Supplementary Information

Material & Methods

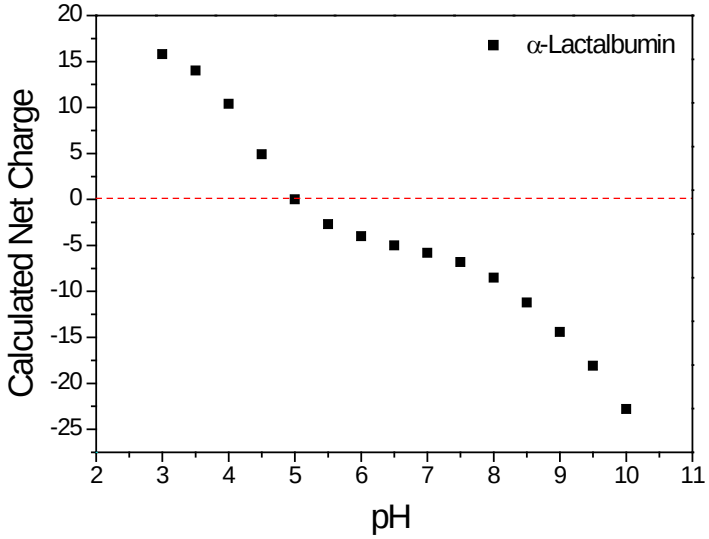
Heterodyne-detected Vibrational Sum Frequency Generation:

The intensity VSFG setup has been described in detail before (S. Strazdaite, J. Versluis, E. H. Backus and H. J. Bakker, *J. Chem. Phys.*, 2014, **140**, 054711.). In short, the laser source of this setup is a regenerative Ti:Sapphire amplifier (Coherent, 800 nm, 35 fs at 1 kHz repetition rate, 3.5 mJ pulse energy). Part of the output is used to pump a home-built OPA and difference-frequency generation stage to produce broadband mid-IR pulses that can be tuned from 2 – 10 μm (10 – 20 μJ energy). A second part of the 800 nm pulse is spectrally narrowed to a bandwidth of ~15 cm^{-1} using an etalon. The resulting narrow-band 800 nm pulse (VIS) and the broadband IR pulse are overlapped in time and space on the sample surface to generate SFG light, which is detected using a monochromator and an Electron-Multiplied Charge Coupled Device (EMCCD, Andor Technologies).

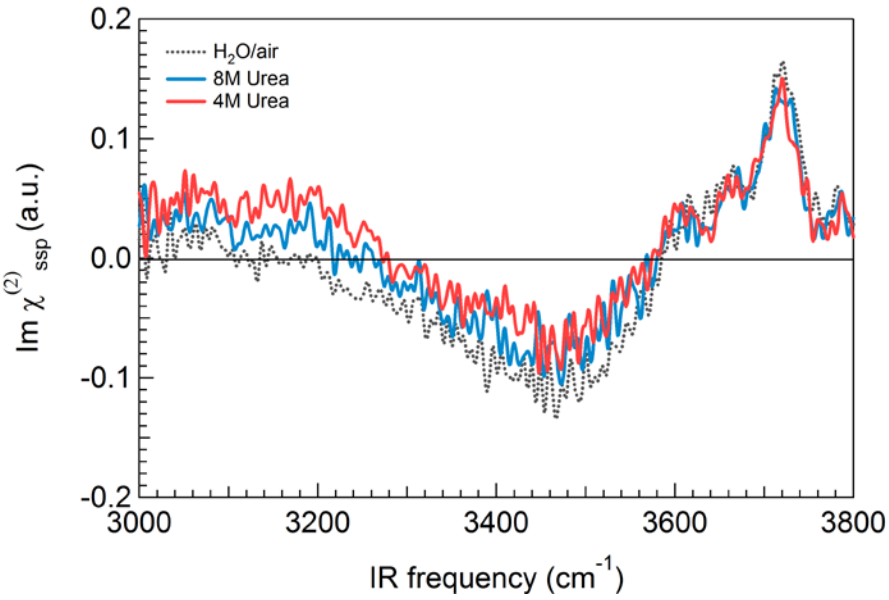
The imaginary and real parts of $\chi^{(2)}$are determined with heterodyne-detected VSFG. In this technique the SFG electric field generated from the sample is combined with a local oscillator (LO) electric field at the same frequency. The LO sum-frequency (LO-SFG) light is generated by first focusing the IR and VIS beams on a metal surface to generate a strong non-resonant $\chi^{(2)}$ SFG signal. This signal is delayed with respect to the IR and VIS beams by passing it through a silica plate (~ 1 mm). The LO-SFG and the IR and VIS beams are refocused using a spherical mirror on the sample surface, where the IR and VIS beam generate the sample SFG. The LO-SFG and sample SFG beams are then collimated using a lens, sent into a monochromator and detected with a CCD. The detected interference pattern contains cross terms from which the real and imaginary $\chi^{(2)}$ can be extracted using Fourier transformation. To obtain Im $\chi^{(2)}$ of the sample, we compare the signal with the HD-VSFG signal of a

reference sample for which the phase of the SFG light is known. We use a z-cut quartz crystal to this purpose. It is essential that the HD-VSFG signal from the reference z-cut quartz crystal is generated at the same height as the sample, as a mismatch in height would result in a phase shift of the recorded spectra and thus in the extracted real and imaginary $\chi^{(2)}$. We control the height of the reference quartz crystal and the sample by monitoring the position of the VSFG signal on the CCD camera. This enables us to define the SFG signal on the camera with a precision of 1 pixel size (16 x 16 μm). Together with our setup geometry this leads to a phase uncertainty of ~π/10 (~20 degrees). All HD-VSFG spectra in this paper were recorded with s-polarized SFG, s-polarized VIS and p-polarized IR. α-Lactalbumin (BioPure) was purchased from Davisco and was used without further purification. Urea (ACS reagent, ≥99.0 %) was purchased from Sigma Aldrich. D_2O (≥ 99.96 %) was purchased from Cambridge Isotope Laboratories. The HD-VSFG measurements were performed in ultrapure water, and the pH/pD was adjusted using NaOH and NaOD (1 M in water, Aldrich) or HCl and DCl (50 % in water, Aldrich).

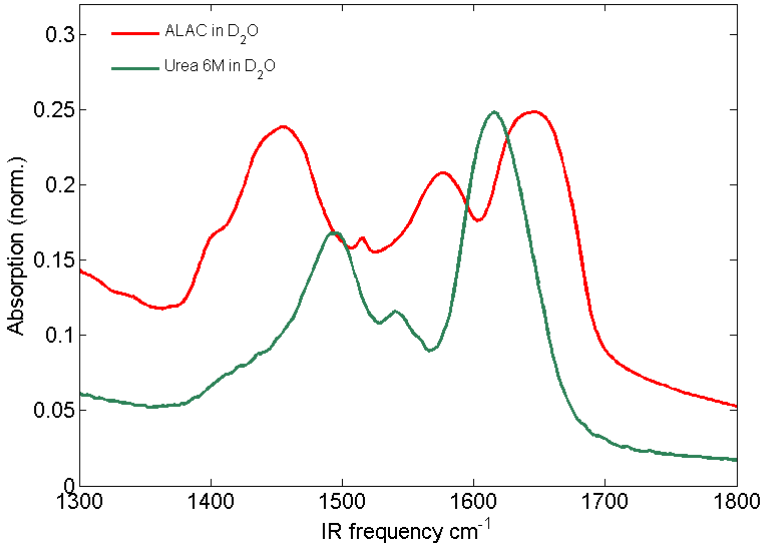
Supplementary Figures



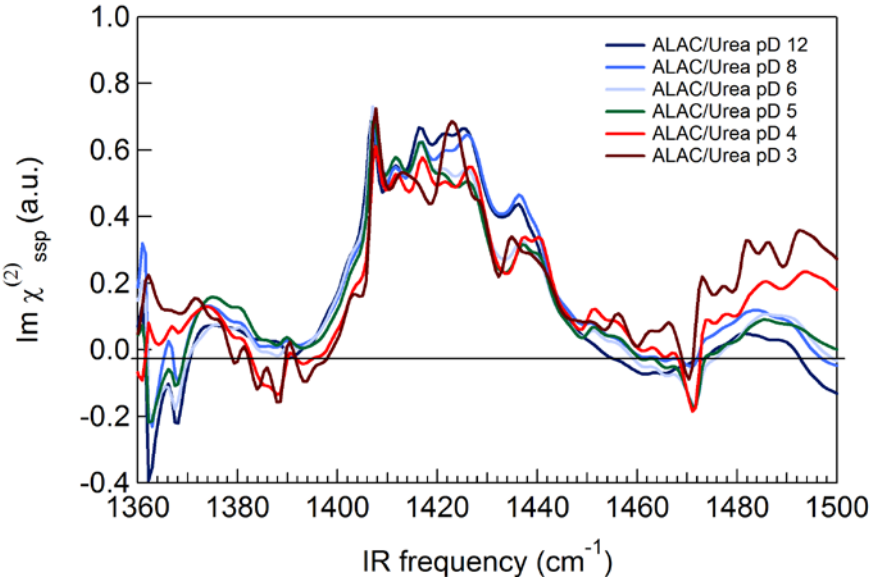
SI 1. Calculated net charge of α-Lactalbumin as a function of pH, which was estimated with ProtParam (E. Gasteiger, C. Hoogland, A. Gattiker, S.Duvaud, M. R. Wilkins, R. D. Appel, A. Bairoch. Protein Identification and Analysis Tools on the ExPASy Server. The Proteomics Protocols Handbook 2005, 571-607) and Protein Calculator v3.4.



SI 2. Imaginary $\chi^{(2)}$ spectra of H_2O and solutions of 4M and 8M urea in H_2O.



SI 3. Linear IR spectra of α-Lactalbumin in D_2O and 6 M Urea in D_2O in the spectral region of the amide I and amide II vibrations.



SI 4. Imaginary $\chi^{(2)}$ spectra of α-Lactalbumin in 6.5 M urea at different pH values in the spectral region of the amide II vibration.