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COMMUNICATION

Two Dimensional Infrared Spectroscopy*

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The development of two dimensional infrared (2D-IR) spectroscopy is reviewed with particular emphasis on the experimental conditions that are required to produce 2D-IR spectra. The 2D-IR spectra of synovial fluid evaporated to dryness on a Teflon® film are presented as a new example of the kinds of experiment feasible using polymer stretching as the source of perturbation, and the potential applications of electric field as a perturbation method are discussed.

KEY WORDS: Two dimensional infrared spectroscopy / Synovial fluid / Teflon / Polymer stretching

INTRODUCTION

The use of infrared spectroscopy provides a common link among scientists engaged in a wide variety of disciplines. This diversity of use has arisen largely through the ingenuity of individuals who customize or refine the technique to address more and more sophisticated problems. From its original role as a molecular fingerprinting technique (a role that remains improtant today, with large databases replacing large volumes of spectra), the infrared spectrometer may now be used in applications ranging from the measurement of wheat protein content to the measurement of vibrational circular dichroism spectra.

With the versatility of infrared spectroscopy established, it is not surprising to learn that yet another hyphenated technique has appeared from the horizon. What is surprising is to find that just at the point when most of us believed the field has really matured there seem to be early signs of a revolution on a scale that few of us would wish to ignore, Infrared spectroscopy is entering a whole new dimension.

In order to do justice to the story of 2-dimensional infrared spectroscopy (2D-IR), it is probably best to start at the beginning. Two gentlemen, new to the city and carpooling to their new research positions at Procter and Gamble's Miami Valley Laboratories, would spend travelling time discussing (what else?) work. Each was probably equally glad to have a captive audience, and -like most of us - was happier at first to talk than to listen. They found common ground around the idea of measuring dynamic properties of polymers using infrared spectroscopy, and a very fruitful collaboration began. They drive their own cars now, but the collabo ation continues...

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POLYMER STRETCHING

2D-IR spectroscopy was devised by Isao Noda and Curtis Marcott of Procter and Gamble (1,2). Combining their expertise in infrared spectroscopy and polymer rheology, they first developed a spectrometer to measure the infrared linear dichroism signal that is induced by uniaxial stretching of polymers (3). This is most conveniently done by subjecting the sample to sunusoidal stress, and measuring the periodic fluctuations in linear dichroism intensity that result.

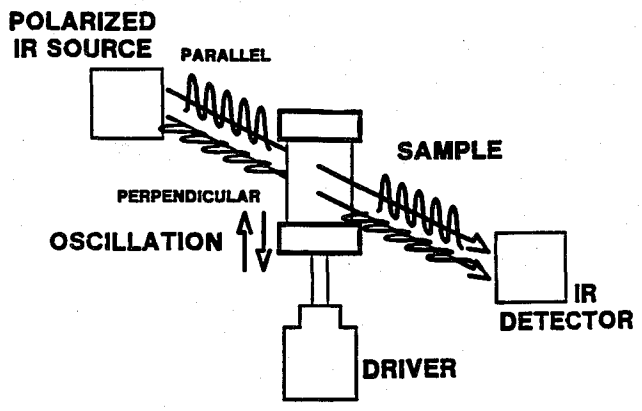

Fig. 1. Experimental setup for the measurement of dynamic infrared linear dichroism spectra (adapted from Reference 3).

The experimental arrangement is shown in Fig. 1. Infrared radiation polarized parallel to the strain axis is passed through the sample, which is subject to mechanical deformation (stretching) at a frequency of ca. 20Hz. The periodic extension and relaxation of the macroscopic sample is accompanied by reorientation of the constituent polymer molecules. This in turn gives rise to a 20 Hz fluctuation in the optical anisotropy of the sample, and hence a 20 Hz variation in the signal falling on the detector. A lock-in amplifire turned in phase with the applied strain then recovers the linear dichroism signal that arises *as a result of the sample deformation*. Optical anisotropy (if any) characteristic of the sample at rest does not contribute to the signal emerging from the lock-in.

The output from the lock-in amplifier contains - in 'raw' form - the change on linear dichroism $\Delta A(\nu)$ associated with stretching of the polymer. The spectrum of $\Delta A(\nu)$ vs. ν is derived by first ratioing the lock-in output against the signal beam energy spectrum measured simultaneously. Further details of the calibration are given in Reference (3).

The lock-in amplifire selectively amplifies signals which fluctuate at the same frequency as that of a reference signal. A second variable in this kind of signal detection is the phase angle of the signal path relative to the reference frequency. For the experiment described above, the phase angle is adjusted such that the lock-in selectively amplifies the signal from

the detector that not only has the same frequency as the mechanical driver, but is also *in phase* with the sample extension/relaxation cycle. The linear dichroism spectrum that emerges is thus called the 'in-phase' spectrum, designated $\Delta A'(\nu)$.

The most intriguing aspect of this measurement is that when set to measure 90 degrees *out of phase* from the reference, there is still signal observed by the lock-in amplifier. Physically, this fact alone tells us that the sample is not behaving in a perfectly elastic manner; for a perfectly elastic sample, all components of the periodic detector signal should be exactly in phase with one another and with the driving frequency. For 'real' sample, sinusoidal perturbation gives rise to *two* signal components, one in phase with the driving frequency (sinusoidal response) and a second component 90 degrees out of phase with the driver reference signal (cosinusoidal response). The linear dichroism spectrum measured at a phase angle of 90 degrees relative to the reference is called the quadrature spectrum, and designated $\Delta A''(\nu)$. The in-phase $\Delta A'(\nu)$ and quadrature ($\Delta A''(\nu)$) components are termed the dynamic infrared linear dichroism (DIRLD) spectra. Typical examples for atactic polystyrene are shown in Fig.2 (see Ref.2).

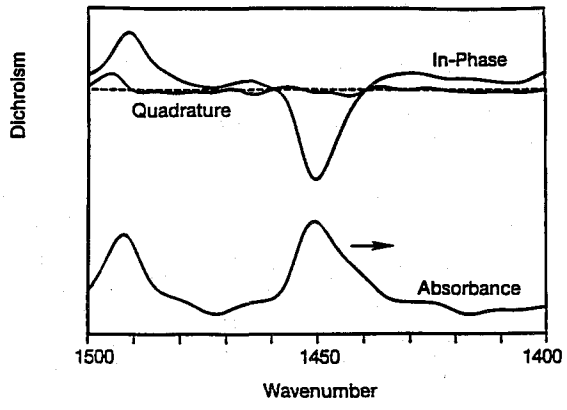

Fig. 2. Absorption and dynamic infrared linear dichroism spectra of atactic polystyrene (adapted from Reference 3).

The above may be represented mathematically by expressing the dynamic variation in the linear dichroism signal intensity $\Delta A(\nu, t)$ as

$$\Delta A(\nu, t) = \Delta A'(\nu)\sin\omega t + \Delta A''(\nu)\cos\omega t \qquad (1)$$

where $\Delta A'(\nu)$ and $\Delta A''(\nu)$ are the in-phase and quadrature spectra, and ω the angular frequency of the (sinusoidal) external perturbation, This expression clarifies the role of the lock-in amplifier in isolating the in-phase (sinusoidal, $\Delta A'(\nu)$) and quadrature (cos-

inusoidal, $\Delta A''(\nu)$) components that define the time dependence of the linear dichroism signal.

These spectra form the ingredients for the 2D-IR spectral representation. Two different 2D-IR spectra, representing the synchronous ($\Phi(\nu_1, \nu_2)$) and asynchronous ($\Psi(\nu_1, \nu_2)$) correlation intensities are derived using the relations

$$\Phi(\nu_1, \nu_2)=1/2[\Delta A'(\nu_1)\Delta A'(\nu_2)+\Delta A''(\nu_1)\Delta A''(\nu_2)] \text{ (Synchronous)} \quad (2)$$

$$\Psi(\nu_1, \nu_2)=1/2[\Delta A'(\nu_1)\Delta A'(\nu_2)-\Delta A''(\nu_1)\Delta A''(\nu_2)] \text{ (Asynchronous)} \quad (3)$$

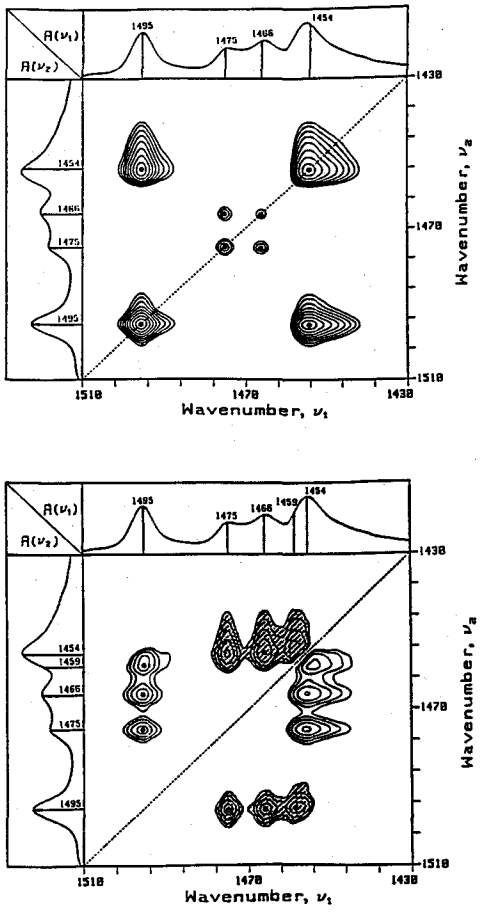

Fig. 3. Synchronous (upper) and asynchronous 2D-IR spectra of a film made of a mixture of atactic polystyrene and low-density polyethylene (adapted from Reference 1).

Figure 3 shows the 2D-IR spectra of a film made of a mixture of atactic polystyrene and low-density polyethylene (1). The asynchronous spectrum demonstrates one clear advantage of the two-dimensional representation, namely the resolution of features that are not obviously resolved in the infrared absorption spectrum. It is worth repeating that the 2D spectra

represents the correlation between *time-resolved* spectra. The in-phase and quadrature spectra reflect the extent of strain and the rate at which the vibrational chromophores return to their equilibrium orientations following removal of that strain. The phase difference of 90 degrees represents a time difference of one quarter of the period of the sinusoidal oscillation. For a typical value of 20 Hz this implies a time lag of ~13 msec separating the two spectra. The enhanced resolution results from the fact that each vibrational chromophore responds differently to the macroscopic perturbation. The two-dimensional spectra reflect these differences in the following properties;

Synchronous spectrum;

1) Off-diagonal peaks relate spectral features (identified by drawing horizontal and vertical lines to the absorption spectra plotted along the axes) corresponding to chromophoers reorient in-phase with one another.

2) The intensity of a diagonal peak reflects the susceptibility of the corresponding chromophore to the perturbation *and* the effectiveness of the spectroscopic probe as a monitor of that susceptibility.

Asynchronous spectrum;

1) Off-diagonal features relate absorptions corresponding to chromophores that do not reorient in-phase with one another.

2) There are no diagonal peaks.

A more complete listing of the properties of 2D-IR spectra is given in Ref.2.

For a blended polymer, different types of constituents may respond quite differently to dynamic stretching. This is evident from the spectra of the polystyrene/polyethylene film (Fig. 3). The synchronous 2D-IR spectrum shows strong off-diagonal peaks connecting chromophores (absorption bands) belonging to the same component, i.e. linking one polystyrene absorption at 1454 cm^{-1} to another at 1495 cm^{-1}, and linking one polyethylene absorption at 1454 cm^{-1} to another at 1466 cm^{-1}, with no off-diagonal bands connecting any polystyrene absorption to any polyethylene absorption (see Fig. 3). The differing rates of reorientation thus form the basis of 2D-IR spectroscopy.

GENERALIZED TWO-DIMENSIONAL SPECTROSCOPY

The conceptual basis of two-dimensional infrared spectroscopy has opened the door to an entirely new realm of experiments. Those of us who are used to seeing our samples as static entities are now able to contemplate any number of ways of learning more about them through their response to appropriate perturbation (s).

The key to fulfilling this goal is to consider exactly what requirements must be met in order to generate the two dimensional spectra. First, the sample must be perturbed in a manner that alters its response to an appropriate spectroscopic probe. Second, the perturbation must be applied and monitored on an appropriate time scale.

The first point is key in realizing the scope of generalized 2D spectroscopy. Any combination of perturbation and spectroscopic probe may be brought together to provide the in-phase and quadrature spectra. Linear dichroism is most appropriate as a monitor of molecular reorientantion induced by polymer stretching. An analogous experiment has been

devised to observe liquid crystal dynamic response to an applied AC electric field (5). The arsenal of perturbations will doubtless grow from the present list of two (mechanical deformation and electric field); obvious candidates are pressure (or acoustic waves), high energy radiation (e.g. visible or UV lasers) and temperature. One practical consideration is that the sample must withstand the repeated cycling of the perturbation that is necessary to achieve a good signal-to-noise level. The polymer experiments typically require several hours to build adequate S/N, as the induced linear dichroism signal is extremely weak ($\Delta A = 10^{-4} - 10^{-6}$ absorbance units). It is likely that other types of perturbation will induce larger signals and hence cut down the measurement time. Moving to shorter wavelength probes (NIR, visible, UV) will also bring more efficient detectors into play, albeit at the expense of the rich detail afforded by the infrared region.

The time scale of the experiment is worth considering carefully as new forms of perturbation come into use, since judicious choice and variations of the perturbation frequency can potentially reveal much more about the sample than any single measurement alone. For example, if the polymer stretching experiment were to be conducted at a *very* low frequency, the only detectable signal would be that in-phase with the deformation cycle. This *elastic* cycling is essentially equivalent to a static experiment measuring the linear dichroism spectrum of the extended polymer. As the oscillation frequency is increased, the *variability* in the *dynamic* responses of different chromophores will start to appear in the form of a quadrature signal. The distinctive time behaviour characteristic of each vibrational mode translates directly to a distinctive phase shift relative to the driving frequency.

What is most intriguing about the prospect of using electric field as the perturbation is the flexibility in both frequency and field strength. For a sample made up of a mixture of compounds ranging in size, it may be feasible to tune the AC frequency so as to select individual components according to the frequency dependence of their susceptibilities. This feature might be used for example to distinguish components of a mixture according to size.

The synchronous spectrum is primarily a reflection of the *extent* of applied strain. The spectrum is non-zero even for completely elastic cycling of the perturbation, i.e. even if the quadrature spectrum shows no features whatsoever (see Eqn. (2)). The incoherence in phase among the different vibrational modes is the fundamentally new information available from these experiments; the asynchronous spectrum shows the 'phase spectrum' as a two-dimensional plot, each cross-peak reflecting the degree of incoherence (i. e. phase angle) between the two relevant chromophores. The appearance of a quadrature spectrum provides the foundation for this plot, tagging those chromophores that move out-of-phase with the driving force and separating them according their phase lag relative to one another. A very good example of this resolution ehhancement is given in Ref.4, a study of the amide I region of human hair keratin deposited on an infrared-transparent substrate; a broad, featureless infrared absorption profile is resolved into at least eight components over the 1620-1680 cm^{-1} region.

A most exciting application of electric field perturbation lies in the ease with which the perturbation frequency can be changed. For a given frequency only a selected group of components will give rise to signals in the asynchronous spectrum, i.e. those components that are responsible for the quadrature spectrum *at that frequency*. A third dimension may easily be added to the experiment by stepping through a range of perturbation frequencies - the

quadrature spectrum will monitor different components/sets of components at each frequency, and thus the third dimension in a stack of asynchronous spectra might be labelled with the axis 'quadrature susceptibility'. The most obvious feature of this experiment is the possibility of screening out (or focussing on) components according to size, and gleaning detailed information about the interactions among the various components.

EXPERIMENTAL CONSIDERATIONS

Early spectra were collected exclusively using modified dispersive instrumentation. More recently, step-can FT-IR spectrometers have been modified for 2D-IR measurements (5-8). The step-scan option is required in order to use an interferometer for the polymer stretching experiments because of the very low frequency of the perturbation (mechanical stretching). For higher frequency perturbations, e.g. electric field or acoustic, it should be feasible to use a conventional rapid-scanning FT-IR spectrometer. The intensity modulation produced by a perturbation of about 25 KHz or higher frequency can be cleanly separated from the Fourier frequencies by using a lock-in amplifier and an appropriate (fast) time constant. This method, termed 'double modulation spectroscopy' was originally devised to measure vibrational circular dichroism spectra using an FT-IR spectrometer. The experiment is described in Reference 9.

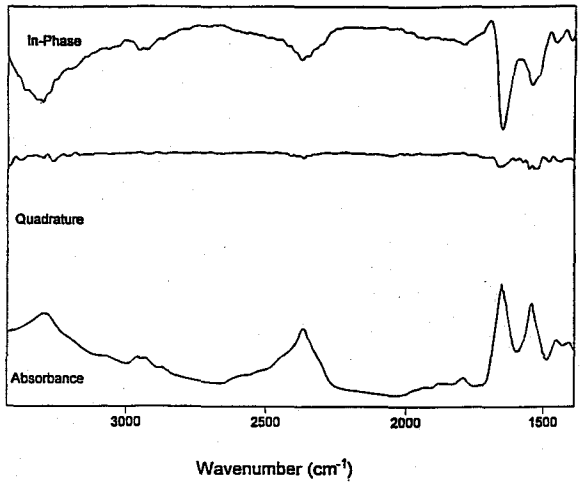

Fig. 4. Absorption and dynamic infrared linear dichroism spectra of synovial fluid dried to a film on a Teflon® substrate.

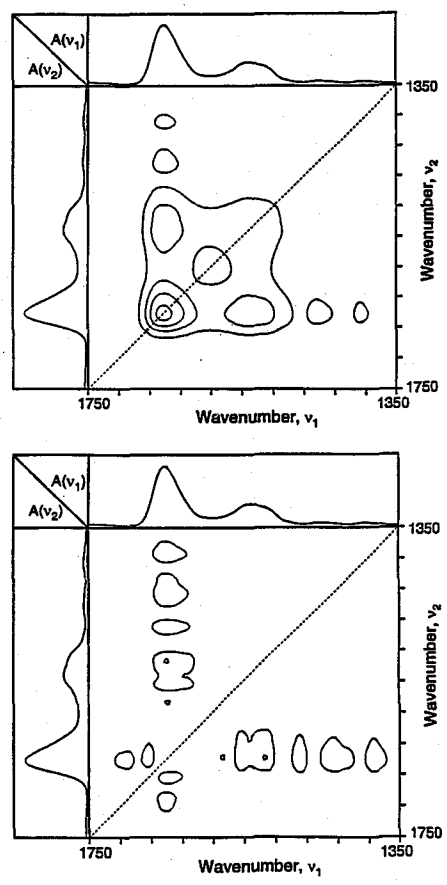

Fig. 5. Synchronous (upper) and asynchronous 2D-IR spectra of synovial fluid dried to a film on a Teflon® substrate.

Figures 4 and 5 display spectra measured recently using the step-scan method. Synovial fluid is a viscous mixture of water and biological macromolecules that serves to lubricate skeletal joints. A spectrum of the nonvolatile components may be measured easily by spreading a film on a CaF_2 window and allowing the water to evaporate (10). In order to cast such a film on a Teflon® surface it was necessary to partially oxidize the surface. This was achieved by placing the Teflon® in a plasma cleaner while admitting small volumes of air. Approxomately 100 μL of the synovial fluid was then spread over an area of 2 cm^2 and permitted to dry, the drying rate moderated by a petri dish placed upside down over the sample. The plotted spectra were obtained by applying 13 Hz sinusoidal displacement to the Teflon® and measuring the DIRLD spectra using a Digilab FTS-60A step-scan spectrometer.

The phase modulation (400 Hz, 2 λ_{HeNe} amplitude) was demodulated using the FTS-60A demodulator, and the output signal fed to a lock-in amplifier (Stanford Research Model SR530) for measurement of the in-phase and quadrature signals. Data were accumulated for a total of approximately twelve hours (overnight) in order to measure the very weak quadrature signal. Spectral reproducibility is good; in-phase spectra, measured for two other films prepared from the same sample, agreed very closely with that shown in Fig.4.

The amide I and amide II protein vibrations provide the most prominent 2D-IR features. Preliminary measurements suggest that the dynamic infrared spectra may discriminate between synovial fluid samples that yield essentially identical infrared spectra.

CONCLUDING REMARKS

The aim of this communication is to give the reader a grasp of the principles underlying two-dimensional spectroscopy, and to convey a sense of the diversity of applications that are likely to come into use as more laboratories acquire the equipment and expertise required to measure the spectra. The reader is encouraged to consult References 1-3 in particular, and references therein, for more detailed accounts of 2D-IR.

We have focussed upon the electric field as a promising type of perturbation appropriate for 2D-IR spectroscopy of liquids and solutions. As a cautionary note, it should be added here that the the electric field may interact with samples in a variety of ways obvious examples being moving charges, orienting dipoles, and inducing dipole moments. The spectra will thus reflect the influence of several factors. It should be possible to distinguish these effects by measuring dynamic changes in both linear dichroism *and absorbance*. Dynamic absorbance spectra may prove useful, for example, as a probe of the binding site (s) of metal ions on polypeptides. The C=O groups involved in complexation might be expected to show large fluctuations in absorption intensity when the sample is subject to an AC field of appropriate frequency.

While the generality of the method is difficult to predict, there can be little doubt that electric field perturbation will prove very useful for certain types of sample. We are in the process of assembling the components necessary to measure electric field induced 2D-IR spectra and looking forward to prospect of applying our spectroscopist's instincts in scanning the realm of (perturbation) frequencies and (field) intensities.

ACKNOWLEDGEMENTS

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