Vibrational Spectroscopy of Biopolymers Under Mechanical Stress: Processing Cellulose Spectra Using Bandshift Difference Integrals

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Received May 11, 2006; Revised Manuscript Received June 29, 2006

Mechanical stretching of covalent bonds, for example when a fibrous polymer is loaded in tension, results in their stretching vibrational bands in the infrared or Raman spectrum being shifted to lower frequency. Conversely stretching a hydrogen bond shifts the stretching vibrational mode of the donor covalent X−H bond to higher frequency. These band shifts are small and difficult to detect in complex regions of the spectrum where differently affected bands overlap. This paper describes a method of integrating the difference spectra (spectrum under tensile strain minus spectrum at zero tensile strain) to recover the shape of the bands that are shifted and the spectral variation in bandshift. The application of this method to two sets of vibrational spectra of cellulose under tension is described. In one example, C−O−C stretching bands of highly crystalline tunicate cellulose were observed to shift to lower frequency under axial strain. In the other example, a group of overlapping O−D stretching bands in partially deuterated cellulose showed varied bandshifts under axial strain, some bandshifts being positive as expected due to extension of axially oriented hydrogen bonds while others were negative. The possibility of constructing spectral plots of bandshift has the potential to clarify the interpretation of overlapped, shifting bands in the vibrational spectra of polymers under tension.

Introduction

When a covalent bond is mechanically stretched it lengthens and becomes less stiff. The reduction in force constant can be measured as a small reduction in the frequency of the corresponding stretching vibrational mode.[1,2] In this way FTIR or Raman spectroscopy or microscopy can be used to probe mechanisms of deformation in complex materials under mechanical stress, providing insights into the molecular origins of their mechanical properties[1]. This approach is widely used in the study of composite materials[3]. It has also been applied, for example, to carbon fibers,[4] nanotubes,[5] and biomaterials such as cellulose[6−9] and silkworm[10] and spider[11] silk.

Bandshifts commonly observed in the midrange infrared are small, commonly of the order of 1 cm^{-1} even under tensile stresses close to the breaking stress of biomaterials. Extracting bandshift information from complex spectra with overlapping, independently shifting bands is challenging,[12] and it is often only possible to consider well-resolved bands of high intensity. Thus, only a fraction of the information content of this type of experiment is readily accessible.

Here we report a method of processing vibrational spectra to extract bandshift information even when the vibrational bands concerned are complex and incompletely resolved.

Theory

When a single band is subjected to a small frequency shift $\Delta\mu_0$ without change in intensity, bandwidth, or band shape, the difference spectrum $I'(\mu) - I(\mu)$ has the form of the first derivative of the band, with negative sign.

$$I'(\mu) - I(\mu) = -\Delta\mu_0 \, dI(\mu)/d\mu$$

where $I(\mu)$ is the original absorbance at frequency μ and $I'(\mu)$ is the shifted absorbance.

Thus, integrating the difference spectrum, from a frequency μ_b at which absorbance is at baseline to frequency μ, allows the original shape of the shifted band to be retrieved and scaled according to the magnitude and sign of the bandshift

$$\int_{\mu_b}^{\mu} I'(\mu) - I(\mu) \, d\mu = -\Delta\mu_0 \, I(\mu)$$

This is illustrated in Figure 1 for a modeled Gaussian band.

It follows that, by dividing the difference integral up to frequency μ by the absorbance $I(\mu)$ at that frequency, the local bandshift at frequency μ can be obtained. In the case of an isolated Gaussian band as shown in Figure 1, the bandshifts thus calculated are constant across the width of the band. Overlapping bands interfere with one another, but their individual bandshifts may be calculated at the original center of each band if the overlap is not excessive. The possibility of comparing a bandshift vs frequency plot with the original spectrum potentially makes the interpretation of complex spectral data easier. Deconvolution of the difference integrals is a more rigorous but less intuitive approach.

In some circumstances, similar macromolecules within a complex material may carry different loads.[6,7,13] The bandshift shown by corresponding vibrational modes will then differ according to the load, and band broadening will result. Band broadening is expected when fibers are randomly oriented within a composite material under unidirectional stress.[2] They may also

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10.1021/bm060457m CCC: $33.50
Published on Web 08/24/2006

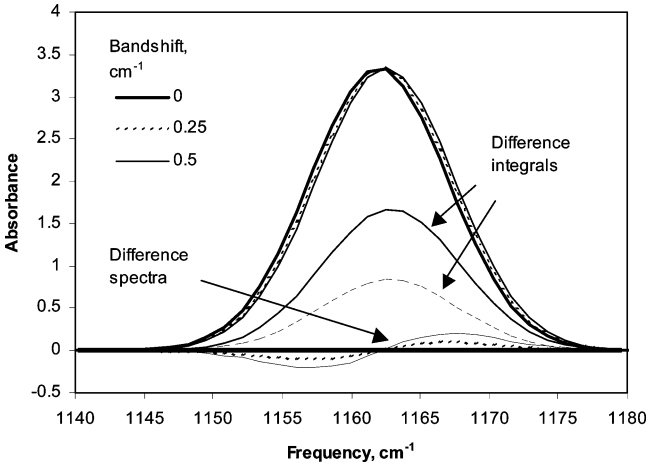

Figure 1. Modeled Gaussian band centered originally on 1162.0 cm^{-1} and shifted to 1162.25 and 1162.5 cm^{-1}. The two resulting difference spectra were integrated numerically (with negative sign) leading to difference integral spectra which reproduce the original band shape but are scaled according to the bandshifts of 0.25 and 0.5 cm^{-1}, respectively. That is, each difference integral spectrum is equal to the product of the original spectrum and the corresponding bandshift.

occur if the microstructure or nanostructure of the material allows strain to be distributed unequally between similarly oriented polymer molecules.[6]

Band broadening due to unequal loading has little effect on the shape of the difference integrals, which still approximately reproduce the shape of the band that is shifted and broadened (Figure 2). Calculation of the bandshift from the difference integral gives the correct mean value at the center of the original band, but the spectral plot of bandshift is characteristically tilted, so that it its magnitude is greatest at the side of the band corresponding to the direction of the shift (Figure 2). This is to be expected because at that side of the band the spectral intensity is dominated by the contribution from the polymer molecules that show the greatest bandshift.

Experimental Section

Cellulose Preparation. Collenchyma strands (~580 mg) were isolated by hand from mature celery plants. Proteins were removed by treatment with 0.2% wt/v Triton X-100 at 3−4 °C for 60 min while stirring occasionally. The Triton solution was then removed with thorough water rinsing. The celery collenchyma strands were hydrolyzed in 1.0 M HCl at ~100 °C for 60 min, followed by a water rinse. Each hydrolyzed collenchyma piece was dried individually at room temperature in a 9 cm Petri dish. Dried collenchyma strands were checked visually for smoothness, orientation, and homogeneity, and only strands with best-conserved original properties (~160 mg) were used further. The preparation of tunicate cellulose has already been described[7]

Raman Spectroscopy of Tunicate Cellulose During Tensile Deformation. These experiments have already been described[7] and only the calculation of the bandshifts from difference integrals is reported here.

FTIR Microscopy of Celery Collenchyma Cellulose During Tensile Deformation. Celery collenchyma cellulose strands were deuterated individually in 0.1 M $NaOD/D_2O$ solution for approximately 20−60 s, then placed onto a flat surface of a glass vessel, left covered for 30 min, and air-dried after a single rinse with analytical grade acetone. Hydroxyls on a small number of cellulose chains within each microfibril are accessible to deuteration under these conditions but not at neutral pH, and only these hydroxyls remain deuterated when the sample is reequilibrated with atmospheric H_2O.[14] Since accessible

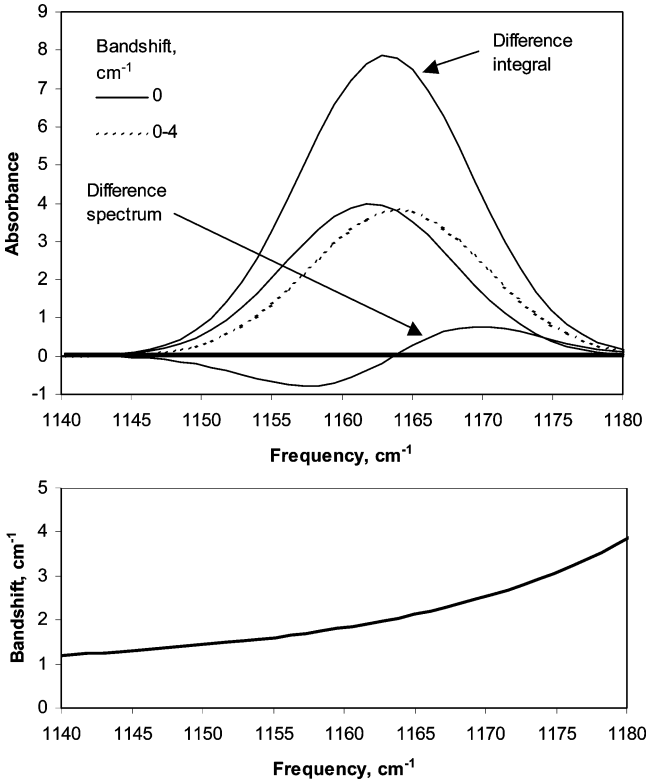

Figure 2. Modeled Gaussian band centered originally on 1162 cm^{-1} (solid line labeled 0 cm^{-1} bandshift). To reproduce the effect of peak broadening simultaneous with bandshift, the original band was divided into three equal components which were shifted to higher frequency by 0, 2, and 4 cm^{-1}, respectively, before being added together again (dotted line labeled 0−4 cm^{-1} bandshift). The resulting difference spectrum was integrated numerically as in Figure 1 and ratioed to spectral intensity to give a spectral plot of calculated bandshift.

surface chains are deuterated under neutral conditions[15] the term "subsurface" deuteration is used for those deuterated in dilute alkali, but their location in the microfibril is not known in detail.

Subsurface-deuterated cellulose fibers from celery collenchyma were attached with cyanoacrylate to a screw-driven tensile strain frame on the stage of a Nicolet Continuum FTIR microscope interfaced to a Nicolet Nexxus FTIR spectrometer. Spectra were obtained at 2 cm^{-1} resolution after averaging 128 scans at each strain level.

Baseline Correction and Normalization. When the spectra are recorded at fixed frequency intervals j, it is straightforward to integrate the difference spectra $\Delta I(\mu)$ numerically by first setting the integral of $\Delta I(\mu_b) = 0$ and then adding $\Delta I(\mu)$ at each frequency step. The integral spectra are multiplied by $-j$.

When the application of this method to real spectra was first attempted, it was found that extremely precise normalization was required and was challenging to achieve. When vibrational spectra are measured under increasing mechanical stress, it is experimentally difficult to return to exactly the same area of the sample for each spectrum. Any inhomogeneity in the sample will then lead to small variations in overall signal intensity which severely distort the difference integral spectra. Similar problems result from dimensional changes controlled by the Poisson ratios of the material. These small variations in overall intensity must as far as possible be removed by normalization before the difference spectra are calculated.

Prior to accurate normalization, accurate baseline correction was necessary. It was preferable to consider only one spectral region at a time (e.g., the O−H stretching region) and to establish well-defined baselines at one or both ends of each region. The aim of the subsequent normalization step is 2-fold. First, heights of significant shifted peaks should be identical, to ensure that their bandshifts calculated from the difference integral spectrum are not distorted. This requirement

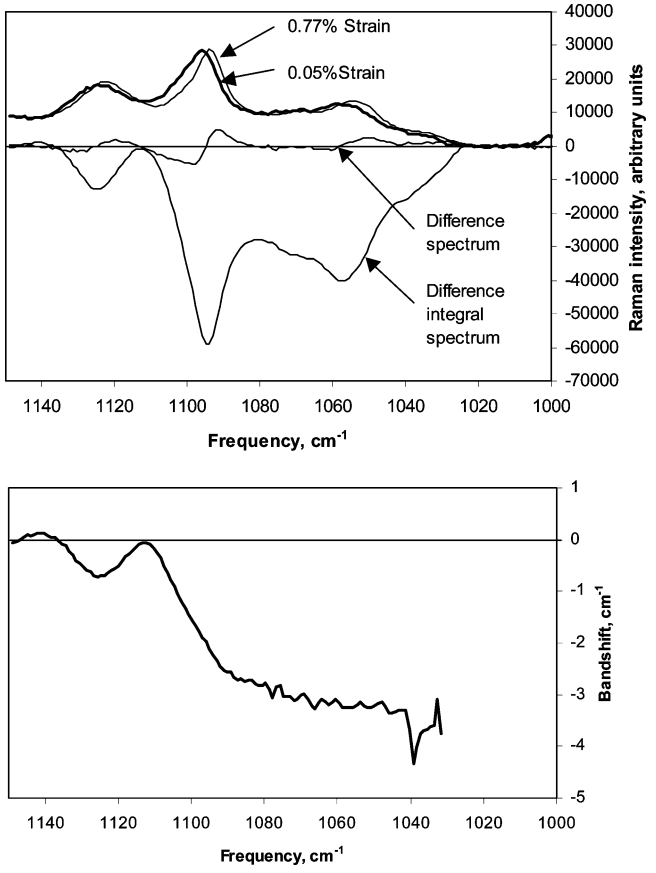

Figure 3. C−O stretching region, Raman spectra of tunicate cellulose fibers embedded with random orientation in epoxy resin.[7] Above: spectra at 0.05% and 0.77% strain, with difference spectrum and difference integral. Below: spectral variation in bandshift calculated from the difference integral. At less than 1030 cm^{-1}, where the intensity of the spectra is very low, the calculated bandshift becomes dominated by noise and is not plotted.

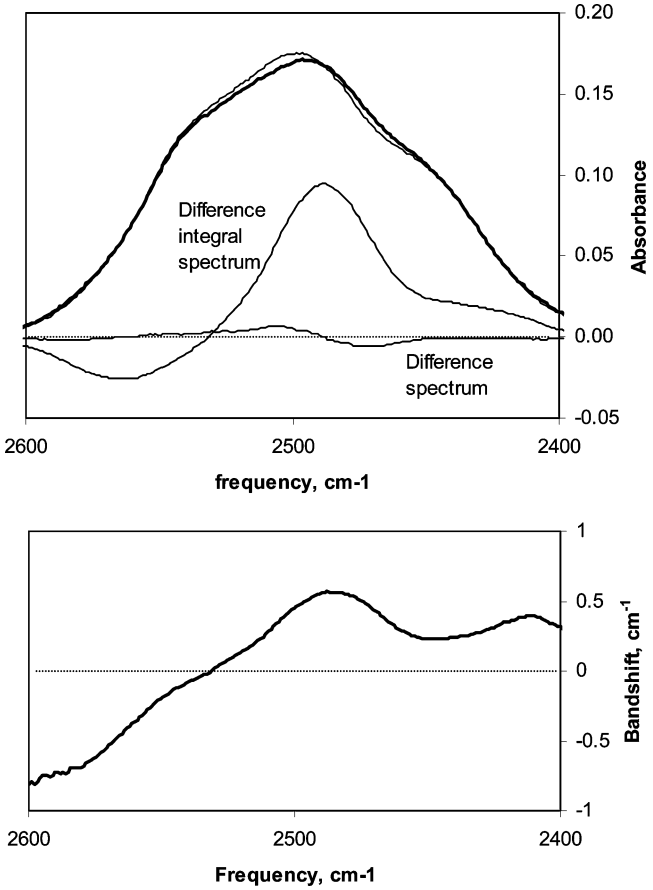

Figure 4. O−D stretching region of partially deuterated celery collenchyma cellulose under axial strain, with difference and difference integral spectra (above) and calculated spectral variation in bandshift (below).

may not be met if changes in bandwidth occur under stress.[2,16] Second, the total areas of the baseline-corrected spectra should be equal after normalization. If not, the difference integral spectrum will be displaced at the high-frequency end of the spectral region studied. Exactly how these requirements are reconciled depends on the experiment. Sometimes normalization to equalize the areas under the baseline-corrected spectra is sufficient to leave the peak heights essentially identical. In the tunicate cellulose example illustrated below, the normalization factor was calculated separately at three points in the spectrum, two peaks and one plateau, and linearly interpolated between these. This left the areas under the spectra approximately, but not exactly, equal. In the second example, the O−D stretching region of subsurface-deuterated celery collenchyma cellulose, only normalization by area was used.

Results

Raman Spectra of Tunicate Cellulose Under Tension: C−O−C Stretching Region. The Raman spectra shown here form part of an experiment published previously.[7] The tunicate cellulose used was of particularly high crystallinity and was dominated by the Iβ allomorph. To increase the signal-to-noise ratio, spectra at three successive strain levels were pooled to generate each of the two spectra used, with mean strains of respectively 0.05% and 0.77%.

The 1000−1200 cm^{-1} region of the cellulose spectrum contains absorption bands from a number of complex vibrational modes to which C−O stretching within the monosaccharide ring and glycosidic C−O−C stretching contribute.[6−9,13] The difference integral spectrum (Figure 3) shows a strong negative peak at 1095 cm^{-1} with a negative bandshift of 2.1 cm^{-1} at its center. Our analysis shows that the magnitude of the negative bandshift is close to 3 cm^{-1} across the 1030−1090 cm^{-1} spectral interval which includes a number of Raman-active modes, some of them with longitudinally aligned transition moments.[7,15] The band at 1125 cm^{-1}, with more weakly axial orientation, shows a smaller negative shift of 0.7 cm^{-1}. Negative bandshifts are expected for covalent bonds aligned and stretched along the fiber axis.

FTIR Spectra of Deuterated Celery Collenchyma Cellulose Under Tension: O−D Stretching Region. When hydroxyl groups are substituted with deuterium, the O−H stretching bands are moved to lower frequency by the factor 1.34, without change in their pattern.[14,15] The alkali-stable "subsurface" deuteration used here led to a pattern of O−D stretching bands resembling the O−H stretching band pattern of crystalline cellulose but with lower resolution due presumably to reduced order. Small bandshifts induced by tensile strain on this material are shown in Figure 2. The corresponding difference spectrum was of low intensity, but when integrated, it showed that there were both positive and negative shifts in overlapping bands, and that their magnitude was less than 1 cm^{-1}. Positive vibrational bandshifts signify increased force constant or decreased length of the covalent O−H bond, attributable to stretching of the hydrogen bond of which the hydroxyl in question is the donor.

Discussion

The examples described above show how detailed bandshift data can be extracted from complex spectra recorded during mechanical deformation, provided that the spectra can be accurately normalized and baseline-corrected. Complex baselines can result from background fluorescence in Raman and scattering in FTIR spectroscopy, but careful linear modeling was sufficient to remove their interference here.

The ability to distinguish the contributions of hydrogen bonding and covalent bonds, through the opposite sign of the resulting bandshifts, is particularly useful. The example of partially deuterated cellulose shows, however, that the hydrogen-bonding patterns in fibrous polysaccharides become distorted under load in quite complex ways.

The interpretation of data from this type of experiment is simplest where the polymer chains in question are aligned with the direction of strain and where bandwidths remain constant. Band broadening introduces some additional complexity, although it can in itself be informative about macromolecular structures and their behavior under stress.[6] Where band broadening results from polymer disorientation, it is possible, in principle, to simplify the interpretation of the data by using incident radiation polarized parallel to the direction of strain.[2] However, FTIR absorbance and Raman scattering intensity vary with $\cos^2 \alpha$, where α is the angle between a polymer chain and the direction of polarization,[15] whereas in an experiment of this kind, the strain on the polymer chain varies with $\cos \alpha$. A fully quantitative solution to this problem remains to be described.

It is becoming evident that the molecular origins of deformation, stiffness, and strength in structural biomaterials differ from those described in more familiar materials such as metals.[17] Vibrational spectroscopy has much to offer toward an understanding of how biomaterials function under mechanical stress, and the use of difference integrals can facilitate the interpretation of the spectroscopic data.

Acknowledgment. This work was financially supported by BBSRC and EPSRC.

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BM060457M