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SHORT COMMUNICATIONS

Reversing-Pulse Electric Birefringence as Applied to Thermal Denaturation of Acid-Soluble Calf Skin Collagen

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The reversing-pulse electric birefringence (RPEB) technique is a powerful means to investigate the electrooptical properties, order-disorder transition, conformations, and dynamics of polymers, colloids, liquid crystals, and bioaggregates. The initial study by O'Konski *et al.* on tobacco mosaic virus[1,2] and the theoretical formulation by Tinoco and Yamaoka[3] have demonstrated the potential use of the RPEB method in the diverse fields of non-ionic and ionized polymers. Only recently, however, this method became widely appreciated.[4-8] We wish to arouse the interest of those who are concerned with the denaturation and conformational transitions of various polymer systems, by showing that the RPEB method can be applied to the thermal denaturation process of collagen. The presence of deep extremum in RPEB signals in the transition temperature region (29—41°C) led to the conclusion that the strand separation of triple-helix collagen to random-coiled gelatin probably occurs by an all-or-none process.

An acid-soluble calf skin collagen sample (Sigma, type III) was purified by repeated dissolution and precipitation with the 0.5 M CH_3COOH–5% NaCl solvent.[9] The melting temperature was 37.4°C at pH 4.0, as determined with the molar ellipticity at 221 nm.[9] The intrinsic viscosity was 15.0 (100 $cm^3 g^{-1}$) in 0.5 M acetic acid. The RPEB signal was measured at 535 nm for a collagen solution (0.132 mg cm^{-3}) in 0.05% acetic acid at a pH of 3.44 on an apparatus with an instrumental time constant of 0.5 μs.[7] A sufficient waiting time (15—30 min) was given at each temperature for the solution to reach the thermal equilibrium, before electric pulses were applied. The birefringence was expressed with the optical phase retardation, δ/d, where d is the path length of the thermostated Kerr cell.

Figure 1 shows some RPEB signals of a collagen solution at different temperatures under a constant field strength of 1.75 kV cm^{-1}. (The Kerr law holds for native collagen up to 2 kV cm^{-1} at 29.2°C.) The signal at 23.8°C exhibits a deep minimum, which almost reaches the baseline, upon reversal of electric pulse. The native collagen molecule should possess a large apparent permanent electric dipole moment μ, since the depth depends on the parameter $\mu^2/\Delta\alpha kT$,[3,5] where $\Delta\alpha$ is the polarizability anisotropy and k is the Boltzmann constant. The RPEB signal in-

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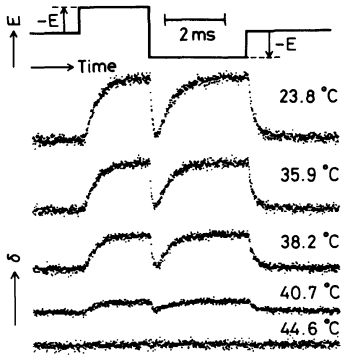

Figure 1. Digitized RPEB signals of collagen in an acidic solution at various temperatures, as specified. Field strength E, 1.75 kV cm^{-1}. Signals are displayed with optical retardation δ in degrees (the sign is positive). Each signal is an average of 8—20 accumulations. Note that the reversing pulse is set longer than the first pulse to assure for the reverse signal to return to the steady state.

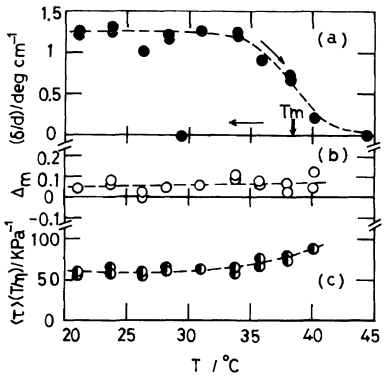

Figure 2. The steady-state phase retardation per path length δ/d (a), the minimum Δ_m of the normalized signal in the reverse process (b), and the corrected relaxation time $\langle\tau\rangle(T/\eta)$ from the decay process (c). In (a), T_m indicates the temperature where δ/d is reduced by half and the thin arrows show the heating and cooling direction of a collagen solution.

tensity begins to decrease at *ca.* 35°C, indicating the incipience of the thermal transition of collagen. The signal at 40.7°C becomes *ca.* one-ninth of the original one, but the minimum is clearly discernible. The signal disappears completely at 44—45°C, but no RPEB signal reappears, when the solution is cooled back to 29°C. By inspecting these signals, we can conclude that the thermal process is irreversible, the denatured single-stranded gelatin molecule gives no RPEB signal at low fields; hence, the native collagen molecule is responsible for the observed signals. It is these signal patterns that make the RPEB method so powerful and convenient in the study of rod-like polymers.[3,8,10]

The quantitative behavior of RPEB signals is clarified by plotting the steady-state value of δ/d in the buildup (Figure 2a) and the normalized minimum Δ_m, defined as $\delta(t_m)/\delta(0)$ (*cf.* Figure 2 of ref 7), in the reverse (Figure 2b) against temperature. Although δ/d value is decreased by one-half at 38.5°C (the melting temperature with a thick arrow), the Δ_m value is almost constant at 0.04—0.07 in the temperature range 21—41°C. This fact suggests that the electric property of native collagen molecule remains unaltered during the denaturation process. From the decay signal, the birefringence-average relaxation time $\langle\tau\rangle$ can be obtained.[5,10] Values of $\langle\tau\rangle(T/\eta)$, corrected for temperature T and solvent viscosity η, remain constant to 35—36°C (Figure 2c). Thus, the hydrodynamic property of the collagen system remains unchanged to the midpoint of the thermal transition region. These results reveal that the collagen solution at a transition temperature contains two species, *i.e.*, the native collagen responsible for the RPEB signal and the denatured gelatin incapable of showing it. It is only the number (more correctly, the mass) of field-orientable collagen molecules that is decreased with increasing temperature; at the same time, the number of unorientable gelatin molecules is increased in a proper proportion. It may then be concluded that the thermal denaturation of the triple-helix collagen to single-stranded gelatin at pH 3.44 is an all-or-none process without any distinct intermediate species.

The large dipole moment of 16000—15000 D (D for debye, 1 D = 3.336×10^{-30} C m) for a native collagen molecule or *ca.* 5000 D per

strand, evaluated from the field-strength dependence of δ/d (to be published), is in good agreement with previous reports.[11-13] This value is too large to be attributed to the sum of the peptide bond moments.[14,15] Hence, the saturating ionic or protonic dipole moment should be induced by the applied pulse field,[3,6] and this moment certainly contributed to the field orientation of ionized collagen molecules at acidic pH.[3,14] With the diameter of 15 Å for a collagen molecule, the overall length of the triple-stranded helix was calculated to be 2800 Å at 29°C with the Broersma equation[16] from the relaxation time extrapolated to zero field strength. This is in surprisingly good agreement with the results obtained by the static[17] and quasi-elastic[18] light scattering methods.

In summary, the RPEB technique could be applied to the thermal denaturation of collagen successfully. The signal patterns reveal the electric property of collagen, which is difficult to obtain by other physical methods. It was shown that only native collagen molecules are responsible for the RPEB signal with a deep extremum. Thus, the transition probably proceeds by an all-or-none process. Detailed electric birefringence work on collagen will be reported shortly.

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