Optical and physical properties of wet-spun films of Na-hyaluronate

Evidence of a phase transition

M. R. Flowers,* R. L. Marlowe,‡ S. A. Lee,* N. Lavalle,* and A. Rupprecht§

*Department of Physics and Astronomy, University of Toledo, Toledo, Ohio 43606 USA; ‡Department of Physics and Astronomy, University of Tennessee at Chattanooga, Chattanooga, Tennessee 37403 USA; and §Division of Physical Chemistry, University of Stockholm, S-106 91 Stockholm, Sweden

ABSTRACT The refractive indices, water content, and volume of wet-spun films of Na-hyaluronate have been measured as a function of relative humidity (*rh*). These data are used with the Lorentz-Lorenz formula to determine the optical polarizabilities of Na-hyaluronate parallel and perpendicular to the helical axis. The analysis reveals a drop in the optical polarizabilities of ~20% between 80 and 88% *rh*, indicating a phase transition.

INTRODUCTION

Hyaluronic acid is a polydisaccharide of the form $(\text{-A-B-})_n$ where A is D-glucuronic acid and B is *N*-acetylglucosamine. Hyaluronic acid must have arisen early in evolution because it is produced by streptococci. Hyaluronic acid is the central organizing component of cartilage. In mammals, this biopolymer is also found in the other connective tissues as well as the vitreous humor. Clearly, this molecule plays a major role in determining the structural and mechanical properties of these connective tissues. Consequently, the structural and mechanical properties of hyaluronic acid are of great biological significance. Recent Brillouin experiments (1) have revealed a dramatic discontinuity (of ~40%) in the speed of sound in wet-spun films of Na-hyaluronate between relative humidities (*rh*) of 84 and 88%. This indicates that the elastic constants of Na-hyaluronate change significantly at this transition. Further elucidation of the mechanism of this transition is required before any useful suggestions about the biological relevance of this transition can be made. In order to provide such additional information, we report measurements of the refractive indices, water content, and the volume of wet-spun films of Na-hyaluronate as a function of *rh*. The refractive indices can be related to the electronic polarizabilities which provide a measure of the response of the electronic degrees of freedom of the system to an external perturbation (the electric field of the light). These polarizabilities are a molecular property[1] and can be used as a probe of changes in the molecule during the phase transition.

Information regarding the polarizabilities of Na-hyaluronate are also of interest due to the observations of permanent macroscopic polarization in another connective tissue: rat-tail tendon (RTT). Freund and coworkers (2–4) have observed optical second harmonic generation (SHG) from RTT. This result is surprising because RTT is composed of equal numbers of up- and down-pointing collagen fibrils (5). The resulting inversion symmetry of RTT should preclude the possibility of SHG. Second harmonic microscopy experiments (3, 4) revealed the existence of a large number of polar, filament-like structures throughout the RTT. To date, no microscopic origin has been suggested for these polar structures in RTT.

X-ray studies (6–11) have indicated that fibers of hyaluronic acid can adopt different structures depending on the conditions of water content, pH, temperature, tension, and sample preparation. For this work, thin film samples were prepared by the wet-spinning technique (12, 13). X-ray diffraction experiments (1) have shown that wet-spun films of Na-hyaluronate are well oriented and highly crystalline below the transition and disordered above the transition. However, these x-ray studies were not able to determine if the conformation of the molecule changes during the transition because the order of the films would be destroyed if the molecules move away from their lattice sites whether or not the conformation of the molecules changes. Our measurements of the optical polarizabilities will provide useful information to determine if the order–disorder phase transition involves a change in the molecular conformation.

EXPERIMENTAL

Na-hyaluronate was provided as a gift from Kabi-Pharmacia AB (Stockholm). Highly crystalline thin films (~30 μm thick) were produced by the wet-spinning technique (12, 13). Electron microscopy studies (14) of wet-spun films of DNA showed that such films are composed of microcrystallites ~3 μm in diameter and macroscopic in length along the helical axes. The microcrystallites are embedded in an amorphous matrix. The volume of these films are found to be ~15% voids (15, 16). The helical axes of the microcrystallites are all aligned in the direction of the stress of the wet-spinning process. The equatorial planes of these microcrystallites are randomly oriented, averaging the properties of the equatorial plane. Though no such studies have been done on wet-spun films of Na-hyaluronate, it is reasonable to assume that these films have a similar morphology: aligned microcrystallites in an amorphous matrix.

The refractive indices were measured via an index-matching technique (15). Small pieces of Na-hyaluronate film were mounted in a sealed sample chamber. The desired *rh* was introduced into the sample chamber by bubbling dry nitrogen gas through the appropriate saturated salt solution (17). The samples were humidified for at least three days. The sample was illuminated with polarized light with wavelength of 500 nm and index-matching fluids were introduced into the

---

[1] The electronic polarizabilities are a molecular property provided that there is no significant contribution from the intermolecular bonds.

0006-3495/92/08/323/04 $2.00

chamber. Photomicrographs were taken of the sample immersed in each index-matching fluid. Wet-spinning produces a film with a rough surface. Due to the focussing effects of the undulating surface, the sample appeared to have light and dark stripes when the refractive indices of the fluid and the sample did not match. The disappearance of these stripes indicate a match of the refractive indices. An Abbe refractometer (Milton Roy, Rochester, NY) was used to measure the refractive index of the fluid. Heptane and 1-chloronaphthalene were mixed (in the appropriate ratios) to produce the index-matching fluids. These materials were chosen for the index-matching fluid since Weidlich et al. (15) found that these fluids do not change the water content of hydrated DNA films.

The water content of these films was determined by using a balance sealed in a glove box. The humidity inside the glove box was controlled by placing the appropriate saturated salt solutions in the glove box. A hygrometer (traceable to the National Institute of Standards and Technology) was used to monitor the *rh*. (The large glove box proved difficult to seal perfectly.) The mass of the film was measured at each *rh*. These measurements were then converted into the number of water molecules per disaccharide by using the molecular weights.

The dimensions of small pieces of Na-hyaluronate were measured by placing samples in a sealed chamber. The samples were mounted on opposing mechanical feedthroughs so that the samples could expand (and shrink) freely. As the dimension of the sample changes, the distance between the two supports was adjusted via the mechanical feedthroughs. Thus, the films were kept nearly flat without any external stress. Saturated salt solutions were used to control the *rh*. Photomicrographs were taken of the films and the size of each film (perpendicular to the axis of the feedthroughs) was measured at each *rh*. These measurements yielded the relative expansion of the film in the directions parallel and perpendicular to the helical axes of the Na-hyaluronate molecules. This same technique has been used in the measurement of wet-spun films of DNA and, when compared to the results of x-ray diffraction, found to provide reliable measurements below 90% *rh* (16, 18).

THEORY

For an isotropic case, the polarizability may be related to the refractive index via the Lorentz-Lorenz equation:

$$\frac{n^2 - 1}{n^2 + 2} = \frac{4\pi N\alpha}{3V}, \tag{1}$$

where n is the refractive index, N/V is the number density, and α is the electronic polarizability. A more general theory with corrections for internal fields was developed by Boettcher (19). The errors due to using Eq. 1 rather than the Boettcher theory are generally small (20) and can be neglected in comparison with our experimental uncertainties. For an anisotropic case, we have (21):

$$\frac{n_i^2 - 1}{\bar{n}^2 + 2} = \frac{4\pi \sum N\alpha_i}{3V}, \tag{2}$$

where i labels the polarization of the incident light (either parallel or perpendicular to the helical axes), and

$$\bar{n}^2 = \frac{2n_\perp^2 + n_\parallel^2}{3}. \tag{3}$$

For any non-zero water content (i.e., at any *rh* above 0%), the films are heterogeneous and consist of Na-hyaluronate and the water of hydration. The water molecules contribute to the refractive indices of the films. The contribution of the water must be subtracted in order to determine the polarizabilities of Na-hyaluronate. Finally, the electronic polarizabilities per disaccharide are given by:

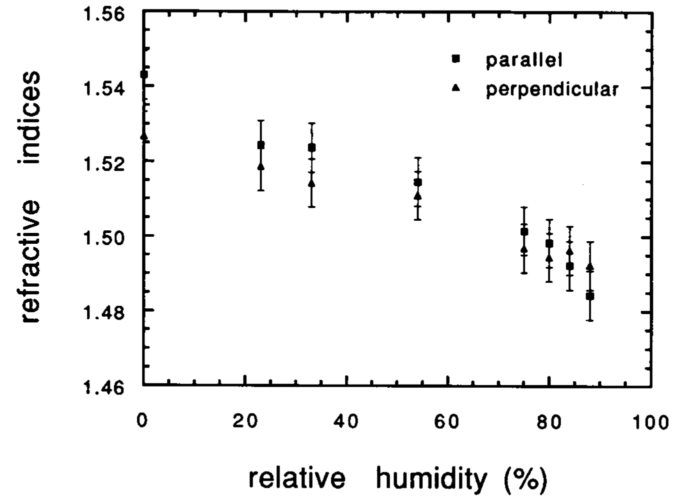

FIGURE 1 The refractive indices of wet-spun films of Na-hyaluronate as a function of relative humidity. The squares (■) and the triangles (▲) are the data for light polarized parallel and perpendicular to the helical axis, respectively.

$$\alpha_{\mathrm{dis},i} = \frac{3(n_i^2 - 1)kV_{\mathrm{dis}}}{4\pi(\bar{n}^2 + 2)N_{\mathrm{dis}}} - N_w\alpha_w. \tag{4}$$

Here $V_{\mathrm{dis}}/N_{\mathrm{dis}}$ is the volume per disaccharide, k is the correction factor for the voids, N_w is the number of water molecules per disaccharide, and α_w is the electronic polarizability of water.

RESULTS AND DISCUSSION

Fig. 1 shows the refractive indices for light polarized parallel and perpendicular to the helical axes as a function of *rh*. Note that there is significant birefringence only at 0% *rh*. For any higher *rh*, the two refractive indices are almost the same. This contrasts with the case of DNA in which the two refractive indices are found to be substantially different (on the order of 0.1 at 0% *rh*) (15). The anisotropy in DNA is attributed to π electrons of the base pairs that can be polarized perpendicular to the helical axis much more than parallel to it. The structure of Na-hyaluronate molecules (7) is significantly more isotropic, as is evident in the refractive indices.

The refractive indices of Na-hyaluronate films decrease in a monotonic fashion (to within the experimental uncertainty) as the *rh* is increased. This decrease is easily understood because more water (with a refractive index of 1.333) is added to the film as the *rh* is increased, lowering the refractive indices of the composite Na-hyaluronate/water film. The addition of water also eliminates the small birefringence seen at 0% *rh*. Note that no anomaly in the refractive indices is noted in the vicinity of the transition (84 to 88% *rh*).

Fig. 2 *a* shows the results of the measurements of the relative changes in the dimensions of small pieces of Na-hyaluronate film parallel ($L_\parallel$) and perpendicular ($L_\perp$) to the helical axes as a function of *rh*. The films are seen to expand by a significant amount perpendicular to the he-

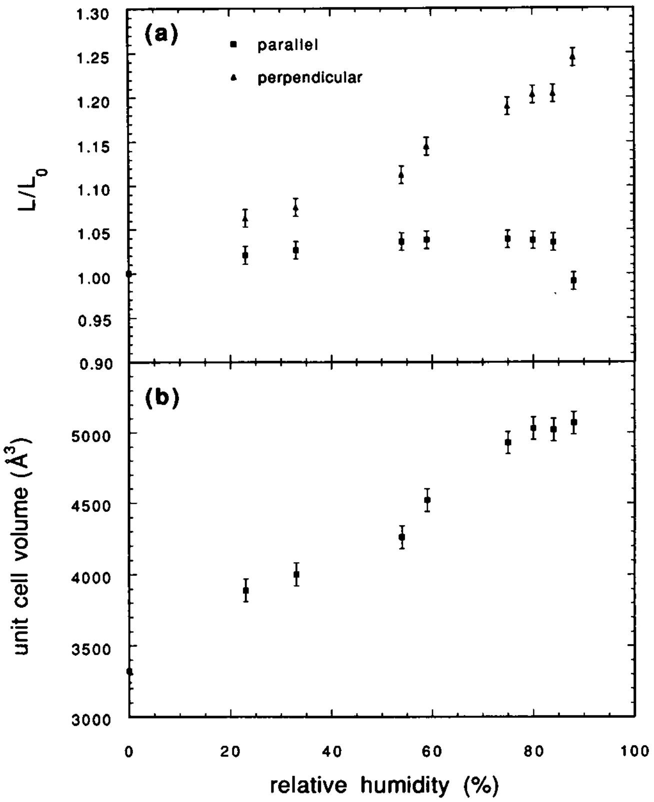

FIGURE 2 (*a*) The relative swelling of a wet-spun film of Na-hyaluronate as a function of relative humidity. The squares (■) and the triangles (▲) are the data for swelling parallel and perpendicular to the helical axis, respectively. (*b*) The volume of the unit cell of Na-hyaluronate as a function of relative humidity, obtained by using $V = L_{\parallel}L_{\perp}^2$. The 0% *rh* volume is then obtained (7).

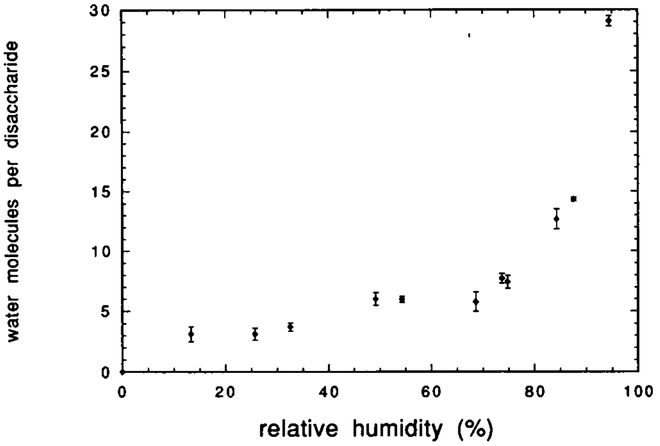

FIGURE 3 The water content of Na-hyaluronate as a function of relative humidity.

lical axes. The expansion parallel to the helical axes is much smaller. At the higher values of *rh*, the films shrink in the parallel direction. At 88% *rh*, $L_{\parallel}$ is actually smaller than at 0% *rh*. Similar effects along the helical axis have been reported from x-ray studies (7, 11). Our measurements can be combined to obtain the relative changes in the volume ($V = L_{\parallel}L_{\perp}^2$). Absolute volumes have been determined by using the reported volume per disaccharide at 0% *rh* (7). The volume per disaccharide as a function of *rh* is shown in Fig. 2 *b* and is seen to expand between 0 and 88% *rh*.

Above 88% *rh*, the films were found to change their physical size in a dramatic fashion: $L_{\perp}$ is about twice its 0% *rh* value while $L_{\parallel}$ is less than half of its 0% *rh* value. The reliability of this simple method to determine volumes is uncertain when such large volume changes occur and we do not report any quantitative results above 88% *rh*. Anomalous swelling in the direction perpendicular to the helical axes has previously been reported in wet-spun films of Na-DNA (14, 22), though the mechanism for such swelling remains controversial. For Na-DNA films, a slight shrinkage was reported parallel to the helical axis. Our observation of a significant shrinkage of Na-hyaluronate films in the direction parallel to the helical axis suggests that a change in molecular conformation is taking place, perhaps a helix-to-coil transition.

Few studies (7, 11) report any information about the size of the crystalline unit cell. As noted earlier, the size of the crystalline unit cell and the conformation of the molecule depend on a variety of parameters during sample preparation. Both Guss et al. (7) and Sheehan and Atkins (11) used fibers, not wet-spun films. However, our expansion data is within the range of reported values.

Fig. 3 shows the water content for these films as a function of *rh*. The data are in substantial agreement with the results of Kleeberg and Luck (23). The water content curve does not show any anomaly at the phase transition, though the water content increases significantly above the transition.

Fig. 4 shows the electronic polarizabilities for the two polarizations of the light as a function of *rh*. At each *rh*,

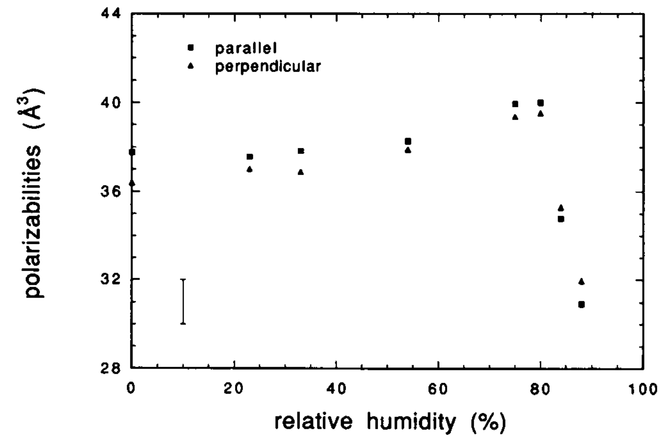

FIGURE 4 The optical polarizabilities of a Na-hyaluronate film as a function of relative humidity. The squares (■) and triangles (▲) are the data parallel and perpendicular to the helical axis, respectively. A typical error bar is shown in the lower left-hand corner.

Flowers et al. Wet-spun Films of Na-Hyaluronate 325

the polarizabilities are nearly the same, reflecting the isotropy of the refractive indices. The most notable feature of this figure is the dramatic drop of the electronic polarizabilities between 80 and 88% *rh*. This is clear evidence of the phase transition first observed by Brillouin spectroscopy (1). The changes in the polarizabilities are ~20%. This is relatively large compared with a 4% change observed at a phase transition in SiO_2 and 0.8% change observed at a phase transition in CsF, but somewhat smaller than the 35% change observed at the A-to-B transition in Na-DNA (15). The 20% change in wet-spun films of Na-hyaluronate indicates that the phase transition greatly influences the electronic degrees of freedom of the molecule. The most likely explanation for these electronic changes is that the conformation of the molecule is changing.

The optical polarizabilities cannot be determined for higher values of *rh* because the measurement of the volume per disaccharide is unreliable above 88% *rh*. Also, at such high water contents, the measurements of the refractive indices are much less reliable. These experiments take between 15 and 30 min to complete. It is unlikely that the loosely bound water added at the highest *rh* values would remain at all its hydration sites over that time scale.

Future studies will include similar measurements on wet-spun films of Li-hyaluronate to determine the role (if any) that the counterion plays in the phase transition.

CONCLUSIONS

The refractive indices, water content, and relative swelling of wet-spun films of Na-hyaluronate have been measured as a function of *rh*. The refractive indices and water content do not show an anomaly at the phase transition. The optical polarizabilities show a dramatic drop (~20%) between 80 and 88% *rh*, indicating that the electronic degrees of freedom are affected in a significant manner at the phase transition. This suggests that the molecular conformation changes during this phase transition. The large shrinkage of the film in the direction parallel to the helical axes also supports the suggestion that the molecular conformation changes at the phase transition.

This work has been supported in part by the Office of Naval Research (N00014-91-J-1457), the Ohio Board of Regents, and the Swedish Medical Research Council.

Received for publication 16 December 1991 and in final form 9 April 1992.

REFERENCES

1. Lee, S. A., W. F. Oliver, A. Rupprecht, Z. Song, and S. M. Lindsay. 1992. Observation of a phase transition in wet-spun films of Na-hyaluronate. *Biopolymers.* 32:303–306.
2. Roth, S., and I. Freund. 1981. Optical second-harmonic scattering in rat-tail tendon. *Biopolymers.* 20:1271–1290.
3. Freund, I., and M. Deutsch. 1986. Second-harmonic microscopy of biological tissue. *Op. Lett.* 11:94–96.
4. Freund, I., M. Deutsch, and A. Sprecher. 1986. Connective tissue polarity. Optical second-harmonic microscopy, crossed-beam summation, and small-angle scattering in rat-tail tendon. *Biophys. J.* 50:693–712.
5. Parry, D. A. D., and A. S. Craig. 1977. Quantitative electron microscope observations of the collagen fibrils in rat-tail tendon. *Biopolymers.* 16:1015–1031.
6. Atkins, E. D. T., and J. K. Sheehan. 1973. Hyaluronates: relation between molecular conformations. *Science (Wash. DC).* 179:562–564.
7. Guss, J. M., D. W. L. Hukins, P. J. C. Smith, W. T. Winter, S. Arnott, R. Moorhouse, and D. A. Rees. 1975. Hyaluronic acid: molecular conformations and interactions in two sodium salts. *J. Mol. Biol.* 95:359–384.
8. Sheehan, J. K., E. D. T. Atkins, and I. A. Nieduszynski. 1975. X-ray diffraction studies on the connective tissues polysaccharides. *J. Mol. Biol.* 91:153–163.
9. Arnott, S., A. K. Mitra, and S. Raghunathan. 1983. Hyaluronic acid double helix. *J. Mol. Biol.* 169:861–872.
10. Mitra, A. K., S. Raghunathan, J. K. Sheehan, and S. Arnott. 1983. Hyaluronic acid: molecular conformations and interactions in the orthorhombic and tetragonal forms containing sinuous chains. *J. Mol. Biol.* 169:829–859.
11. Sheehan, J. K., and E. D. T. Atkins. 1983. X-ray fibre diffraction study of conformational changes in hyaluronate induced in the presence of sodium, potassium and calcium cations. *Int. J. Biol. Macromol.* 5:215–221.
12. Rupprecht, A. 1970. A wet spinning apparatus and auxiliary equipment suitable for preparing samples of oriented DNA. *Biotechnol. Bioeng.* 12:93–121.
13. Rupprecht, A., and B. Forslind. 1970. Variation of electrolyte content in wet-spun lithium- and sodium-DNA. *Biochim. Biophys. Acta.* 204:304–316.
14. Lewen, G. D., S. M. Lindsay, N. J. Tao, T. Weidlich, R. J. Graham, and A. Rupprecht. 1986. A mechanism for the large anisotropic swelling of DNA films. *Biopolymers.* 25:765–770.
15. Weidlich, T., S. M. Lindsay, and A. Rupprecht. 1987. The optical properties of Li- and Na-DNA films. *Biopolymers.* 26:439–453.
16. Lee, S. A., S. M. Lindsay, J. W. Powell, T., Weidlich, N. J. Tao, G. D. Lewen, and A. Rupprecht. 1987. A Brillouin scattering study of the hydration of Li- and Na-DNA films. *Biopolymers.* 26:1637–1665.
17. Washburn, E. W., editor. 1926. International Critical Tables of Numerical Data, Physics, Chemistry and Technology, Vol. 1. McGraw-Hill, New York. 67–68.
18. Lavalle, N., S. A. Lee, and A. Rupprecht. 1990. Counterion effects on the physical properties and the A to B transition of calf-thymus DNA Films. *Biopolymers.* 30:877–887.
19. Boettcher, C. J. F. 1973. Theory of Electric Polarization. Elsevier Scientific Co., New York.
20. Hermans, P. H. 1946. Contributions to the Physics of Cellulose Fibers. Elsevier Publishing Co., New York.
21. Vuks, M. F. 1965. Determination of the optical anisotropy of aromatic molecules from the double refraction of crystals. *Opt. Spectrosc.* 20:361–364.
22. Edwards, G. S., L. Genzel, W. L. Peticolas, and A. Rupprecht. 1986. Measurements of a large anisotropy in the swelling of oriented DNA films in aqueous solution. *Biopolymers.* 25:223–227.
23. Kleeberg, H., and W. A. P. Luck. 1979. Infrared-spectroscopic investigations on the hydration of glycosaminoglycans. Glycoconjugates, Proceedings of the Fifth International Symposium. R. Schauer, P. Boer, E. Buddecke, M. F. Kramer, J. F. G. Vliegenthart, and H. Wiegandt, editors. Thieme, Stuttgart. 98–99.