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Spectroscopic studies of the physical properties of hyaluronate films: the origin of the phase transition

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The elastic constants of wet-spun films of Li- and NaHA were evaluated as a function of relative humidity (RH) for both the directions parallel and perpendicular to the helical axis. The elastic constants were found to decrease over the entire range of RH. The ultraviolet (UV) absorption of a NaHA film was measured both before and after being cycled through the phase transition between 84 and 92% RH. The changes in the UV spectrum due to the phase transition were found to be irreversible.

INTRODUCTION

Hyaluronic acid (HA) is a polydisaccharide with a repeat unit consisting of D-glucuronic acid and *N*-acetyl-glucosamine, as shown in Guss *et al.* (1975). The HA molecule has helical symmetry. In mammals, this molecule is found in connective tissues and in cartilage where it is the central organizing molecule of the structure. HA is also present in the synovial fluid of the eye. HA must have arisen very early in evolution since it is found in the encapsulating structure of *Streptococcus.*

The HA polymer has a negative charge of −1e for each disaccharide unit. A positive counterion is required for charge neutrality: H^+ in the case of the acid itself or positive counterions such as Li^+ or Na^+ for the salts of the acid. Along with the net charge of each disaccharide, there are regions of positive or negative charge within the two glucose rings. Water molecules (with their very large dipole moments) are attracted by the charged sites to form hydration shells. These water molecules 'screen' the local charges and lower the Coulombic interactions between different segments of the HA molecule. Thus, increasing the amount of water present in the hydration shells should have the effect of lowering the mechanical stiffness of the HA molecule. The X-ray experiments of Guss *et al.* (1975) have shown that the counterions form strong intermolecular interactions with neighboring HA molecules. The water of hydration should also screen the intermolecular bonds mediated by the positive counterions, and increasing the amount of hydration of a solid film should also decrease the mechanical stiffness of the film.

The primary water of hydration (i.e., the tightly bound water) has been studied by Brillouin spectroscopy by Lee *et al.* (1993). They found that the relaxation time of the water relaxation mode is approximately 50 ps in both Li- and NaHA. Lee *et al.* (1993) also found that the microscopic coupling constant between the polymer and the primary water of hydration is approximately $3.0 \times 10^{-50}\,\mathrm{kg\,m^4\,s}$ in the direction parallel to the helical axis and approximately $7.0 \times 10^{-50}\,\mathrm{kg\,m^4\,s}$ in the perpendicular direction. These values are very similar to those observed by Tao *et al.* (1988) for NaDNA: a relaxation time of approximately 40 ps with microscopic coupling constant being approximately $4.2 \times 10^{-50}\,\mathrm{kg\,m^4\,s}$ and $5.5 \times 10^{-50}\,\mathrm{kg\,m^4\,s}$ for the parallel and perpendicular directions, respectively. These similarities suggest that the properties of the primary hydration shell and its coupling to the biopolymer has little dependence on the biopolymer itself. This is reasonable since such coupling would be expected to be a local property, determined primarily by the identity of the atoms involved. Since the same basic atoms are involved (carbon, oxygen, nitrogen and hydrogen) for both biopolymers, one would expect similar behavior, as is observed. It should be noted that the anisotropy for the coupling constant for HA is approximately 100% which is significantly larger than the coupling constant observed in DNA (approximately 30%).

One of the important functions of cartilage is to absorb the jarring motion accompanying movement of

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living vertebrate organisms. The kinetic energy associated with this shock is believed to be dissipated by doing work to move the water of hydration. Applying a stress to a section of cartilage is believed to force some of the water out of the hydration shells, thereby decreasing the amount of screening and, consequently, stiffening the molecule. We study the effect of the water of hydration on the physical properties of HA in order to elucidate the exact mechanism of this stiffening.

Brillouin spectroscopy has been used to study the speed of sound in wet-spun films of both Li- and NaHA as a function of relative humidity (RH) (Lee *et al.*, 1992, 1993). These studies revealed two important observations: (1) the speed of sound along the helical axis in HA films is anomalously large (~5 km/s) at low relative humidity (RH); and (2) a discontinuous drop in the speed of sound is observed between 84 and 88% RH. Since the speed of sound is a measure of the mechanical stiffness of the film, such a high speed of sound indicates that these films are remarkably stiff. Indeed, this speed of sound is a little larger than the speed in 'soft' metals such as copper (Weast, 1969). X-ray studies (Guss *et al.*, 1975; Mitra *et al.*, 1983; Arnott *et al.*, 1983; Sheehan & Atkins, 1983) have shown that crystalline phases of HA have many intra- and intermolecular hydrogen bonds as well as very strong intermolecular bonds mediated via the positive counterion. The very high speed of sound is most likely related to the very strong bonding found in these samples.

The discontinuity in the speed of sound between 84 and 88% RH is clear evidence of a phase transition. Since the high sound speed is believed to be connected to the strong bonding, the discontinuity in the speed of sound is probably an indication of significant changes in the bonding within the sample. An X-ray study (Lee *et al.*, 1992) on the same wet-spun samples as used in the Brillouin experiments found evidence of a phase transition as well: films of NaHA were observed to give crystalline diffraction patterns at 90% RH but only rings characteristic of a disordered sample at 93% RH. It must be emphasized that this order-to-disorder transition was observed at a higher RH than the discontinuity in the speed of sound. This suggests that the weakening of the crystalline network is a two-step process: (1) a discontinuous weakening in the strength of the bonding takes place between 84 and 88% RH (causing the discontinuous drop in the speed of sound); and (2) a further weakening of the network occurs between 90 and 93% RH and the bonds become too weak to maintain crystalline order. This suggests that the phase transition observed in these films is complex and interesting. The results of the different experimental probes of this phase transition are summarized in Table 1.

Table 1. Summary of phase transition in HA films

Property	RH (%)	Water content (WMPD)	Type
Sound speed	84/88	14/17	Discontinuous
Crystallinity	90/93	21/26	Discontinuous
Optical polarizabilities	⩾75	⩾10	Continuous
Ultraviolet absorption	⩾80	>11	Continuous

Sources: The sound speed and crystallinity observations are from Lee *et al.* (1992), the optical polarizabilities observations are from Flowers *et al.* (1992) and Lee *et al* (1994); and the ultraviolet absorption observation is from Lee *et al.* (1994).

Though the measurements are made by controlling the RH to which the sample is exposed, the more natural variable is the number of water molecules per disaccharide (WMPD). Water content has been measured for wet-spun films of Li- and NaHA (Flowers *et al.*, 1992; Lee *et al.*, 1994) and found to have the same basic shape as had been found earlier in NaDNA (Lee *et al.*, 1987). Of particular importance is the fact that there is no anomalous increase in the water content between 84 and 88% RH. This confirms that the discontinuous drop in the speed of sound is due to a change associated with the HA biopolymer itself.

Given the anomalies in the speed of sound and degree of crystallinity, it is of interest to determine the status of the electronic degrees of freedom in these films as a function of RH. Flowers *et al.* (1992) and Lee *et al.* (1994) have measured the optical polarizabilities of Li- and NaHA at 500 nm. They measured the refractive indices, volume per disaccharide and water content as a function of RH and then extracted the optical polarizabilities (a measure of how easily the electrons of the molecule are polarized) via a Lorentz–Lorenz analysis. As shown in Table 1, these studies showed that the polarizabilities for wet-spun films of both Li- and NaHA decrease continuously, beginning at approximately 75% RH. This suggests that the electronic states are sensing the phase transition at an even lower water content than the speed of sound and degree of crystallinity.

The optical polarizabilities are related to the real part of the dielectric function of this material. As is well-known from linear response theory, the real and imaginary parts of the dielectric function are connected by a Kramers–Kronig relation. Consequently, measuring the imaginary part of the dielectric function would provide important confirmation of the changes observed in the real part. Such measurements have recently been made by Lee *et al.* (1994) who measured the UV absorption of wet-spun films of NaHA. They observed significant changes in the UV absorption beginning at approximately 80% RH, consistent with the optical polarizability measurements.

In this paper we address two important questions: (1) Do long-range interactions of the electronic states show any changes at a lower RH than 75%; and (2) Is the phase transition reversible? In order to address the first question we evaluated the elastic modulii of these films. For the second question we measured the ultraviolet (UV) absorption of NaHA films as the films are cycled back and forth through the phase transition.

MATERIALS AND METHODS

NaHA was provided as a gift from Kabi-Pharmacia AB. Highly crystalline thin films ($\sim 30\,\mu$m thick) were produced by the wet-spinning technique (Rupprecht, 1970; Rupprecht & Forslind, 1970). Such films are composed of long microcrystallites which are well-oriented in the helical direction but randomly oriented in the direction perpendicular to the helical axis. The spun fibers wound up on a cylinder were bathed in an ethanolic solution with 0.01 M NaCl, and in a subsequent drying procedure the fibers merged into a film of ordered NaHA. X-ray diffraction experiments (Lee *et al.*, 1992) showed that such NaHA films were crystalline at and below 90% RH and non-crystalline at 93% RH.

UV absorption spectra were measured between 220 and 400 nm with a Varian DMS UV-Visible Spectrophotometer. Small pieces of NaHA film were brought to the desired RH by mounting them over a hole 2.00 mm in diameter in an aluminum plate inside a sealed quartz cuvette along with the appropriate saturated salt solution (Washburn, 1926). An identical aluminum plate was placed in a cuvette in the reference beam of the spectrometer. NaHA films were allowed to equilibrate for at least 2 days at each RH value before the UV spectrum was measured. The UV spectrum was first measured at 33% RH and then the saturated salt solution was replaced with the one for 92% RH. After 2 days the UV spectrum was measured again. Again the salt solution was replaced and the sample was humidified at 33% RH for a further 2 days. The UV absorption was measured again at 33% RH.

The films were not of perfect optical quality: striations were visible. These striations diffract the incident light, causing an essentially constant offset of the measured amount of transmitted light. This constant offset was subtracted for the spectra reported here. At the highest water contents of these experiments (above 88% RH), the films changed size significantly: they expanded by roughly a factor of two in the direction perpendicular to the helical axis and shrank by roughly a factor of three parallel to the helical axis. These changes were not perfectly uniform; the surfaces of the films (which were relatively flat and parallel below 88% RH) then bulged noticeably. This resulted in a focusing effect which scattered light out of the optical axis of the spectrophotometer, causing a further increase in the baseline of the detected light at the highest water contents. These shifts in the baselines were subtracted from the raw data.

RESULTS AND DISCUSSION

Lee *et al.* (1992, 1993) have performed Brillouin scattering experiments on both Li- and NaHA films as a function of RH. They used different scattering geometries to probe longitudinal acoustic phonons (i.e., sound waves) propagating both parallel and perpendicular to the helical axis of their oriented films. For the parallel direction, their equal-angle geometry permitted them to extract the speed of sound directly from the measured Brillouin shift. However, for the perpendicular direction, they used a different scattering geometry for which the measured Brillouin shift is directly proportional to the product of the refractive index and the speed of sound. Consequently, additional experimental data are needed in order to determine the perpendicular speed of sound. Recently, the refractive indices of Li- and NaHA films have been measured by Lee *et al.* (1994) and Flowers *et al.* (1992). These data on both the Brillouin shifts and the refractive indices were combined to extract the speed of sound in the perpendicular direction for both Li- and NaHA films, as given in Table 2.

These speed of sound (v_s) measurements were combined with the measured densities (ρ) of the films (Flowers *et al.*, 1992; Lee *et al.*, 1994) to yield two of the elastic moduli (c_{ij}) via $c_{ii} = \rho v_s^2$ for high symmetry directions. This result was exactly true along the helical axis, corresponding to c_{33}. For the direction perpendicular to the helical axis, our samples were effectively isotropic since the microcrystallites were randomly oriented in their equatorial plane. The work of Guss *et al.* (1975) showed that NaHA is tetragonal at 0% RH and orthorhombic at 75% RH. As discussed in Nye (1985), $c_{11} = c_{22}$ for tetragonal crystals and the randomness of the equatorial plane in our samples is irrelevant; our experiments measure c_{11}. For orthorhombic crystals $c_{11} \neq c_{22}$, meaning that our

Table 2. The measured Brillouin shifts of sound waves, refractive indices and speeds of sound for the direction perpendicular to the helical axis as a function of relative humidity (RH) for both Li- and NaHA

RH (%)	NaHA			LiHA		
	$\nu_\perp$ (GHz)	$n_\perp$	$V_\perp$ (km/s)	$\nu_\perp$ (GHz)	n	$V_\perp$ (km/s)
0	22.9	1.527	3.897	23.7	1.530	3.897
23	22.4	1.519	3.833	23.7	1.520	3.832
33	22.2	1.514	3.801	23.2	1.517	3.801
54	21.2	1.511	3.636	22.4	1.505	3.635
75	20.2	1.497	3.495	20.7	1.493	3.495
80	18.9	1.495	3.282	16.2	1.489	3.281
84	19.2	1.496	3.321	16.2	1.478	3.321
88	13.4	1.492	2.323	13.4	1.464	2.354

The Brillouin data are from Lee *et al.* (1993) and the refractive indices data are from Flowers *et al.* (1992) for NaHA and Lee *et al.* (1994) for LiHA.

measured elastic moduli is an average of c_{11} and c_{22} over all equatorial angles which we denote c_{11}^{eff}. Insufficient structural studies have been carried out to date in order to determine the range over which NaHA is in the tetragonal and orthorhombic forms. Consequently, we will denote the elastic modulus perpendicular to the helical axis as c_{11}^{eff} for all values of water content. In addition, the LiHA samples lacked crystalline order, though the helical direction was well defined. Consequently, our data can be used to determine c_{33} and c_{11}^{eff} for LiHA. The elastic moduli was determined by the strength of the bonding between the atoms of a material which, in turn, was determined by the electronic properties. Thus, changes in elastic moduli reflected changes in the electronic properties. Our evaluation of c_{33} and c_{11}^{eff} provided a quantitative measure of these changes.

As shown in Figs 1 and 2, the speed of sound in both the parallel and perpendicular directions decreased with increasing water content for both Li- and NaHA. There are two possible origins of this decrease: (1) the effect of 'mass-loading' (a mechanical effect); and (2) weakening of the bonding between atoms (an electronic effect). 'Mass-loading' refers to the increase of the effective mass of the molecular components due to the addition of the water of hydration. If the water is strongly bound to the binding site, then the water molecules will move along with the components of the HA molecule, effectively increasing the mass of the vibrating element and thereby decreasing the speed of sound. Brillouin scattering experiments (Lee *et al.*, 1993) have shown that the water of primary hydration is strongly bound to the HA molecule, implying that this water will effectively increase the mass of the HA molecule. The water of secondary hydration appears to be much less strongly bound and these water molecules presumably do not couple strongly enough to change the effective mass of the HA molecule.

In our calculations of c_{33} and c_{11}^{eff} for both Li- and NaHA the primary water of hydration (up to 80% RH) was assumed to change the effective mass of the HA molecule while the secondary water of hydration was assumed to have no effect on the effective mass. If the decrease of the speeds of sound (and, consequently, the elastic moduli) were caused solely by a mass-loading effect due to the primary water of hydration, then this calculation would yield elastic moduli which were independent of the water content. In contrast, Fig. 3 shows that both c_{33} and c_{11}^{eff} decrease as the water content increases for both Li- and NaHA over the entire range of hydration. This is strong evidence for a weakening of the effective force constants since mass-loading due to the primary water of hydration has been included via the described method.

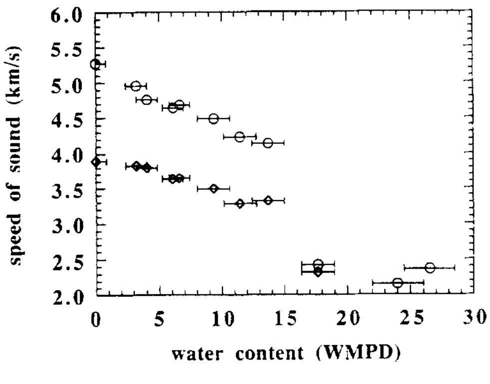

Fig. 1. The speed of sound for NaHA films for the directions parallel (open circles) and perpendicular (open diamonds) to the helical axis are shown as a function of water content. The data are derived from the Brillouin scattering data of Lee *et al.* (1993).

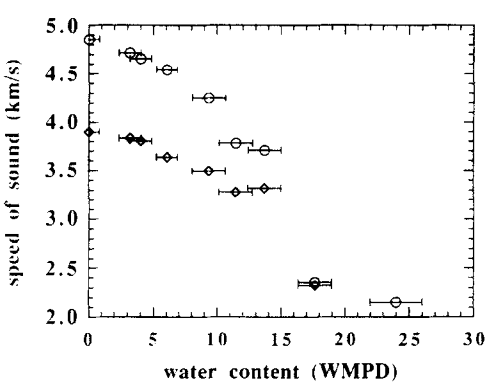

Fig. 2. The speed of sound for LiHA films for the directions parallel (open circles) and perpendicular (open diamonds) to the helical axis are shown as a function of water content. The data are derived from the Brillouin scattering data of Lee *et al.* (1993).

As evidence in support of this method of accounting for mass-loading due to the water of hydration, it should be noted that it has been successfully applied to DNA (Lavalle *et al.*, 1991; Wittlin *et al.*, 1986). As discussed earlier, the hydration mechanisms of HA and DNA appear to be very similar, implying that a method which correctly accounts for hydration effects in DNA should be useful for HA also.

Since c_{33} and c_{11}^{eff} are given by ρv_s^2, the elastic moduli are also sensitive to the volume of the unit cell via the density ρ. As discussed in Flowers *et al.* (1992) and Lee *et al.* (1994), the volume of the unit cell of both Li- and NaHA changes over the range of water content. Such changes in the unit cell can occur only if the bonding between the molecules of the unit cell changes. X-ray diffraction experiments (Mitra *et al.*, 1983; Arnott *et al.*, 1983; Sheehan & Atkins, 1983) have shown that the bonding involves intermolecular hydrogen bonding as well as long-range Coulombic interaction. Consequently, changes in the size of the unit cell reflect changes in the electronic states of the system.

The elastic moduli c_{33} and c_{11}^{eff} decrease as the water content increases for both Li- and NaHA, as shown in Fig. 3. Since the effect of mass-loading has been

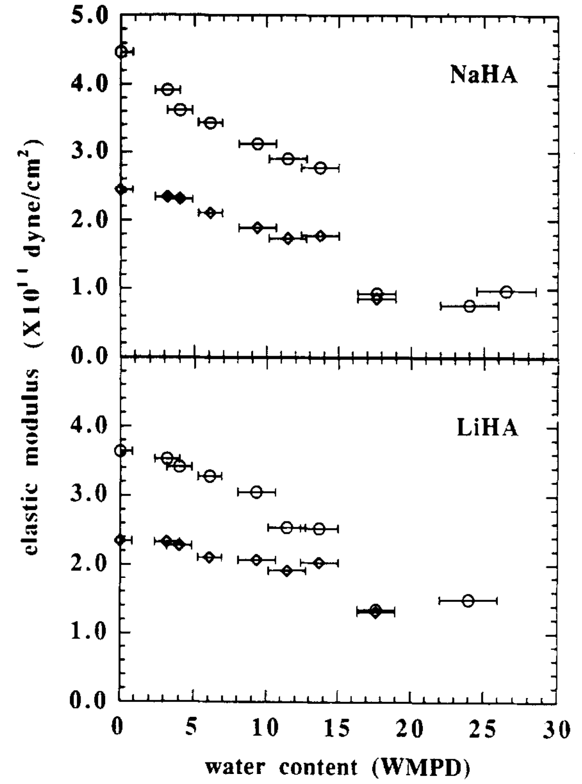

Fig. 3. The elastic moduli c_{33} and c_{11}^{eff} of NaHA and LiHA wet-spun films are shown as functions of water content. The empty circles denote c_{33} and the empty diamonds denote c_{11}^{eff}.

included, the observed decrease is clear evidence of changes in the electronic states of this system. These changes were seen to be continuous over the entire range of hydration except for the discontinuous drop at approximately 15 WMPD. These results support the interpretation of the changes observed in these wet-spun films of Li- and NaHA: the observed structural and sound speed discontinuities are driven by changes in the electronic states.

Figure 3 reveals several interesting features about the softening of the bonding in the network. For water contents below the phase transition (below approximately 14 WMPD), the elastic moduli decreased monotonically for both Li- and NaHA. The elastic modulus along the helical axis (c_{33}) decreased by approximately 30% between 0 and 14 WMPD for both Li- and NaHA while c_{11}^{eff} decreased by approximately 20%. This suggested that the interactions in the helical direction were more sensitive to water content than the interactions perpendicular to the helical direction. Since the long-range Coulomb interaction was very sensitive to the screening effects of the water of hydration, this suggested that Coulombic interactions were more important along a molecule than between neighboring molecules.

As shown in Table 1, UV absorption experiments (Lee *et al.*, 1994) and optical polarizability measurements (Flowers *et al.*, 1992) showed continuous changes beginning at approximately 75% RH. In contrast, the elastic constants showed changes at all water contents. This difference in the dependencies on the water content reflected the different spatial scales probed by these different measurements. The UV absorptivity involved measurements of the excitation of highly localized electrons within the two constituents of HA (Na-glucuronate and *N*-acetyl-glucosamine). Lee *et al.* (1994) have provided evidence that the UV absorption involves the C=O group of Na-glucuronate. The optical polarizabilities also involve highly localized electronic states. The fact that both the absorptivity and the optical polarizabilities are not affected by the first 8 or 9 WMPD (below about 75% RH) indicates that the electronic states involved in these processes are relatively insensitive to the primary water of hydration. This is certainly what is to be expected for the electrons involved in covalent bonds of, for instance, the C=O group (probably involved in the observed UV absorption). It is very likely that the UV absorption of NaHA is sensitive to the water content in an indirect manner as is the case with DNA: the water of hydration causes structural changes of the molecule (destacking of the bases in the case of DNA) and it is these structural changes which cause a change of the absorptivity. In contrast, measurements of the elastic moduli via Brillouin scattering, probe very large length scales (approximately 3000 Å). This is due to the fact that all of the atoms within a unit cell move in phase in a sound wave. Consequently, the short-range forces between atoms within a unit cell are altered very slightly. The effective restoring force felt by the atoms is most probably due to the long-range (Coulombic) forces acting on the scale of the wavelength of the sound wave. These long-range Coulombic forces are very sensitive to the screening effects of all of the water of hydration, so the elastic moduli start to decrease as soon as any water is added to the system.

Figure 4 shows the results of UV absorption measurements between 220 and 400 nm of a NaHA film. Curve 1 shows the uncycled film at 33% RH. Note that the only significant feature of curve 1 is a large absorption which goes off-scale at 220 nm. This absorption is so intense that its peak exceeds the measurement ability of our spectrophotometer. Curve 2 shows the same film at 92% RH (above the phase transition). There are three differences between curves 1 and 2: the edge of the intense absorption at 220 nm has shifted to higher wavelengths and two new absorption peaks are observed (at approximately 270 and 320 nm). For curve 3, the same film was then re-exposed to 33% RH; that is, this film had been cycled through the transition. Note that curve 3 shows two features observed in curve 2: the edge of the intense absorption at 220 nm is still shifted to higher wavelengths and the 270 nm absorption peak is still present, though at slightly lower intensity. It is difficult to deduce whether

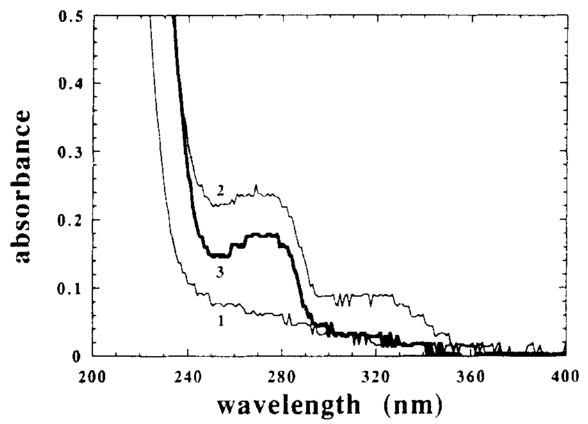

Fig. 4. The UV absorption spectrum of a NaHA film is shown for the range 220–400 nm. Curve 1 shows the spectrum of an uncycled film at 33% RH. Curve 2 shows the spectrum of the same film at 92% RH (above the transition). Curve 3 shows the spectrum of the same film back at 33% RH (after having been cycled through the transition).

or not the 320 absorption peak is still present. The intensity of the 320 peak is definitely lower in curve 3 than in curve 2. However, it is possible that the peak is present but cannot be resolved from the experimental noise. The important point is that curve 3 is not identical to curve 1. This is strong evidence that the phase transition is irreversible.

Another possibility is that the phase transition is reversible but has significant hysteresis. Our present experimental results cannot eliminate this interpretation. Even if this is the case, such hysteresis is characteristic of first order phase transitions.

As discussed by Lee *et al.* (1994) the UV absorptions near 270 and 320 nm most likely involve the C=O group of Na-glucuronate. The oscillator strength for these two absorption peaks is non-zero above the phase transition. The fact that these peaks first become evident above the phase transition suggests that local changes in the geometry of the molecular unit occur at the phase transition. The fact that these peaks are observed in a sample at low humidity after being cycled through the transition implies that the changes in local geometry are irreversible. The sample can absorb UV light at low humidity because the appropriate constituents of the molecule are still in the 'new' position. It is most likely that the conditions present during the wet-spinning process (i.e., degree of hydration and the amount of applied tension) are sufficient to foster the adoption of the original crystalline form. Once the crystalline pattern is destroyed by the phase transition, the molecule is unable to recover its original state.

This combination of the different experimental studies of the phase transition in Li- and NaHA allows a picture of the mechanism of this transition to be developed. Measurements of the speed of sound and the density of these films yield the elastic moduli which depend on the bonding strength between and within the molecules of the network. This bonding is determined by the electronic states of the system. Hence, changes in the elastic moduli represent changes in the electronic states. The observed decrease in the elastic moduli c_{33} and c_{11}^{eff} indicate that the bonding in the network weakens as the water content is increased, due to screening of the charged sites. Similarly, the degree of crystallinity of the sample depends on the nature of the bonding between neighboring molecules which must be strong enough to maintain crystalline order. The loss of crystalline order in NaHA between 20 and 26 WMPD (between 90 and 93% RH) indicates that the relevant bonding has become too weak to maintain the crystalline network; entropic forces are then able to destroy the crystalline regularity.

SUMMARY

Two measurements of electronic states have been reported here: (1) the elastic constants of wet-spun films of Li- and NaHA; and (2) UV absorption experiments on NaHA films before and after cycling through the phase transition. The elastic moduli of Li- and NaHA decrease over the entire range of hydration. This indicates that the long-range Coulombic interactions are very sensitive to the water content. Weakening of the electronic interactions appears to drive the phase transition observed between 84 and 92% RH. The irreversible changes in the UV absorption support the conjecture that changes occur in the local geometry of the molecular components at the phase transition.

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