Supplementary Information for

Direct Observation of Intrachain Hydrogen Bonds in Aqueous Hyaluronan

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Data Analysis Method

Fit of the linear infrared absorption spectra at different DCl concentrations. We fit the linear infrared spectra using a global fitting procedure as a function of frequency and concentration of added DCl ($[D^+_{added}]$), which is based on the minimization of the square error

$$\sum_{i,j} \left(S\left(\omega_j, [D^+_{added}]_i\right) - S^{exp}\left(\omega_j, [D^+_{added}]_i\right) \right)^2$$

(1)

where S is the fitted spectrum and S^{exp} the experimental spectrum. Assuming that the experimental spectra are a linear combination of the spectra at neutral (S_n) and acidic (S_a) pH, we expect that

$$\forall i, for\ \omega_{\{min\}} \leq \omega_J \leq \omega_{\{max\}}$$
$$S\left(\omega_j, [D^+_{added}]_i\right) = c_n S_n\left(\omega_j, [D^+_{added}]_i\right)^{neutral} + c_a S_a\left(\omega_j, [D^+_{added}]_i\right)^{acid}$$

(2)

where c_n and c_a are the coefficient for the neutral and acid spectra, respectively.

Calculation of the pK$_a$ value in heavy water. We determine the pD and the fraction of protonated/depronated carboxylic acid groups of hyaluronan as a function of added DCl using the acid-base equilibrium and mass balance equations:

    i. $c_a = [DA] + [A^-]$
    ii. $[D^+_{added}] = [DA] + [D^+]$

    iii. $pK^D{}_a = pD^+ - \log_{10} \frac{[A^-]}{[DA]}$
    iv. $pK^D{}_w = pD^+ + pOD^-$

(3)

The first equation represents the conservation of the analytical concentration c_a of hyaluronan, that at every pD has to be preserved as the sum of neutral hyaluronic acid $[DA]$ and negatively charged hyaluronan $[A^-]$. The second equation represents the conservation of the total concentration of D^+. The third equation is the acid-base equilibrium equation for hyaluronic acid. The fourth equation is the heavy water dissociation equilibrium, where $pK^D{}_w$ indicates the heavy water dissociation constant. Note that the acid dissociation constant in heavy water solutions, pK$_a$^{D}, is different from that in water solution. In order to extract the pK$_a$^{D}, we fit the fractions of $[DA]^{ext}$ and $[A^-]^{ext}$ (extracted from the global fit of the linear spectra), by using a global fitting procedure as a function of added DCl, $[D^+_{added}]$. This procedure is based on the minimization of the least-square error of the following expression:

$$\sum_i ([DA]^{ext}([D^+_{added}]_i) - [DA]([D^+_{added}]_i, pK_a^D))^2$$

$$+ \sum_i ([A^-]^{ext}([D^+_{added}]_i) - [A^-]([D^+_{added}]_i, pK_a^D))^2$$

(4)

where pK_a^D is the only free parameter. $[DA], [A^-]$ are obtained by solving the system of equations in Eq.(2) with known c_a, pK_w^D as a function of $[D^+_{added}]_i$ and pK_a^D. By assuming $pK_w^D = 14.95$, we obtain from the fit a value of $pK_a^D = 3.3 \pm 0.2$ that is in good agreement with the expected value from literature assuming a pK_a in water of 2.9.

pD to pH conversion. By using a glass electrode we read the apparent pH* of a heavy water solution. Since we calibrate the pH-meter with water buffers, due to the different hydrogen activity on the surface of the glass electrode between water and heavy water, we need to use the following correction to obtain the pD [1]:

$$pD = pH^* + 0.44$$

(5)

The pD values can thus be converted to the pH values of a solution of water of similar acidity using[1]:

$$pH = pD * 0.929$$

(6)

Fit of the 2DIR slices. We fit the 2DIR using a global fitting procedure as a function of probe frequency and time which is based on the minimization of the square error

$$\sum_{i,j} \left(S(\omega_j, t_i) - S^{exp}(\omega_j, t_i) \right)^2$$

(7)

where S is the fitted spectrum and S^{exp} the experimental spectrum. Assuming that the experimental spectra are a linear combination of the residual diagonal signal, indicated by S_{COO-}, and the cross-peak signal, indicated by S_{CP}, we expect that

$$\forall i, for\ \omega_{\{min\}} \leq \omega_J \leq \omega_{\{max\}}$$
$$S(\omega_j, t_i) = c_{COO-}(t_i)S_{COO-}(\omega_j) + c_{CP}(t_i)S_{CP}(\omega_j) + c_{heating}(t_i)S_{heating}(\omega_j)$$

(8)

where c_{COO-}, c_{CP} and $c_{heating}$ are the time-dependent amplitudes of the diagonal, cross-peak and heating, respectively. The heating signature $S_{heating}(\omega_j)$ corresponds to the 2DIR signal at late time delay (Tw= 10 ps).

The spectral signatures S_{COO^-} and S_{CP} are defined as the sum of two Lorentzian-shaped peaks that correspond to the bleach and the esa of the diagonal peak ($l_{COO_-}^{bleach}$ and $l_{COO_-}^{esa}$) and of the cross-peak (l_{CP}^{bleach} and l_{CP}^{esa}),

$$S_{COO-}(t_i, \omega) = c_{COO-}(t_i)[-l_{COO_-}^{bleach}(\omega) + factor_{COO-} * l_{COO}^{esa}(\omega)]$$

(9)

And

$$S_{CP-}(t_i, \omega) = c_{CP}(t_i)[-l_{CP}^{bleach}(\omega) + factor_{CP} * l_{CP}^{esa}(\omega)]$$

(10)

where $factor_{CP}$ and $factor_{COO^-}$ represent the relative amplitude of the excited state absorption with respect to the bleaching signal.

We thus rewrite equation (7) as follows:

$$\sum_{i,j} \left(c_{CP}(t_i)[-l_{CP}^{bleach}(\omega_j) + factor_{CP} * l_{CP}^{esa}(\omega_j)] + c_{COO-}(t_i)[-l_{COO_-}^{bleach}(\omega_j) \right.$$
$$\left. + factor_{COO-} * l_{COO}^{esa}(t_i)(\omega_j)] + c_{heating}(t_i)S_{heating}(\omega_j) - S^{exp}(\omega_j, t_i) \right)^2$$

Where c_{COO-} and $c_{CP}(t_i)$ are delay-time-dependent amplitudes, and the widths, centre frequencies, and relative amplitude (factor) are global parameters.
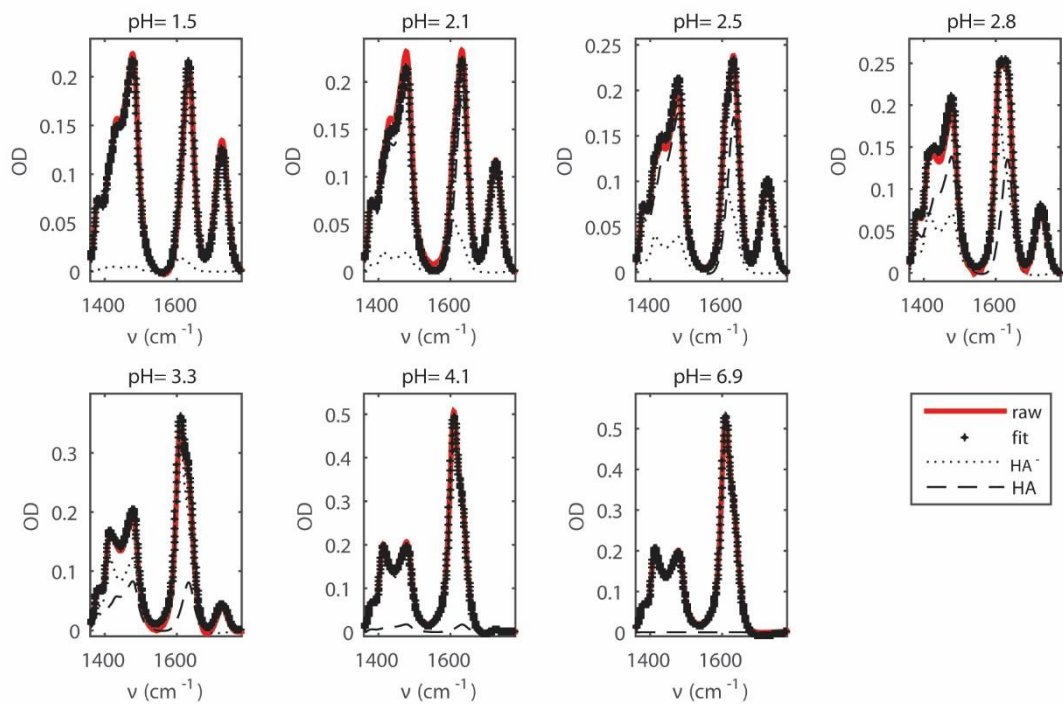
The result of the fit is shown in Fig.S3.

Fig.S1: Measured raw linear spectra (red) with fits (symbols) of hyaluronan at 20 mg/ml at different pH values (see legends) in heavy water solutions, and the resulting inferred spectra for HA and HA⁻.
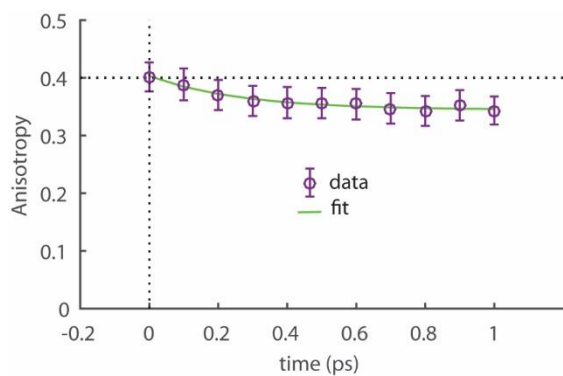
Fig.S2. Anistropy decay of the anti-symmetric stretching vibration of the carboxylate anion group. The data are fitted to an exponential decay with an offset. We obtain a decay time constant of 0.250 ± 0.150 ps, and an offset of 0.37 ± 0.04.
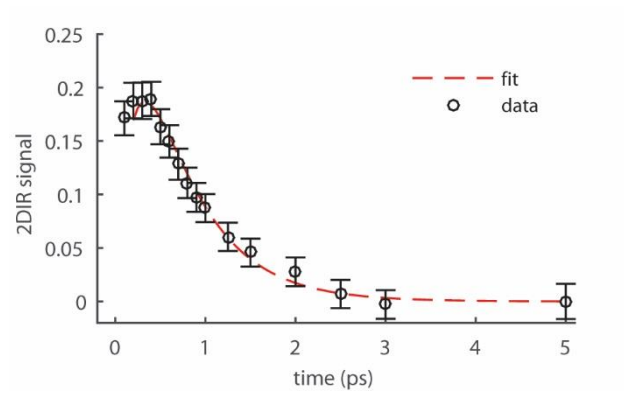
Fig.S3. 2DIR signal obtained directly from the 2DIR spectrum reported in Fig.2b at the cross-peak frequencies (excitation at 1614 cm^{-1}, detection at 1637 cm^{-1}). The data are fitted to a double exponential function with a rise time constant of 0.2 and a decay time constant of 0.6 ps.

Fig.S4: 2DIR signal as a function of probe frequency at T_w =0.5 ps. The 2DIR signal is obtained by averaging over excitation frequencies between 1600 and 1620 cm^{-1} of the 2DIR spectrum, where we subtracted the parallel 2DIR spectrum from three times the perpendicular 2DIR spectrum.
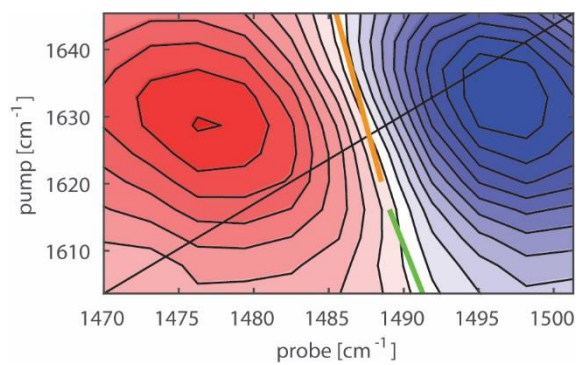
Fig. S5: 2DIR spectrum in parallel polarization of N-acetyl-glucosamine in D_2O at a concentration of 8% wt and at a pH 7 at a waiting time of 0.3 ps.
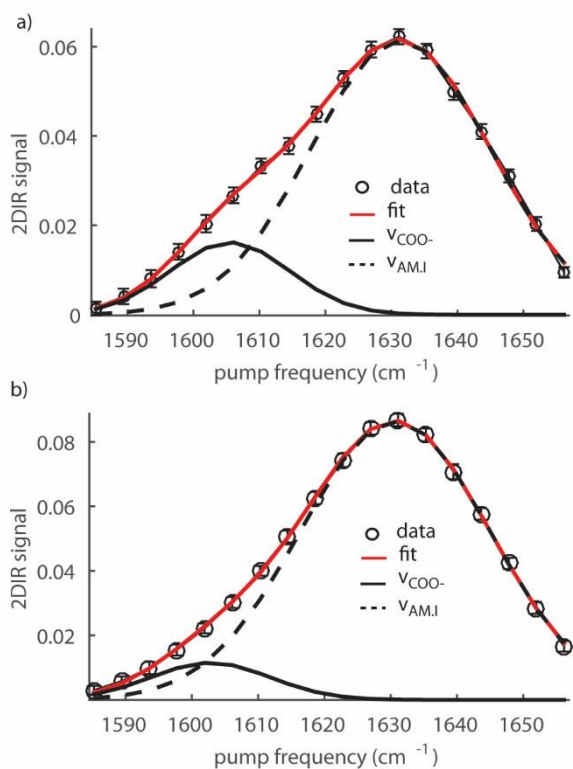
Fig.S6: a) Transient spectra obtained by averaging the 2D-IR spectra over the probe-frequency range between 1488 and 1492 cm^{-1} for parallel *a)* and perpendicular *b)* polarization configuration, for a solution of 15 mg/ml hyaluronan in heavy water at a pH of 6.8. The waiting time is 0.3 ps. By extracting the ratio between the parallel and perpendicular spectra, we obtain an anisotropy of $R_{am,I-am,II}$ = -0.1±0.04 and $R_{COO-am,II}$ = 0.1±0.1 for the $v_{AM.I}$ and v_{COO-} vibrations, respectively. From these anisotropy values, we extract the angle between the transition dipole moments of the amide I and amide II vibration, and the angle between the transition dipole moments of the anti-symmetric carboxylate stretching vibration and the amide I vibration.

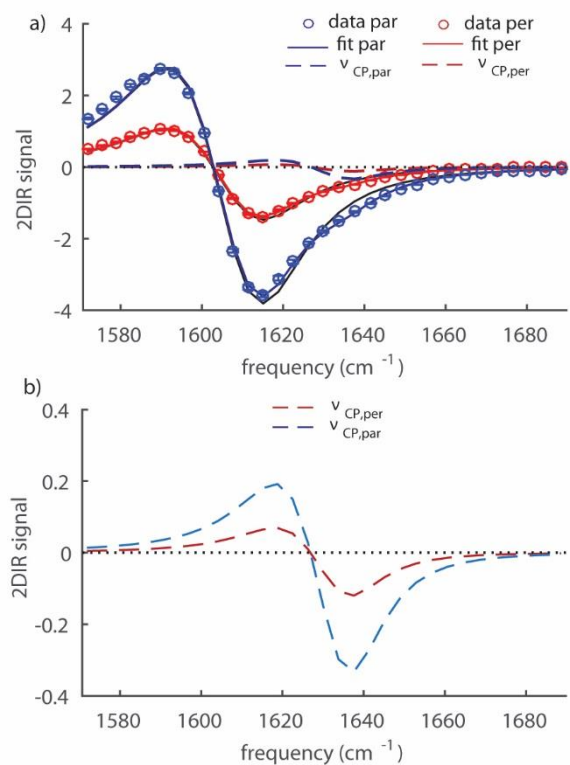Using $\theta = arcos(\sqrt{\frac{5R+1}{3}})$ we thus obtain angles of 67±5° and 45°±10°, respectively.

Fig.S7: a) Measured transient absorption spectra for a pump frequency of 1607 cm^{-1} for parallel (blue circles) and perpendicular (red circles) polarization together with fits (blue and red solid lines) , for a solution of 20 mg/ml hyaluronan in heavy water at pH=6.8. The waiting time is 0.3 ps. The contributions of the cross-peak signal with the amide I vibration to the parallel and the perpendicular transient absorption spectra are represented with the blue and red dashed lines. b) Cross-peak signals obtained from the fits to the transient absorption spectra shown in a). From the ratio of the parallel and perpendicular cross-peak signals, we obtain an anisotropy of R=0.32±0.05. From this anisotropy value, we can extract the angle between the transition dipole moments of the amide I and anti-symmetric stretching using $\theta = arcos(\sqrt{\frac{5R+1}{3}})$. We thus obtain an angle of 15°±10°.
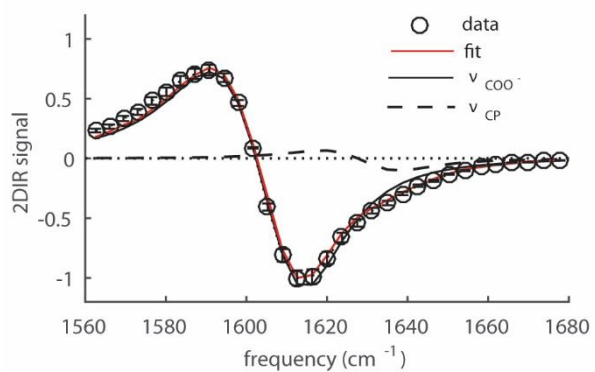
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Fig.S8: Measured transient absorption spectrum obtained for a pump frequency of 1607 cm^{-1} (black circles) together with a fit (red line), for a solution of 20 mg/ml hyaluronan in heavy water at pH=6.8. The waiting time is 0.3 ps. The solid line represents the diagonal response of the anti-symmetric vibration of COO$^-$, and the dashed line represents the spectral response of the cross-peak signal with the amide I vibration, i.e. the signal of this vibration following excitation of the anti-symmetric stretch vibration of COO$^-$.
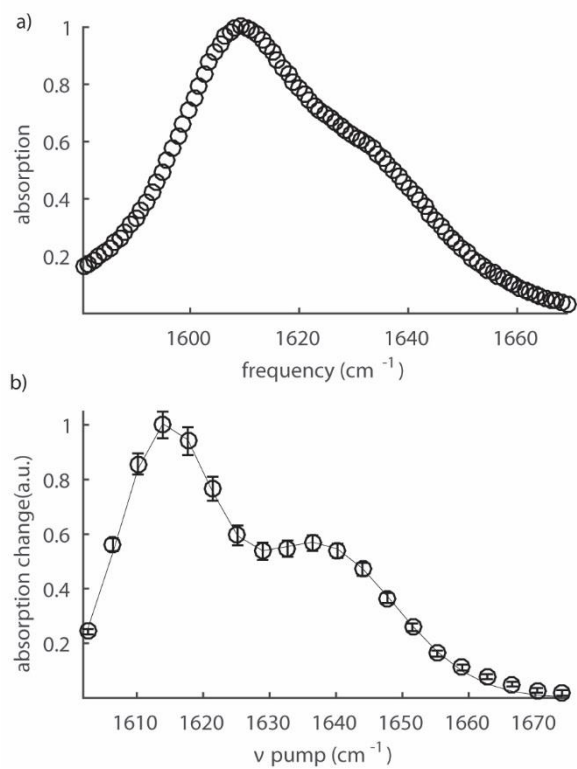
Fig.S9: a) Normalized measured linear IR spectrum of a solution of hyaluronan at 20 mg/ml at pH=6.8. b) Normalized isotropic transient absorption spectrum of a solution of 20 mg/ml hyaluronan in heavy water at pH=6.8. The waiting time is 0.3 ps. The similarity in the shape of the spectra indicates that the amide I vibration and the anti-symmetric stretch vibration of COO$^-$ have a similar absorption cross section.
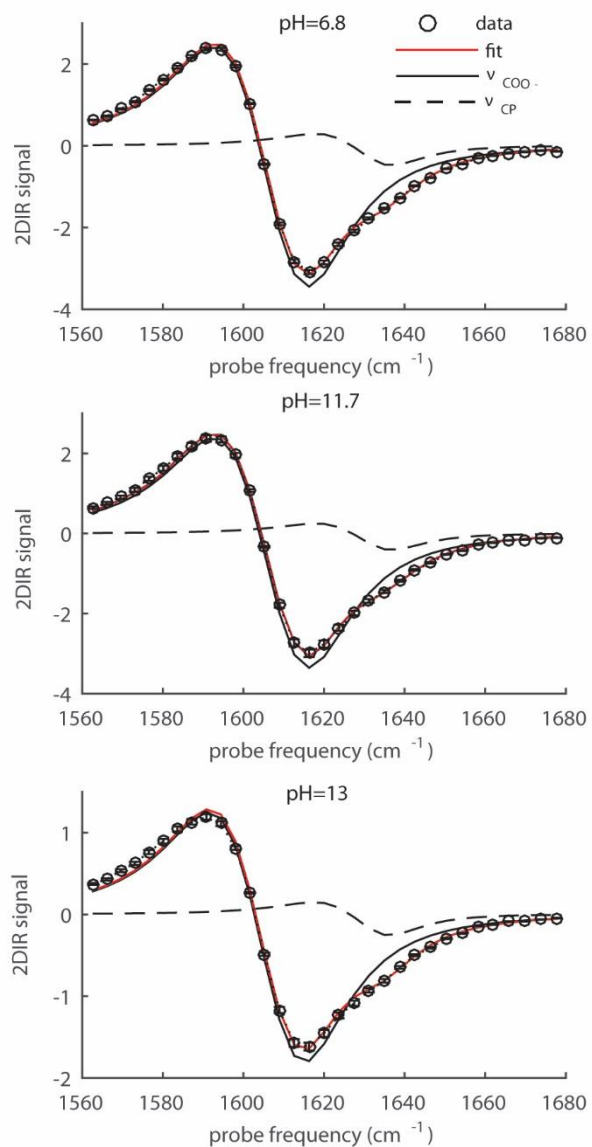
Fig. S10: Measured isotropic transient absorption spectra obtained by averaging the 2DIR signals over a pump-frequency range between 1607 and 1613 cm^{-1}, for a 20 mg/ml solution of hyaluronan in heavy water at different pH values, which were obtained by adding different amounts of NaOD to the solution. The waiting time is 0.3 ps. The data are represented by the circles, and the fits to the spectra by the red solid lines. The black solid line represents the diagonal signal of the anti-symmetric vibration of COO$^-$, and the dashed black line represents the cross-peak signal with the amide vibration, i.e. the response of the amide vibration following excitation of the anti-symmetric vibration of the COO$^-$ group.
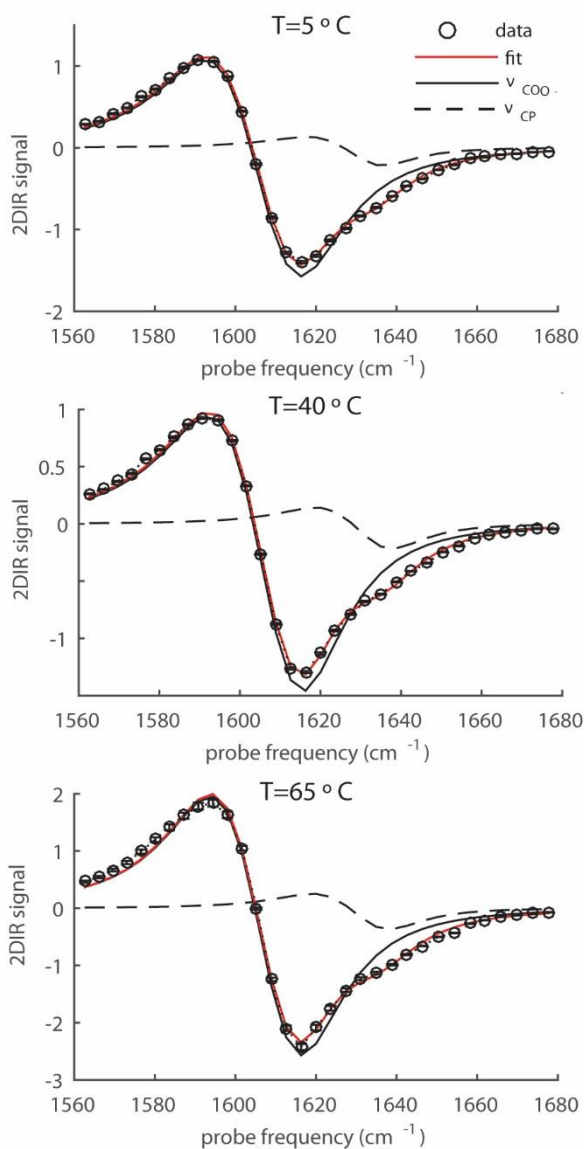
Fig. S11: Measured isotropic transient absorption spectra obtained by averaging the 2DIR signals over a pump-frequency range between 1607 and 1613 cm^{-1}, for a 20 mg/ml solution of hyaluronan in heavy water at different temperatures. The waiting time is 0.3 ps. The data are represented by the circles, and the fits to the spectra by the red solid lines. The black solid line represents the diagonal signal of the anti-symmetric vibration of COO^-, and the dashed black line represents the cross-peak signal with the amide vibration, i.e. the response of the amide vibration following excitation of the anti-symmetric vibration of the COO^- group.

Supplementary Bibliography

(1)   Krężel, A.; Bal, W. A Formula for Correlating PKa Values Determined in D2O and H2O. *J. Inorg. Biochem.* **2004**, *98* (1), 161−166. https://doi.org/10.1016/J.JINORGBIO.2003.10.001.