Brillouin-scattering study of hyaluronic acid: Dynamic coupling with the water of hydration and phase transitions

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Brillouin spectra have been measured from wet-spun films of Li and Na hyaluronate (NaHA) between 0% and 93% relative humidity (RH). The speed of sound is very high (about 5 km/s) in both Li and NaHA films at low values of RH. The Brillouin spectra show substantial coupling between the longitudinal-acoustic phonons and a relaxation mode of the water of hydration. A coupled-modes model is used to interpret these data. This analysis shows that the relaxation time of the water of hydration (about 50 ps) is constant over the range of hydration. The microscopic coupling constant also appears to be independent of hydration. Between 84% and 88% RH, the uncoupled phonon frequency for both Li and NaHA is found to drop by $\sim 40\%$, and $\sim 25\%$ for phonons propagating in the parallel and perpendicular directions, respectively, indicative of a phase transition.

INTRODUCTION

Hyaluronic acid (HA) is a polydisaccharide of the form $(—A—B—)_n$ where A is glucuronic acid and B is N-acetylglucosamine. Hyaluronic acid must have arisen early in evolution since it is found in streptococci. This molecule is the central organizing component of cartilage and is found in other connective tissues. The mechanical properties of this molecule are of great interest in order to gain a more complete understanding of the mechanical function of such connective tissues.

As with other biopolymers, local concentrations of electrical charge along the HA molecule attract water molecules to form a hydration shell. The water content curve (the number of water molecules per disaccharide repeat unit as a function of relative humidity) has been measured [1,2] and found to be similar in shape to the water content curve of DNA [3,4]. The presence of the water molecules at the charged sites of hydration modifies the intramolecular Coulombic interactions, and therefore changes the properties of the HA molecule.

Our initial Brillouin study of wet-spun films of NaHA [5] revealed two surprising results. First, the speed of sound along the helical axis in NaHA is greater than 5 km/s at low values of relative humidity (RH). This is an extraordinarily large speed of sound for a polymer and is comparable to the speed of sound in such "soft" metals as copper [6]. In contrast, the speed of sound along the helical axis in NaDNA [7] is about 3.5 km/s at low values of RH. Second, the speed of sound along the helical axis in

NaHA was found to drop by $\sim 40\%$ between 84% and 88% RH, indicative of a phase transition.

Given the biological importance of the mechanical properties of this molecule, an understanding of the microscopic origin of this phase transition is very important. One likely explanation of this phase transition is that it is driven by a change of the conformation of the HA molecule. Another possibility is that the transition has its origin in the crystallinity of the lattice, such as the A to B transition in DNA. Lindsay et al. [8] have shown the importance of strong intermolecular bonds of the crystalline lattice in stabilizing the A conformation of DNA. It is possible that the phase transition observed in NaHA might also be connected to the crystallinity of the sample. In order to probe the origin of this transition, we report in this article our Brillouin experiments on both crystalline NaHA films and *noncrystalline* LiHA films as a function of RH. The data from LiHA are essentially identical to the results from NaHA, indicating that the crystallinity of the sample does not play any role in the phase transition. This is consistent with the hypothesis that a molecular conformation change drives the phase transition.

Very recent measurements have been reported of the refractive indices and optical polarizabilities of wet-spun films of NaHA [2] and showed that the polarizabilities drop by about 17% between 80% and 88% RH. This change in the molecular polarizabilities also provides support for the hypothesis that a *molecular* conformational change drives this phase transition.

The interpretation of the Brillouin data is not straight-forward since these films are heterogeneous (consisting of HA and water) at all values of RH above 0%. The effects of coupling between the acoustic phonons and the relaxation mode of the water of hydration can modify the observed Brillouin spectra. Our data are interpreted via a coupled-modes model which has been used successfully to describe similar coupling in NaDNA by Tao and co-workers [9,10]. This analysis permits us to determine the changes in the HA molecule itself, independent of any effects due to the coupling of the two modes. Changes in the "uncoupled" molecule near the phase transition between 84% and 88% RH are of particular interest.

We compare the results of our study of Li and NaHA to the results for the better-studied biopolymer, DNA. For NaDNA, Tao and co-workers [9,10] found that both the relaxation time τ (≈ 40 ps) of the primary hydration shell and the microscopic coupling constants β are independent of RH. The values of β reported were 4.2×10^{-50} kg m^4/s and 5.5×10^{-50} kg m^4/s for phonons propagating parallel and perpendicular to the helical axis, respectively. We find similar behavior in both Li and NaHA. Tao and co-workers also found that the primary hydration shell maintains its integrity even after the secondary hydration shell has been filled.

EXPERIMENT

Films of oriented Li and NaHA with low contents of LiCl and NaCl, respectively, were prepared with a wet-spinning method described in detail elsewhere [11,12]. NaHA of bacterial origin, a generous gift from KABI-Pharmacia AB, was spun by analogy with the wet spinning of K hyaluronate in Ref. [13]. X-ray-diffraction experiments [5] showed that the NaHA films were crystalline below the phase transition and disordered above the transition. To obtain LiHA films, NaHA was first dialyzed twice against 1.5M LiCl and thereafter spun as LiHA in an analogous fashion. It was observed that LiHA did not spin quite as efficiently below the spinneret as NaHA. Although x-ray-diffraction patterns from films of LiHA obtained with this procedure did not show any spots characteristic of crystalline samples, the LiHA molecules should be well oriented. However, due to the less efficient spinning of LiHA the degree of orientation might be somewhat inferior to that of NaHA. For the Brillouin-scattering experiments, small pieces of the films were mounted in sealed cuvettes. The relative humidity of the cuvettes was controlled by placing a small amount of the appropriate saturated salt solution [14] in the bottom of the cuvette. The samples were allowed to equilibrate for at least three days before any Brillouin experiments were performed.

The Brillouin spectra were recorded at room temperature using a tandem Fabry-Pérot interferometer [15] with 5 mW of 5145-Å light from an argon-ion laser at the sample chamber. The laser was focused to a spot about 50 μm in diameter on the sample. This power level does not disrupt the Brillouin spectra observed from DNA [16]. The wave vectors of the phonons being probed in these experiments were either parallel or perpendicular to the

helical axes of the HA molecules. For the parallel measurements, an equal angle (45°-45°) forward-scattering geometry was used with the film bisecting the incident and scattered beam directions. For the perpendicular measurements, a near backscattering geometry (with an external scattering angle of 165°) was used. The measured linewidth data were corrected for instrumental broadening by deconvolution and aperture broadening by integrating over the aperture [17].

Brillouin scattering (inelastic light scattering via the creation or annihilation of acoustic phonons) gives rise to peaks shifted in frequency by an amount

$$v_B = \frac{2 n V_s \sin\left[\dfrac{\theta}{2}\right]}{\lambda_L} , \tag{1}$$

where v_B is the frequency of the acoustic phonon involved, n is the refractive index, V_s is the speed of sound, θ is the internal scattering angle, and λ_L is the wavelength of the incident laser light. Equation (1) is valid for optically isotropic materials and for light scattering from longitudinal acoustic phonons in optically anisotropic materials such as HA. For such materials with an equal-angle forward-scattering geometry, Eq. (1) can be rewritten (by using Snell's law):

$$v_B = \frac{2 V_s \sin\left[\dfrac{\Psi}{2}\right]}{\lambda_L} , \tag{2}$$

where Ψ is the external scattering angle.

If the sound wave is attenuated with an energy decay constant α, then the Brillouin peaks have a half width $\Gamma_{1/2}$, given by

$$\Gamma_{1/2} = \frac{\alpha V_s}{2\pi} . \tag{3}$$

The normalized phonon attenuation is given by

$$\alpha \lambda_s = \frac{2\pi \Gamma_{1/2}}{v_B} , \tag{4}$$

and may be determined directly from the Brillouin spectra.

COUPLED-MODES MODEL

The coupled-modes model has been successfully used for the study of soft modes in a number of ferroelectrics [18]. Tominaga et al. [19] used Barker and Hopfield's [20] description for the coupling between a low-frequency optic mode of DNA and the relaxation mode of the hydration shell. In recent work by Tao and co-workers [9,10] this model has been applied to study the effects of hydration on the acoustic phonons of DNA. This model assumes displacive coupling between the two modes and incorporates both the changes in the coupling strength as water is added and the wave vector of the sound wave q in a natural way. The essential features of this phenomenological model are given here to clarify the interpreta-

tion of the physical parameters; the reader is referred to Refs. [9] and [10] for further details. The equations of motion are

$$m'(\omega_w^2 - \omega^2 - 2i\omega\Gamma)Q_r + k_{12}Q_p = f_w \ , \tag{5}$$

$$k_{12}Q_r + \frac{\rho}{q^2}(\omega_0^2 - \omega^2 - 2i\omega\gamma)Q_p = f_p \ . \tag{6}$$

Here m' is the mass of water per unit volume, ω_w and Γ are the resonant frequency and linewidth of the water relaxation mode, respectively, and k_{12} is the coupling between the generalized displacements of the relaxation mode Q_r and the phonon Q_p. Note that the exact nature of the relaxation mode displacement is unknown. The "generalized driving forces" on the water mode and the acoustic mode are f_w and f_p, respectively. The mass density of the disaccharide is ρ, the phonon wave vector is q, and ω_0 and γ are the resonant frequency and linewidth of the uncoupled phonon, respectively. Tao and co-workers found that the resulting phonon susceptibility T_p is given by

$$T_p = \frac{Q_p}{f_p} = \cfrac{1}{\omega_0^2 - \omega^2 - 2i\omega\gamma - \cfrac{k_{12}^2 q^2}{m'\rho\omega_w^2(1 - i\omega\tau)}} \ . \tag{7}$$

Here the relaxation time of the water of hydration τ is given by

$$\tau \equiv \frac{2\Gamma}{\omega_w^2} \ . \tag{8}$$

We re-express the coupling constant as

$$\delta^2 \equiv \frac{k_{12}^2 q^2}{m'\rho\omega_w^2} \ . \tag{9}$$

The strength of the coupling constant k_{12} between the water relaxation mode and the HA phonon should depend (to first order) on the product of the number densities of both the water of hydration n_w and the disaccharide repeat units n_d

$$k_{12} = n_w n_d \xi \ . \tag{10}$$

Here ξ is a proportionality constant. We also have that $m' = n_w m_0$ (where m_0 is the mass of a single water molecule) and $\rho = n_d m_d$ (where m_d is the mass of the disaccharide repeat unit). Also the number densities n_d and n_w will be connected by the water content N_w (the number of water molecules per disaccharide repeat unit) as $n_w = N_w n_d$. Equation (9) can be rewritten as

$$\delta^2 = \frac{n_d^2 N_w \xi^2 q^2}{m_0 m_d \omega_w^2} = \frac{n_d^2 N_w \beta^2 q^2}{m_0 m_d} \ , \tag{11}$$

where $\beta^2 \equiv \xi^2/\omega_w^2$. This coupling constant β is expected to be approximately independent of water content for the assumptions made. Tao and co-workers [9,10] found that β was roughly independent of water content for NaDNA over a very wide range of water contents. They found that only the water of hydration in the primary shell contributed to the coupling of the two modes.

From the fluctuation-dissipation theorem the Brillouin spectra is proportional to the imaginary part of the phonon susceptibility: $S(\omega) = (kT/\hbar\omega)\text{Im}(p^2 T_p)$ where p is the photoelastic constant. We have used the imaginary part of Eq. (7) to fit our experimental data, as discussed in the next section. The adjustable parameters of these fits are ω_0, γ, δ, and τ. We have constrained τ to be constant for all values of RH. The width and height of the central feature in our spectra are particularly sensitive to the value of the coupling constant δ.

RESULTS AND DISCUSSION

Raw Brillouin spectra and the fits of the coupled-modes model [Eq. (7)] for phonons propagating parallel to the helical axis of LiHA are shown in Fig. 1. Experiments performed for several different values of the wave vector q (by varying the scattering angle) for the LiHA sample at 88% RH confirmed that the observed peak is due to an acoustic phonon. The raw data can be used to extract both the speed of sound V_s (Fig. 2) and the normalized phonon attenuation $\alpha\lambda_s$ (Fig. 3), as described above. Figure 2 shows the speed of sound in the parallel direction for both Li and NaHA as a function of RH,
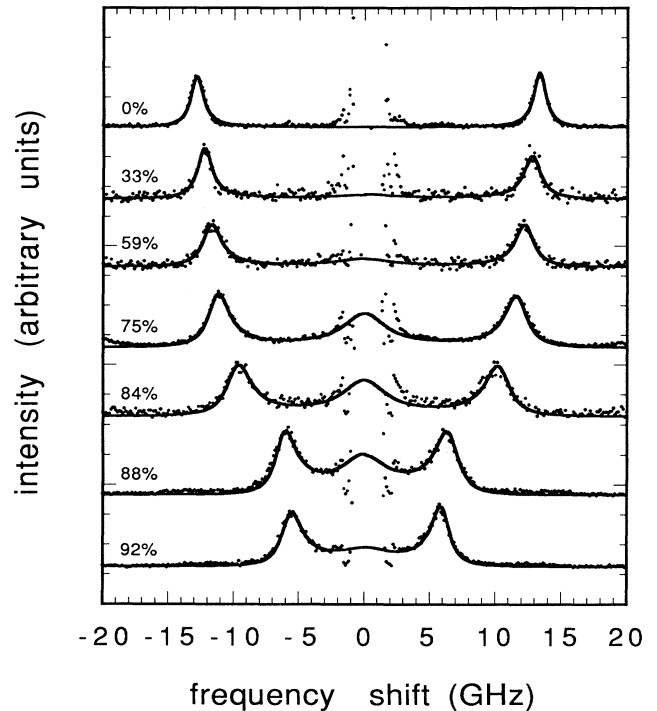

FIG. 1. Raw Brillouin data (the solid circles) for phonons propagating in the parallel direction in LiHA are shown for 0, 33, 59, 75, 84, 88, and 92% RH. The theoretical fits [from the imaginary part of Eq. (7)] are the solid lines. Note the dramatic change in the phonon frequency between 84 and 88% RH, indicative of phase transition. Values for the parameters used in the theoretical fits are given in Table I.
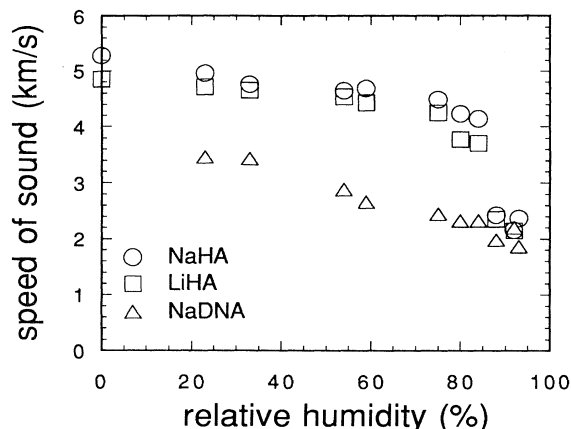
FIG. 2. Speed of sound (extracted directly from the experimental data) in the parallel direction for NaHA (the open circles), LiHA (the open squares), and NaDNA (the open triangles) as a function of relative humidity. The speed of sound in both Li and NaHA drops by ~40% between 84 and 88% RH, indicative of a phase transition.

along with data from NaDNA [7]. The speed of sound in the parallel direction is very similar in both Li and NaHA at all values of RH. In particular, the speed of sound is very high at low values of RH (of the order of 5 km/s) and it drops by about 40% between 84% and 88% RH for both Li and NaHA. The fact that the phase transition is observed both in our crystalline samples (NaHA) and our noncrystalline samples (LiHA) strongly supports the conjecture that the underlying mechanism for this phase transition involves a molecular conformation change.

Figure 2 does show two small differences between Li and NaHA. First, below the transition, the speed of sound in LiHA appears to be consistently smaller than the speed of sound in NaHA. Second, the change in the speed of sound at the transition is ~37% in LiHA while it is ~42% in NaHA. This difference could be due to ei-

ther the lack of crystallinity in the LiHA samples or the identity of the counterion. As we discuss later, other evidence supports the former interpretation. Above the transition the speed of sound is very similar for LiHA, NaHA, and NaDNA.

Figure 3 shows $\alpha\lambda_s$ (the normalized phonon lifetime) extracted directly from the experimental data for both Li and NaHA as a function of RH. The results for the two counterions are very similar. Evidence for the phase transition is seen in the dramatic increase of $\alpha\lambda_s$, though this quantity changes in a somewhat less abrupt manner than did the speed of sound.

The values for the fitting parameters δ, γ, and ω_0 of the coupled-modes model are listed in Table I for all of the data from both Li and NaHA. We find that τ (the relaxation time of the water mode) is about 50 ps at all values of water content. This value is close to the value of about 40 ps determined for NaDNA by Tao and co-workers [9,10], suggesting that the hydration shells for these two biopolymers are similar.

Since this coupled-modes analysis yields the uncoupled phonon frequency ω_0, we can evaluate the degree of softening intrinsic to the HA molecule. Figures 4(a) and 4(b) show the uncoupled phonon frequency in the perpendicular and parallel directions, respectively, for both Li and NaHA as a function of RH. These figures show *significant and discontinuous* softening between 84% and 88% RH. This clearly shows that dramatic changes are occurring in the HA molecule and to the bonding to neighboring molecules in this range of values of RH. At the phase transition, ω_0 drops by ~40% and ~25% for phonons propagating in the parallel and perpendicular directions, respectively, for both Li and NaHA. In other words, the transition as measured by phonons propagating in the perpendicular direction is less abrupt than for the parallel direction. As noted earlier, the speed of sound in the parallel direction in the LiHA samples showed a less abrupt transition than in NaHA. Figure 4(a) shows a similar effect in the phonons propagating perpendicular to the helical axis in the crystalline samples of NaHA. This supports the interpretation that the

TABLE I. The values of the uncoupled phonon frequencies ω_0, the coupling constant δ, and the uncoupled phonon linewidth γ as determined by fitting the raw Brillouin spectra for phonons propagating in the parallel and perpendicular directions for both Li and NaHA. The theoretical expression for the Brillouin spectra is proportional to the imaginary part of Eq. (7).

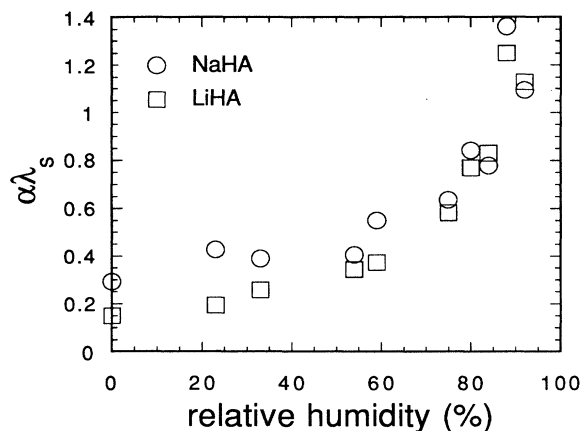|	LiHA						NaHA					
RH	$\omega_{0\parallel}$ (Grad/s)	$\delta_\parallel$ (Grad/s)	$\gamma_\parallel$ (Grad/s)	$\omega_{0\perp}$ (Grad/s)	$\delta_\perp$ (Grad/s)	$\gamma_\perp$ (Grad/s)	$\omega_{0\parallel}$ (Grad/s)	$\delta_\parallel$ (Grad/s)	$\gamma_\parallel$ (Grad/s)	$\omega_{0\perp}$ (Grad/s)	$\delta_\perp$ (Grad/s)	$\gamma_\perp$ (Grad/s)
0	82.4		3.58	149.5		3.37	89.9		5.72	142.6		3.52
23	80.5	25.0	3.32	148.8	50.0	3.19	85.0	27.5	5.70	142.5	66.5	3.01
33	79.3	28.5	3.62	148.4	50.0	3.14	81.5	31.0	5.70	141.0	72.5	3.02
54	77.5	30.0	3.50	141.0	70.0	3.50	79.5	32.5	5.70			
59	75.9	34.5	3.40	140.5	85.0	3.18	79.9	37.5	5.70	133.0	75.0	3.40
75	73.1	47.3	2.90	131.5	90.0	4.00	76.9	43.0	4.50	129.5	85.0	3.00
80	65.5	43.5	2.30	120.8	95.0	5.00	72.3	43.0	5.00	122.5	90.0	5.00
84	63.8	40.0	3.50	121.2	96.0	5.50	70.5	40.0	5.00	122.0	85.0	4.50
88	40.8	26.0	3.50	93.2	70.0	11.0	41.7	24.0	5.00	86.3	81.0	10.5
92	37.6	19.3	3.35	85.3	65.0	10.0	37.5	18.0	4.00	80.0	42.0	14.0
93							39.0	19.0	6.50	84.0	47.5	12.8

FIG. 3. Normalized phonon attenuation $\alpha\lambda_s$ (extracted directly from the experimental data) for phonons propagating in the parallel direction for both NaHA (the open circles) and LiHA (the open squares) as a function of relative humidity.
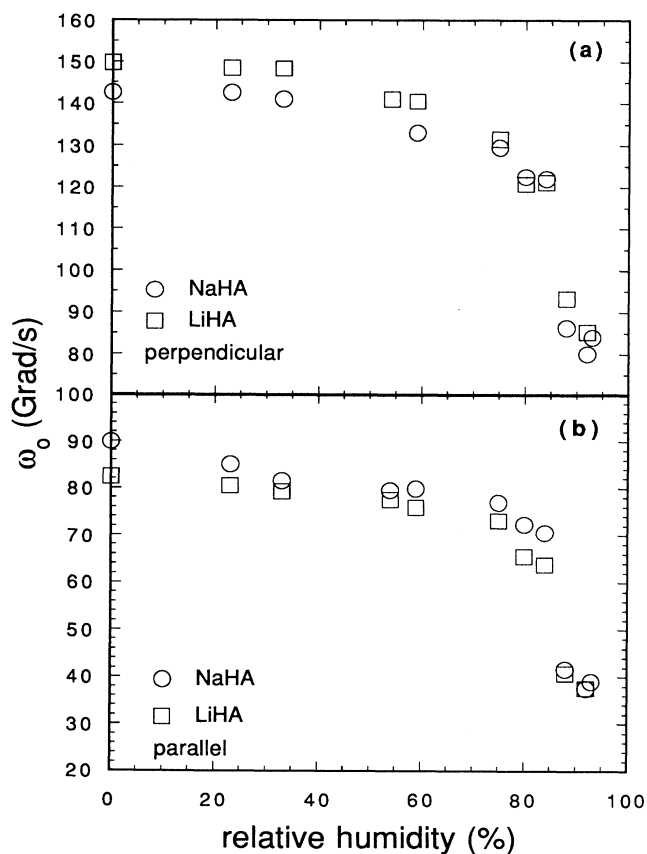

FIG. 4. Uncoupled phonon frequency ω_0 for both NaHA (the open circles) and LiHA (the open squares) for phonons propagating in the (a) perpendicular and (b) parallel directions as a function of relative humidity. Discontinuities in ω_0 of $\sim 25\%$ in the perpendicular direction and $\sim 40\%$ in the parallel direction are observed at the phase transition.

lack of crystalline order in the LiHA samples caused the less abrupt drop in the parallel speed of sound shown in Fig. 2. It is important to emphasize that this coupled-modes analysis has allowed us to evaluate the softening intrinsic to the "uncoupled" HA molecule. The fact that the discontinuous drop is observed in both crystalline NaHA and noncrystalline LiHA shows conclusively that this transition is not due to an effect of the crystalline network. Given the molecular evidence of Flowers et al. [2] these Brillouin data provide strong support for the hypothesis that the phase transition is driven by a molecular change.

Falk, Hartman, and Lord [3] performed the first measurements of the water content of DNA and they found that the water content increased significantly above an RH of about 80%. This led to the suggestion of two kinds of hydration: "primary" (up to $\sim 80\%$ RH) and "secondary" (above $\sim$ 80% RH). Temperature-dependent infrared studies by Falk, Poole, and Goymour [21] showed that the primary hydration is different from bulk water since it does not freeze into a crystalline ice lattice. Raman [22] and Brillouin [9,10] experiments of Tao and co-workers showed that the water of primary hydration is tightly bound to DNA, while the water of secondary hydration is bound much more loosely. The coupled-modes analysis of Tao and co-workers [9,10] showed that the primary hydration shell retains its identity even under conditions of secondary hydration. The water content data for HA [1,2] also show a significant increase above an RH of about 80%. This suggests that the hydration process is also composed of primary and secondary hydration in HA. Evidence for such a hydration in HA has been given by Hirohisa et al. [23] and by Joshi and Topp [24] using differential scanning calorimetry. Part of the water of hydration was found to be very strongly bound to the HA. This should correspond to the primary hydration referred to above. NMR work [25] by Lahajnar and Rupprecht also showed a hydration in HA similar to that observed in DNA.

We assume that the coupling between the acoustic phonons and the water of hydration in HA involves only the primary hydration (the case observed in DNA). By using Eq. (11) and the data of Flowers et al., β, the microscopic coupling constant, is determined, as shown in Table II. The coupling constant is found to be approximately independent of water content and shows very little sensitivity to the identity of the counterion. For NaHA, β is found to be 3.4×10^{-50} kg m^4/s and 7.7×10^{-50} kg m^4/s for longitudinal acoustic phonons propagating parallel and perpendicular to the helical axis, respectively. For LiHA, β is found to be 2.9×10^{-50} kg m^4/s and 6.4×10^{-50} kg m^4/s for longitudinal acoustic phonons propagating parallel and perpendicular to the helical axis, respectively. As in the case of NaDNA, the perpendicular coupling constant is larger than the parallel coupling constant, though the anisotropy is only $\sim 30\%$ in NaDNA and $\sim 100\%$ for both Li and NaHA.

Several similarities in the results from DNA and HA are worthy of note since the composition (and, to some extent, the structure) of these biopolymers are substantially different. First, the relaxation time of the water re-

TABLE II. The values of the water content N_w, the number density n_d of disaccharide repeat units, and the coupling constant β for phonons propagating in both the parallel and perpendicular directions in both Li and NaHA. The water content N_w and number density n_d of disaccharide repeat units are for NaHA and taken from Ref. [2]. The coupling constant β is determined by using Eq. (11). For LiHA the values of N_w and n_d for NaHA are used.

			LiHA		NaHA	
RH (%)	N_w	n_d (10^{26} m^{-3})	$\beta_\parallel$ $(10^{-50} \text{ kg m}^4/\text{s})$	$\beta_\perp$ $(10^{-50} \text{ kg m}^4/\text{s})$	$\beta_\parallel$ $(10^{-50} \text{ kg m}^4/\text{s})$	$\beta_\perp$ $(10^{-50} \text{ kg m}^4/\text{s})$
23	3.1	2.57	2.97	5.95	3.16	7.68
33	3.8	2.50	2.99	5.28	3.31	7.81
54	5.3	2.35	2.64	6.16	3.16	7.28
59	5.6	2.21	3.10	7.63	3.76	7.52
75	8.0	2.03	3.79	7.26	3.96	7.86
80	8.5	1.99	3.18	6.94	3.94	8.24
84	8.5	1.99	2.93	7.00	3.67	7.76
88	8.5	1.97	1.93	5.17	2.23	7.50

laxation mode is very similar: 40 ps for DNA and 50 ps for HA. Also, the microscopic coupling constant β has similar values for both DNA and HA: $\beta_\parallel$ is $\sim 3.0 \times 10^{-50}$ kg m^4/s for HA and $\sim 4.2 \times 10^{-50}$ kg m^4/s for DNA and $\beta_\perp$ is $\sim 7.0 \times 10^{-50}$ kg m^4/s for HA and $\sim 5.5 \times 10^{-50}$ kg m^4/s for DNA. These similarities suggest that the properties of the hydration shell and its coupling to the biopolymer have only a small dependence on the identity of the biopolymer itself. Finally, at and above 88% RH, the speed of sound in the parallel direction is virtually the same for both DNA and HA. Given the intrinsic differences between these two biopolymers, the fact that the speeds of sound are so similar is either a remarkable coincidence or suggests "universal" behavior of heavily hydrated biopolymers.

SUMMARY

Brillouin spectra have been measured from wet-spun films of Li and NaHA between 0% and 93% RH. These spectra show substantial coupling between the longitudinal acoustic phonons and a relaxation mode of the water of hydration. A modified coupled-modes analysis of these data shows that the relaxation time of the water of hydration (about 50 ps) and the microscopic coupling constant are constant over the range of hydration studied. The relaxation time τ is very similar to the value reported for DNA, suggesting that the two hydration shells are similar in nature. The microscopic coupling constant β shows significant anisotropy in HA. Also, β is approximately the same for both HA and DNA. A phase transition is observed between 84% and 88% RH for both noncrystalline LiHA and crystalline NaHA. The uncoupled phonon frequencies drop by $\sim 40\%$ for phonons propagating in the parallel direction and $\sim 25\%$ for phonons propagating in the perpendicular direction. The observation of this transition in both the crystalline and noncrystalline samples argues for a molecular origin of the phase transition.

ACKNOWLEDGMENTS

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