactions between these molecules and proteins or lipids can also be monitored. Membranes can be studied as model systems, as isolated membranes, in cells or as subcellular fractions. The macromolecular structure and composition of tissue, whole cells and bacteria can be used to follow disease. Until the 1980s, infrared spectroscopy was generally carried out using dispersive instruments and the time taken to record spectra of adequate sensitivity precluded easy investigation of biological systems. With the advent of fast computing, the Fast Fourier Transform, new detectors and the earlier work concentrated on the high energy X-ray end of the spectrum where the total available photon flux results in large advantages over traditional laboratory sources. The introduction of devices such as 'undulators' and 'wigglers' that are designed to produce highly intense beams of radiation in specific high energy spectral regions has lead to the third generation synchrotrons such as that being built by the Australian Synchrotron Project. For many years the low energy synchrotron output, particularly the infrared portion was ignored because the total power in the mid infrared (MIR) is less than that of a high quality traditional black body source such as a globar source operating at 2000K. The early work was restricted to the far infrared (FIR) region where globar sources are particularly weak and the synchrotron has an advantage in output power of an order of magnitude or greater. Major advances in IR instrumentation have been occurring since the 1980s, and over the last 10 years significant advantages have also been recognised in the MIR region.

Advantages of a synchrotron source
There are essentially four advantages of a synchrotron IR source:

Brightness Although the MIR power from a synchrotron source is less than that from a globar source, it is the synchrotron's brightness that gives it a major advantage. Brightness is the power per unit area x solid angle and for a synchrotron, brightness is some 300 times higher than for the globar source. Hence, for small samples, the synchrotron has a great advantage. An infrared microscope with a globar source operates by introducing the radiation into the microscope through an aperture with most of the IR photons discarded in the process. Consequently, spectra of samples below 30-40 μm in size are not usually of sufficient sensitivity to be useful. The sample size under which a synchrotron source gives greater sensitivity is typically 90 μm, although this depends on the particular synchrotron attributes and can be much smaller.

Pulsed beam The nature of the synchrotron beam gives rise to pulses of radiation that are typically in the range between ~50-500 ps duration with repetition rates anywhere between a 50-500 MHz. Previously, time-resolved broad band IR spectroscopy has been restricted to times of a few ns with μs being standard. With a synchrotron source this time scale can be pushed to around 100ps, the timescale where laser sources are normally required.

Synchrotron Infrared Spectroscopy
Although synchrotron sources produce radiation over the whole of the electromagnetic spectrum, all the early work concentrated on the high energy X-ray end of the spectrum where the total available photon flux results in large advantages over traditional laboratory sources. The synchrotron has a great advantage. An infrared microscope with a globar source operates by introducing the radiation into the microscope through an aperture with most of the IR photons discarded in the process. Consequently, spectra of samples below 30-40 μm in size are not usually of sufficient sensitivity to be useful. The sample size under which a synchrotron source gives greater sensitivity is typically 90 μm, although this depends on the particular synchrotron attributes and can be much smaller.

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1 MIR: 4000-500cm-1 (2.5-20 μm)
2 FIR: 500-10cm-1 (20-1000 μm)
Synchrotron Infrared Spectroscopy in Biology and Biochemistry

Infrared Microscopy and Imaging The major use of synchrotron sources has to date been in microscopy and has been primarily concerned with biological samples. By coupling a standard FTIR microscope to a synchrotron source, it is possible to obtain spectra down to the diffraction limit of the light. For confocal microscope systems, a spatial resolution of $\lambda/2$ is possible. Hence apertures of 5 $\mu$m allow spectroscopy to 1000 cm$^{-1}$. Fig. 1 compares the spectrum obtained for a single red blood cell of diameter ~7 $\mu$m with synchrotron and globar sources. It is easily seen that with the globar the spectrum is essentially useless whereas with the synchrotron source the spectrum above 1000 cm$^{-1}$ is of useful quality and shows the normal strong bands from macromolecules with the intense amide bands of protein standing out. Over the last six years, an increasing number of papers have appeared dealing with biological systems; with recent examples studying cell apoptosis and necrosis (2), protein changes in Alzheimer’s disease (3), bone demineralisation on osteoporosis development (4), protein structural changes in prion-infected tissue (5), microscopic scarring in the cardiomyopathic heart (6), and secondary structure changes in immobilised enzymes (7).

Since the development of IR microscope systems, it has been possible to generate spectral images that can be used to follow macromolecular changes in tissue samples. In particular, they provide a way of determining changes in diseased or treated tissue to give molecular based pathological probes, and we used such a technique to investigate cervical tissue (8). The drawback of these systems is the many hours required to collect the data and the limited spatial resolution. Recent advances in IR imaging array detectors allow for such images to be recorded in minutes with far greater spatial resolution. Straight chemical images can be useful for determining macromolecular content and can also be used in other ways after treatment with multivariate statistical methods. The images shown in Fig. 2 are from a liver biopsy. The infrared chemical images b and c, taken using a focal plane array infrared imaging instrument are generated from signals due to collagen (1200-1300 cm$^{-1}$) and glycogen (960-1175 cm$^{-1}$) and correlate well with fibrotic and normal regions respectively. The image in Fig. 2d has been generated via hierarchical cluster analysis (HCA) and the various cell types colour coded. The respective colour coded spectra are given in Fig. 2e and the coding again correlates well with regions of high collagen and high glycogen. Examination of the plots shows that the imaging instrument does not resolve individual cells, even though the pixels in the imaging array are 5.5 $\mu$m square.

Polarisation Synchrotron light can be either linearly or circular polarised depending on how it is extracted from the synchrotron ring. It thus provides a source of bright broadband polarised light for dichroism, grazing angle reflectance and ellipsometry experiments on small samples that are not amenable to standard laboratory-based experiments.

High Power FIR Radiation At energies below 100 cm$^{-1}$ (wavelength 100 $\mu$m), a synchrotron can produce a greater flux than a standard mercury arc lamp, the benchtop source in this region. At these wavelengths, however, the radiation is not always extracted because synchrotron light essentially fans out as a function of wavelength. The long wavelength light is produced at larger angles requiring rather large holes in the synchrotron vacuum chamber to extract the FIR flux. Synchrotron engineers show a great reluctance to provide such large holes!
Synchrotron-based microspectrometers should give diffraction limited resolution and we have tested this by recording images of diseased epithelial cervical tissue using a variety of infrared imaging techniques. Fig. 3 shows a comparison between a synchrotron-derived HCA map and an infrared imaging array HCA map.

The non-pyknotic nuclei indicated by the arrows in the haemotoxylin and eosin section in Fig. 3 are just detected in the synchrotron map but are not evident in the map generated with the Stingray imaging spectrometer. These nuclei are approximately 10 μm in diameter, which is close to the wavelength dependent spatial resolution limit and also the aperture size used in the synchrotron measurements. The nuclei locations were confirmed by superimposing the visible image over the infrared image. This shows that the synchrotron image, albeit collected over some 40 times longer, has more spatial information than the image from the imaging array system. In the near future, it is expected that a combination of small imaging arrays that match the beam size of the collimated synchrotron source can be used. This will combine the benefits of the enhanced spatial resolution of synchrotron sources and the rapid collection time advantage of imaging arrays.

**Time Resolved Experiments** Broadband FTIR has to date mainly been restricted to following reactions in the μs timescale, although it is used in combination with very fast lasers to probe protein folding, using such methods as laser-induced temperature jump relaxation spectroscopy (10). Probing reactions on time scales of ns and below has usually been restricted to laser-based monitoring methods where the major restriction is the single frequency nature of the laser. Broadband spectroscopy however does provide great advantages in that all the chemical species can be monitored simultaneously. Hence, the pulsed synchrotron broad source opens up a whole new range of possibilities in such areas as protein folding and photochemical reaction centers. The combination of this with the advantages gained in single cell work through a synchrotron source promises much. Such ’pump laser probe’ FTIR experiments have been possible for quite some time but the development of free electron lasers (FEL) based on synchrotron sources now allows for the synchrotron to provide the pump in time resolved studies. A number of current FEL sources are in operation, with new FEL systems, termed fourth generation light sources, able to provide broadly tunable femtosecond high intensity pulses. Flavell et al. have summarised the benefits of a new system to built at Daresbury, UK (11). The high intensity will allow very high resolution spectral imaging applications, using near-field imaging. In the IR regime, subdiffraction limited resolution of the order of 30-50 nm should become possible, allowing the subcellular structure of living cells to be examined. The combination of high brilliance with short pulse lengths from multiple sources will allow development of techniques that probe the nature of biomolecule interactions with surfaces.

**Fig. 2. Human fibrotic liver.**

a) Haemotoxylin and eosin stained liver section.

b) Image generated from collagen bands (1200-1300cm⁻¹).

c) Image generated from glycogen bands (960-1175 cm⁻¹).

d) Image generated from cluster analysis with five clusters.

e) Spectra of the five clusters.

The black pixels indicate regions where the software deemed the signal to be of poor quality (in b, c and d).
Polarisation Based Experiments Polarised light is useful for probing the surface structure and orientation of thin layers using techniques such as ellipsometry and for dichroic experiments. Recently, both circular dichroism and ellipsometry have been extended into the infrared region where the chemical structural information available significantly increases the usefulness of the techniques. The synchrotron provides a ready source of polarised light for these experiments with the added advantage of the brightness allowing for experiments on extremely small samples.

Far Infrared and Terahertz Spectroscopy The FIR region has been a notoriously difficult regime in which to work because of the difficulty of obtaining intense sources of radiation and the necessity of operating under vacuum or purge to eliminate strong transitions from water. The absorptions in this region are weak and tend to arise mainly from large amplitude bending motions, stretches between heavy nuclei and phonon modes that are useful in materials research. Much of the early work was restricted to the gas phase and the area has not been generally well-explored. The powerful flux of radiation produced by synchrotrons makes spectroscopy in this region more appealing; but the brightness of the source in this region does not allow for true microscopy because of the long wavelength of the radiation and the resultant diffraction limit of 100 µm at 100cm⁻¹. The spectrum below 100 cm⁻¹ has become known as the terahertz (THz) region and it is here that synchrotron sources present real advantages. It is only lately that femtosecond laser techniques have provided radiation sources in this region, allowing for the characterisation of biological and biochemical samples. Signals thought to result from large amplitude motions in peptide backbones and DNA have been observed and there is much excitement amongst THz engineers over its possible uses in biological spectroscopy and imaging. The synchrotron provides an alternative source of THz radiation for these experiments and recently it has been shown that THz fluxes many orders of magnitude greater than those presently available are possible (12). THz spectroscopy and imaging, particularly of biosamples, is in its infancy. A useful reference for exploring the possible biochemical uses of this spectral region arose out of the recent THz Bridge Workshop in Capri, Italy 2002, with the papers published in a special issue of the Journal of Biological Physics (13).

The Infrared Beamline on the Australian Synchrotron

The beamline on the Australian synchrotron will use a novel beam extraction technique to produce two synchrotron IR beams directed to two separate instruments. One of these instruments will be a FTIR microspectrometer capable of diffraction limited spatial resolution in the MIR region and capable of ATR spectroscopy. The other will most likely be an FTIR instrument based on a focal plane or linear imaging detector, allowing for efficient diffraction limited infrared imaging in the MIR region.

References