

# Supporting Information: The molecular structure and surface accumulation dynamics of hyaluronan at the water/air interface

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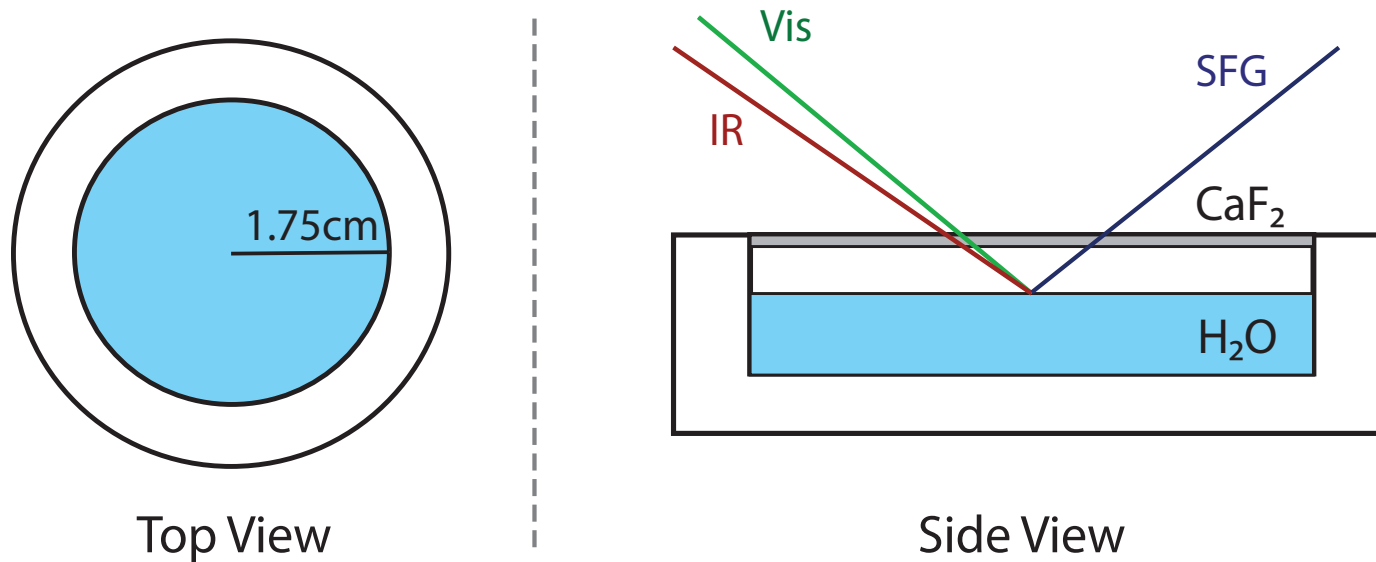
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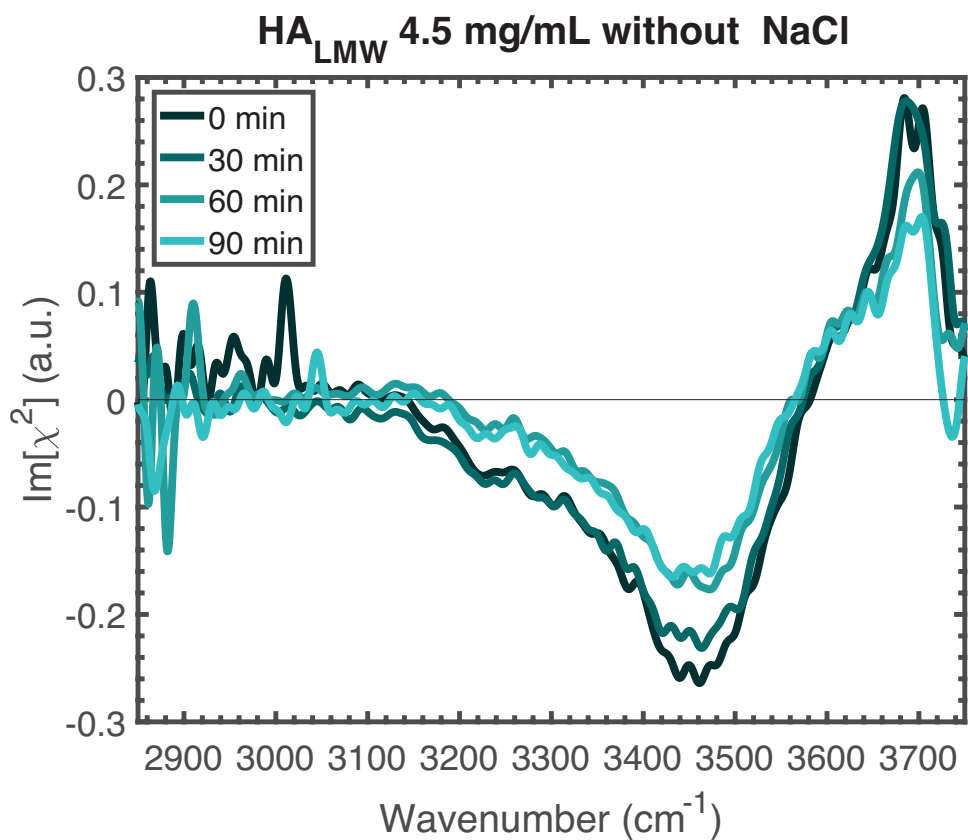
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Supporting Figure 1: Schematic of the custom built sample cell made of Teflon and covered by a calcium fluoride window. The sample cell has a diameter of 3.5 cm and can hold a sample volume of 4ml.

## Effect of the salt concentration in the solvent on the accumulation of hyaluronan at the surface

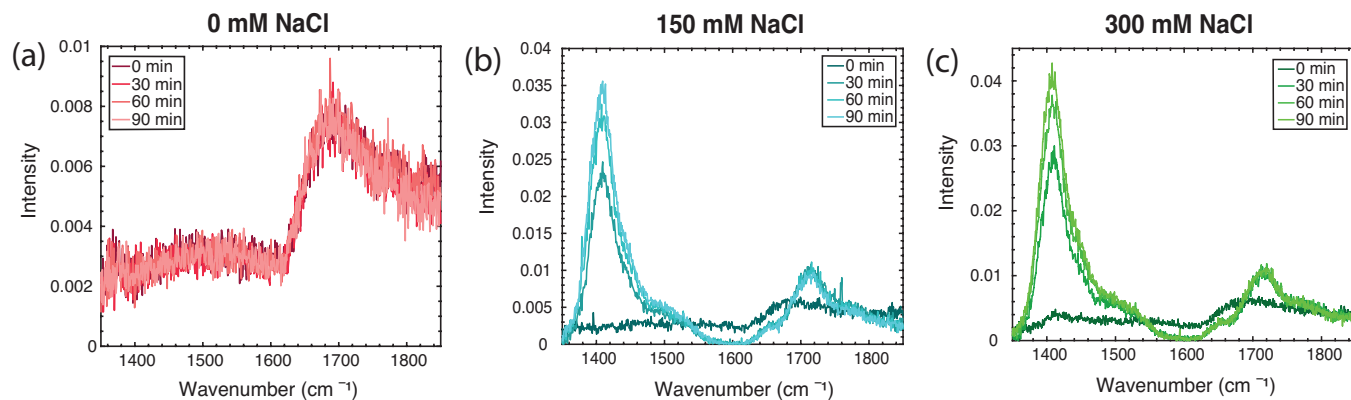
In Supporting Figure S2 we show  $\text{Im}[\chi^{(2)}]$  spectra in the region of the OH stretch vibrations of 4.5 mg/mL  $\text{HA}_{\text{LMW}}$  in pure water without salt added to the solution in a time range of 0 - 90 min with a pH of 7. It is seen that in the absence of salt,  $\text{HA}_{\text{LMW}}$  does not come to the surface. To confirm the depletion of  $\text{HA}_{\text{LMW}}$  from the surface by removing salt from the solvent, we performed additional measurements in the frequency region of the carboxylate anion and carbonyl vibrations of hyaluronan at  $1350\text{ cm}^{-1}$  -  $1850\text{ cm}^{-1}$ .



Supporting Figure 2:  $\text{Im}[\chi^{(2)}]$  spectra of  $\text{HA}_{\text{LMW}}$  solutions with a concentration of 4.5 mg/ml with pH 7 and no NaCl added to the solution in the time frame of 0 min - 90min.

In Supporting Figure S3, we show intensity VSG spectra of 4.5 mg/mL HA<sub>LMW</sub> solutions with different concentrations of NaCl in a time frame of 0 - 90 min. Figure S3 (a) show the intensity VSG spectra of HA<sub>LMW</sub> without any additional salt added to the solution. The spectra show the response of the water bending mode at 1650 cm<sup>-1</sup> that is identical to the line shape that is observed for a neat water surface (see Figure S9).<sup>1,2</sup> This observation confirms the observation of Supporting Figure 2 that HA polymers do not adsorb to the water/air interface if there is no salt present in the solution. When 150 mM NaCl is added to the 4.5 mg/mL HA<sub>LMW</sub> solution, two prominent features appear in the spectrum (Figure S3(b)) at 1420 cm<sup>-1</sup> and 1720 cm<sup>-1</sup>, which we assign to the vibration of the symmetric mode of the -COO<sup>-</sup> group and to the vibration of the carbonyl mode of the -COOH group, respectively.<sup>3,4</sup> The presence of these two peaks indicates that for the salt containing solution, HA molecules are able to accumulate at the surface. Increasing the NaCl concentration further does not lead to additional changes in the adsorption process (Figure S3(c)). Figure S3 (b) and (c) show that the peak height at 1420 cm<sup>-1</sup> increases over time, while the peak at 1720 cm<sup>-1</sup> stays more or less constant. The absence of a time-dependence of the -COOH band can be explained from the higher surface propensity of the protonated carboxyl groups of the HA polymers. Previous surface tension studies showed that the surface activity for protonated fatty acids is higher than for their deprotonated analogues.<sup>5</sup> It should be noted that the rise of the signal of HA coming to the surface in Figures S3 (b) and (c) is somewhat delayed compared to the result of Figure 2 (d) of the main manuscript where we probed the signal of water for a highly similar solution containing salt and 4.5 mg/ml of HA<sub>LMW</sub>. This difference can be explained from the fact that Figure 2 of the manuscript and Figure S3 represent experiments in different frequency regions and with different acquisition times. In the experiment of Figure S3 we used an infrared pulse centered at 6 μm which yields a slightly different local temperature than the experiment of Figure 2 where we used a 3 μm pulse that is much more strongly absorbed by water. In addition, Figure S3 represents an intensity VSG experiment with a longer acquisition

time than the HD-VSFG experiment of Figure 2. These differences do not affect the overall trend, being that for a solution containing 4.5 mg/ml of HA<sub>LMW</sub> and a sufficient amount of salt, HA<sub>LMW</sub> comes to the surface within ~30 minutes, but they do lead to differences in the observed early-time dynamics.

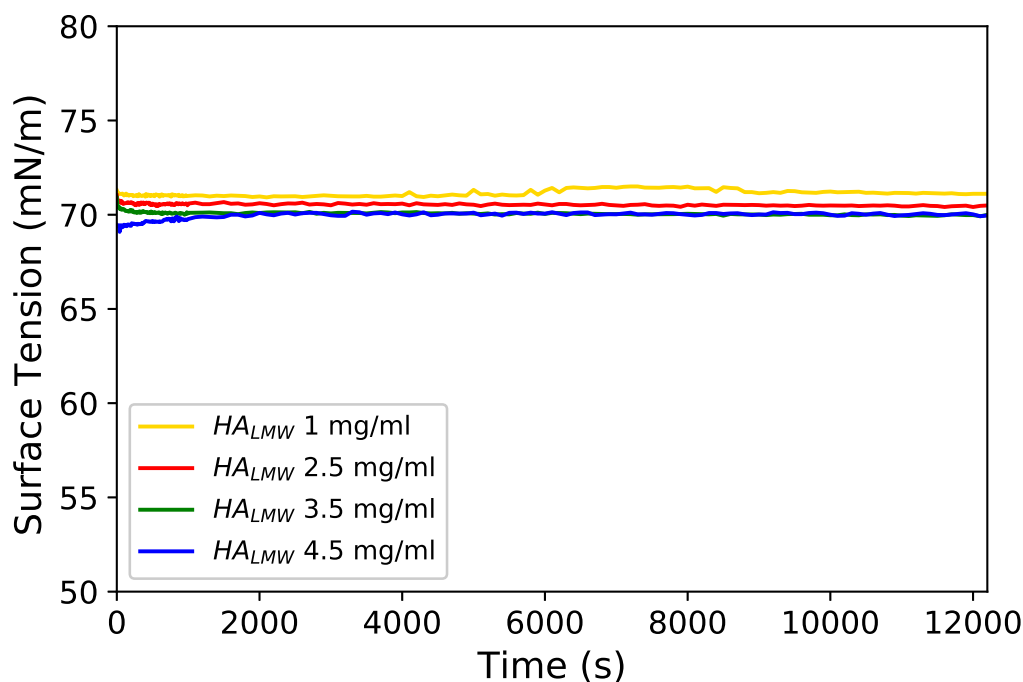


Supporting Figure 3: Intensity SFG spectra of 4.5 mg/ml HA<sub>LMW</sub> solutions with different NaCl concentrations of (a) 0 mM (red), (b) 150 mM (blue) and (c) 300 mM (green).

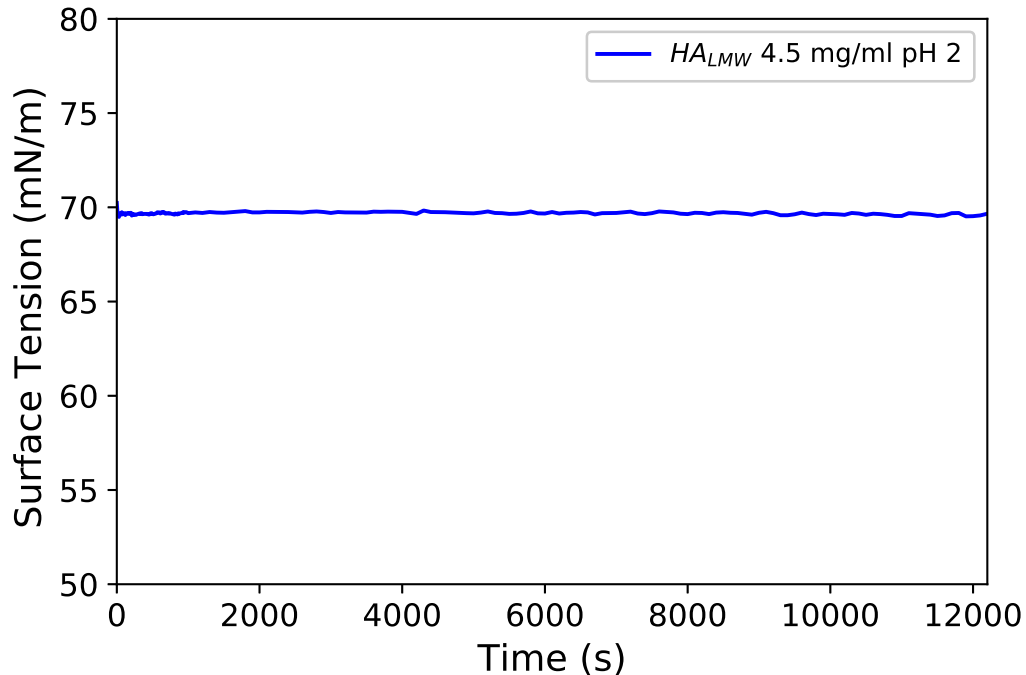
The enhanced surface accumulation of hyaluronan in the presence of salt can likely be explained from the competition of the ions with the solvent molecules. Salt ions interact strongly with water molecules,<sup>6,7</sup> thereby excluding the polymers from the bulk and pushing the polymer chains towards the surface. This phenomenon was described before for proteins and is well known under the name "salting up" effect.<sup>8</sup> An additional effect may be that the cations shield the negative charges of the carboxylate anion groups. As a result, these groups have a less strong interaction with water and the surface propensity increases. Hence, the presence of ions makes it more favorable for HA to accumulate at the surface. Without ions, the bulk-surface equilibrium is strongly shifted towards the bulk.

## Methods: Surface Tension

The effect of hyaluronic acid on the water-air surface tension is measured with the pendant drop method. We performed the pendant drop measurements by using a DSA 30S drop shape analyzer (Kruss, Germany) and analyzed the measurements with the Kruss Advanced software. For each measurement, a  $15\ \mu\text{l}$  droplet of hyaluronic acid solution is formed with a rate of  $2\ \mu\text{l}$  per second using an automated dosing system from a hanging glass syringe with needle diameter of 1.060 mm (Hamilton). After the droplet reaches its final volume, images are taken over the course of four hours to follow the evolution of the droplet's shape. From these frames, the droplet contour is detected automatically and fitted with the Young-Laplace equation to yield the interfacial tension. All measurements are performed in a closed and humid environment to minimize water evaporation from the droplet.<sup>9</sup>



Supporting Figure 4: Surface tension measurements of  $HA_{LMW}$  in a sodium phosphate buffer (137 mM NaCl, 10mM  $Na_2HPO_4$ , 2.7 mM KCl) with a pH of 7.4 for different concentrations of 1 mg/ml, 2.5 mg/ml, 3.5 mg/ml and 4.5 mg/ml.

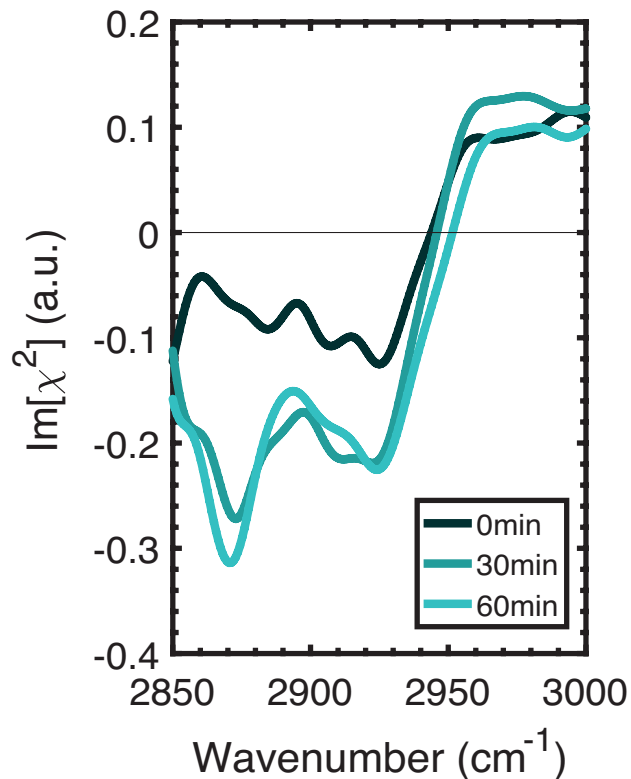


Supporting Figure 5: Surface tension measurements of HA<sub>LMW</sub> with a concentration of 4.5mg/ml and 150mM of NaCl with a pH of 2.

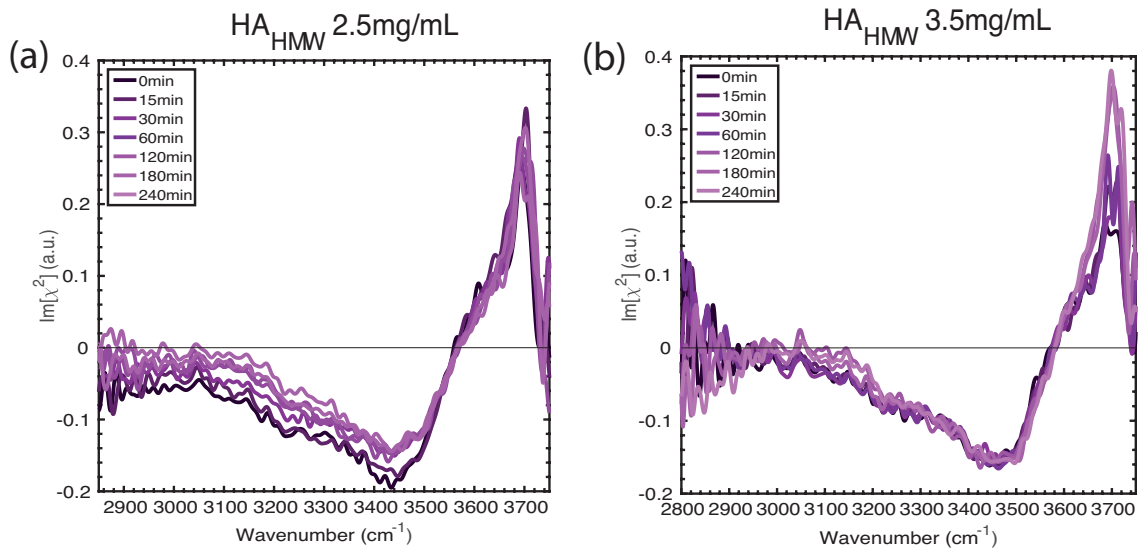
The surface tension measurement show that for HA<sub>LMW</sub> in a sodium phosphate buffer (137 mM NaCl, 10mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl) with a pH of 7.4, the surface tension stays constant at a value of ~70 mN/m over a time frame of 240 min (Supporting Figure 4). This notion applies to all studied concentrations of 1 mg/ml, 2.5 mg/ml, 3.5 mg/ml and 4.5 mg/ml. Furthermore, we performed surface tension measurements for HA<sub>LMW</sub> with a concentration of 4.5 mg/ml and 150 mM of NaCl at a pH of 2 (Supporting Figure 5). We again observe no change of the surface tension within 240 min. It should be noted that this does not mean that HA<sub>LMW</sub> does not come to the surface. For a polyelectrolyte solution the tendency of the polyelectrolyte to come to the surface is not necessarily associated with a decrease of the surface tension. A polyelectrolyte will have a quite favorable (Coulombic) interaction with water, so the gain in free energy of coming to the surface is much smaller for a polyelectrolyte surfactant than for instance for a neutral surfactant. In addition, a polyelectrolyte has

counter-ions for which an increase of the surface area leads to a negative free-energy change, because a larger number of these ions is forced in the near surface area where the hydration interactions with these ions are less favorable. Hence, for a polyelectrolyte solution compared to pure water, an increase in surface has both a positive effect on the free energy in making more room for the polyelectrolyte, but also a negative effect in forcing more of the counter ions into the near surface region. These two opposing effects can compensate each other, with the result that the surface tension of a polyelectrolyte solution is similar to that of pure water and does not change when the concentration of the polyelectrolyte (and thus of its counterions) is increased.<sup>10</sup> The HD-VSFG measurements show very clearly that HA<sub>LMW</sub> does come to the surface. An essential characteristic of HD-VSFG is that it measures the vibrational spectrum of the molecules at the surface, which constitutes a far more specific probe compared to the surface tension.

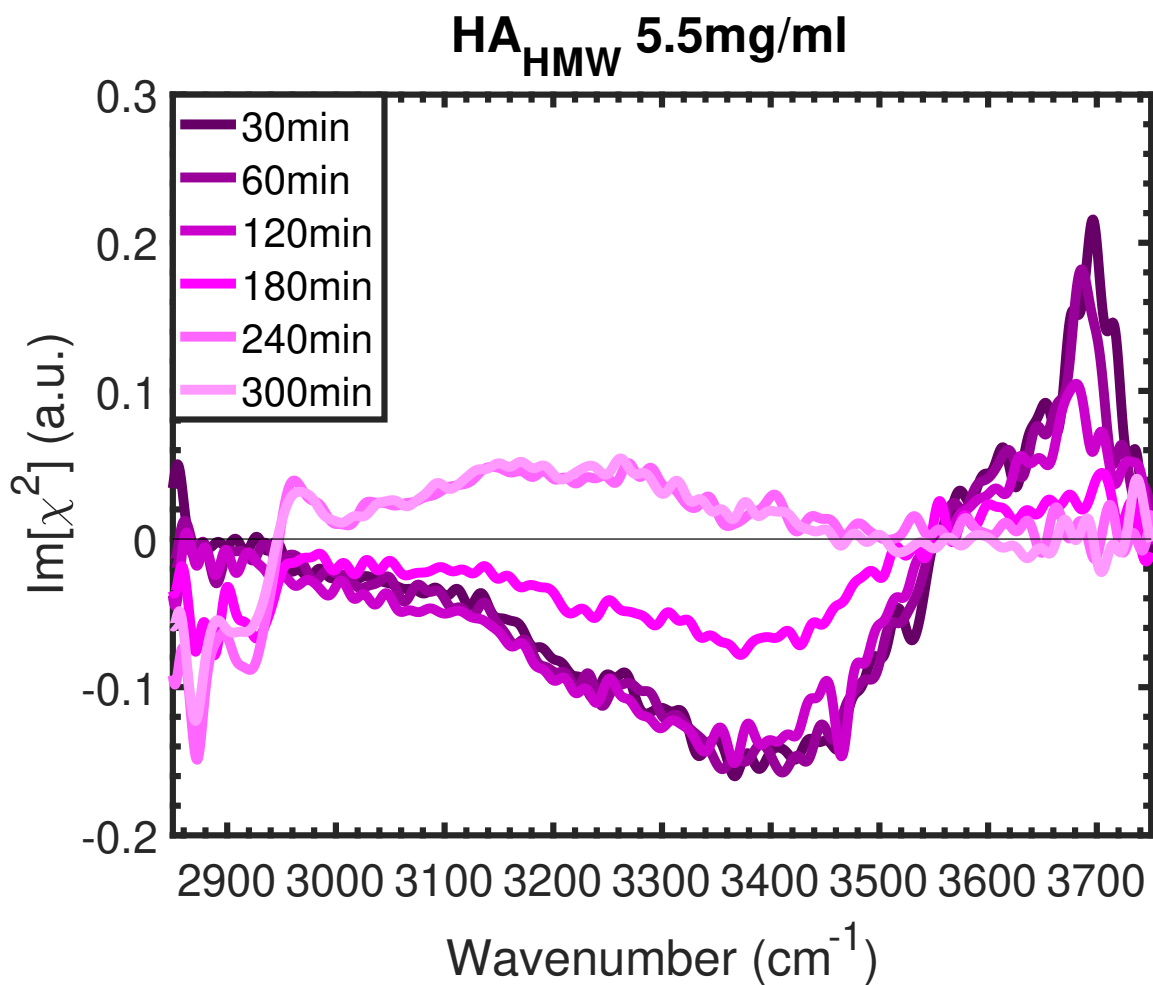




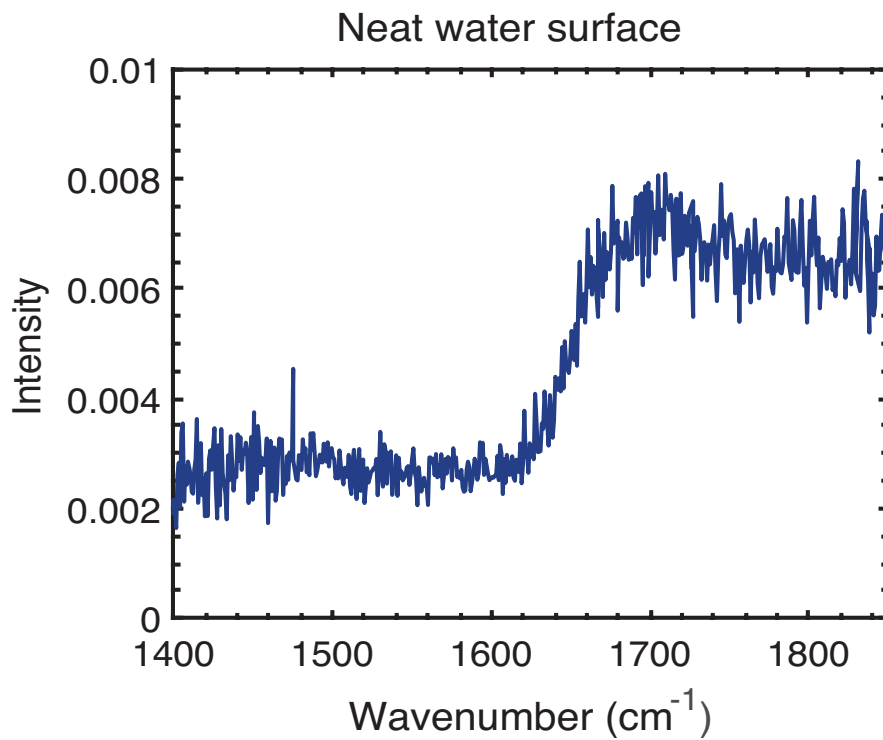
Supporting Figure 6:  $\text{Im}[\chi^{(2)}]$  spectra of 4.5 mg/ml  $\text{HA}_{\text{LMW}}$  solutions measured over a time range of 0 - 60 min in the frequency region of the CH vibrations. The spectrum at 30 min shows the rise of the response of the symmetric stretch vibration of the methylene group ( $\nu_{\text{CH}_2, \text{SS}}$ ) overlapping with the Fermi resonance of the methyl group at  $2940 \text{ cm}^{-1}$ . After 30 min the peak at  $2880 \text{ cm}^{-1}$  of the symmetric stretch vibration of the methyl group becomes dominating. This latter change of the spectrum indicates a reorganization of the HA molecules at the surface.



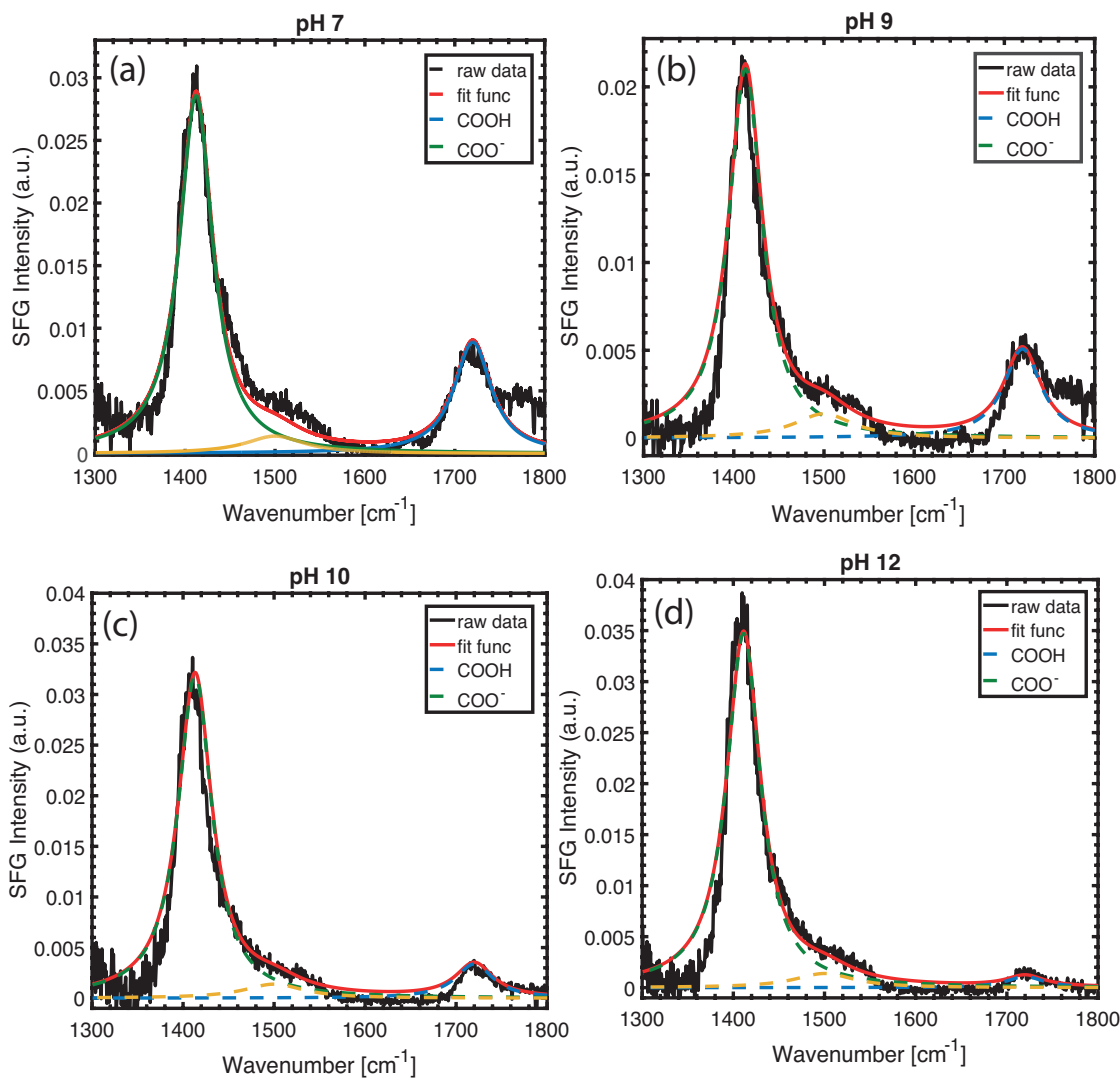
Supporting Figure 7:  $\text{Im}[\chi^{(2)}]$  spectra of (a) 2.5 mg/ml and (b) 3.5 mg/ml  $\text{HA}_{\text{HMM}}$  solutions in a time frame of 0 - 240 min. For both concentrations the spectra are identical to the spectrum of a neat water surface. This means that  $\text{HA}_{\text{HMM}}$  does not accumulate at the solution surface within a time of 240 min for this concentrations.



Supporting Figure 8:  $\text{Im}[\chi^{(2)}]$  spectra of 5.5 mg/ml HA<sub>HMW</sub> solutions in a time frame of 0 - 300 min. HA<sub>HMW</sub> accumulates at the solution surface after very long waiting times of more than 240 min and shows the same vibrational features as HA<sub>LMW</sub>. This measurement shows that the difference in surface accumulation of HA<sub>LMW</sub> and HA<sub>HMW</sub> is due to kinetic effects



Supporting Figure 9: Intensity SFG spectra of a neat water surface in the frequency range of 1400 - 1850  $\text{cm}^{-1}$ . The measurement was taken in SSP polarization configuration (s-SFG, s-VIS, p-IR).



Supporting Figure 10: Shows the fitting procedure with lorentzian curves of the intensity VSFG spectra of  $HA_{LMW}$  with a concentration of 4.5 mg/ml at different pH values at 120 min.

In the Supporting Figure 10 the fitting procedure with lorentzian curves of the intensity VSFG spectra of  $HA_{LMW}$  with a concentration of 4.5 mg/ml at different pH values at 120 min is shown. We fit the spectra with the sum of three different lorentzian shape like curves centered at  $1420\text{ cm}^{-1}$ ,  $1520\text{ cm}^{-1}$  and  $1720\text{ cm}^{-1}$ . The peak at  $1420\text{ cm}^{-1}$  is assigned to the symmetric stretch vibration of the carboxylate anion ( $\nu_{ss,COO^-}$ ) of HA, and the narrow peak at  $1720\text{ cm}^{-1}$  to the carbonyl stretch vibration of the carboxylic acid group ( $-COOH$ ) of HA. In addition to the two dominant peaks at  $1420\text{ cm}^{-1}$  and  $1720\text{ cm}^{-1}$  we extract another

peak centered at  $1520\text{ cm}^{-1}$ . Here we suggest that this peak, that appears like a shoulder of the  $1720\text{ cm}^{-1}$  feature in the VSG intensity spectra, can be assigned to the antisymmetric stretch vibrations of the carboxylate anion. To make a quantitative assessment about the surface propensity of  $\text{HA}_{\text{LMW}}$  at the surface we only take the sum of the vibrational modes of the stretch vibrations of the carboxylate anion ( $\nu_{ss,\text{COO}^-}$ ) and the carbonyl stretch vibration of the carboxylic acid group ( $-\text{COOH}$ ).

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