Traversing the free-energy pathways of intricate biomolecular processes

Enhanced simulation development and applications

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Publication date
2021

Citation for published version (APA):
The selective binding mechanism of ion-responsive rigidity in hyaluronan

The biological functions of natural polyelectrolytes are strongly influenced by the presence of ions, which bind to the polymer chains and thereby modify their properties. Although the biological impact of such modifications is well-recognized, a detailed molecular picture of the binding process and of the mechanisms that drive the subsequent structural changes in the polymer is lacking. Here, we study the molecular mechanism of the condensation of calcium, a divalent cation, on hyaluronan (HA), a ubiquitous polymer in human tissues. By combining two-dimensional infrared spectroscopy (2DIR) experiments with molecular dynamics (MD) simulations, we find that calcium specifically binds to HA at millimolar concentrations. Because of its large size and charge, the calcium cation can bind simultaneously to the negatively charged carboxylate group and the amide group of adjacent saccharide units. MD simulations and single-chain force spectroscopy measurements provide evidence that the binding of the calcium ions weakens the intra-molecular hydrogen-bond network of HA, increasing the flexibility of the polymer chain. We also observe that the binding of calcium to HA saturates at a maximum binding fraction of 10-15 mol %. This saturation indicates that the binding of Ca$^{2+}$ strongly reduces the probability of subsequent binding of Ca$^{2+}$ at neighboring binding sites, possibly as a result of enhanced conformational fluctuations and/or electrostatic repulsion effects. Our findings provide a detailed molecular picture of ion condensation, and reveal the severe effect of a few, selective and localized electrostatic interactions on the rigidity of a polyelectrolyte chain.

3.1. Introduction

Polyelectrolytes are charged polymers, which are widely present in nature and in man-made materials for applications ranging from wound dressing to oil-recovery [1, 2]. Because of their charged nature, the conformation and physical properties of polyelectrolyte chains strongly depend on the solution pH and on the salt conditions. The electrostatic repulsive forces among the charges along the chain enhance the polymer rigidity. Usually, dissolved ions are mobile, and can thus screen the charges along the chain, thereby reducing the total persistence length that is a measure of the chain rigidity [3]. Localized electrostatic interactions between ions and the charges on the chain (normally referred to as Manning condensation) can occur if the distance between the charges on the chain is less than the Bjerrum length ($\lambda_B$) [4]. One possible consequence is that the polymer backbone wraps around bound ions [5, 6]. Condensation of multivalent ions has also been reported to entail local ion “jackets”, and consequently, a reduction in persistence length of polyelectrolyte chains can also happen without creating local wrapping of the chain [7]. Although the effect of condensation on the configuration of polyelectrolytes has been thoroughly studied in previous work [5–8], the molecular details of the complexes formed between cations and polyelectrolytes, and the molecular mechanisms underlying the conformational changes that follow from the ion binding, are still unknown.

Among all the natural polyelectrolytes, a specific class of extracellular matrix polysaccharides, the glycosaminoglycans (GAGs), is arguably one of the most important for animal life. In the human body, GAGs are critically important in many biological processes, such as proliferation, anticoagulation [9, 10], inflammatory responses [11, 12], and the immune response to external pathogens [13]. Hyaluronan (HA) is the structurally simplest member of the GAG family. Alone or together with other extracellular matrix macromolecules (e.g., collagen), it dictates tissue elasticity, hydration and permeability, and it also directs cell behavior through multivalent engagement with cell surface receptors, such as CD44 [14]. These properties allow HA to mediate diverse functions in a wide range of physiological and pathological processes, including development [15, 16], mammalian reproduction [17], inflammation [18], and tissue lubrication [19, 20]. Diseases such as cancer or osteoarthritis are correlated with changes in the average molecular weight, supramolecular organization and concentration of HA [21–23]. Thanks to its biocompatibility, HA is also widely applied as a building block for responsive and biocompatible hydrogels [24–26].

An interesting feature of HA is the sensitivity of its mechanical properties to a particular divalent cation, calcium. Calcium ions have been found to show an unusually strong effect on the thickness of HA brushes (i.e. dense arrays of HA chains grafted with one end to a surface) designed to emulate certain properties of the glycocalyx of cells [27]. Moreover, at a concentration of a few mM of calcium ions, HA solutions show a decrease in viscosity that is much more drastic than in the presence of a similar concentration of sodium or magnesium [28, 29]. Consistent with this finding, a previous study also showed that calcium ion levels in the low mM range cause a reduced translational diffusivity of small solutes, such as glucose
3.2. Experimental results

3.2.1. Complexation of Ca^{2+} ions with hyaluronan

We used linear and nonlinear IR spectroscopy to characterize the interaction between calcium ions and HA (see Supplementary Methods of the publication on which this chapter is based [31] for details). In Fig. 3.1b we report linear infrared absorption spectra of a solution of HA at a concentration of 20 mg/ml, where we vary the CaCl_2 concentration from 0 to 300 mM. In the frequency region between 1580 and 1680 cm\(^{-1}\), we observe two bands, one at 1609 cm\(^{-1}\) and the other at 1633 cm\(^{-1}\). Following the literature, we assign the band at 1609 cm\(^{-1}\) to the absorption band of the antisymmetric stretching mode of the carboxylate anion group (\(\nu_{\text{antisym}}\)), and the band at 1633 cm\(^{-1}\) to the absorption band of the amide I vibration (\(\nu_{\text{AM.I}}\)) of the amide group [32]. Upon addition of CaCl_2, we observe a small increase of the absorption around 1590 cm\(^{-1}\), and a decrease of the absorption near 1607 cm\(^{-1}\), indicating that the calcium affects the molecular vibrations of the carboxylate anion group. Most notable, however, is the enhanced absorption in the high-frequency region of the spectrum, corresponding to the high-frequency wing of the amide I band (1650 cm\(^{-1}\)). In the absence of calcium, the amide group is hydrated on average by two water molecules, and each hydrogen bond induces a red-shift of the amide I absorption band of 10 to 20 cm\(^{-1}\) [33, 34]. The observed partial blue shift of the amide I band from 1633 cm\(^{-1}\) to 1650 cm\(^{-1}\) thus suggests that calcium ions dehydrate

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1 Obtained by our experimental collaborators. Linear IR and 2DIR measurements were performed by G. Giubertoni and analyzed together with H.J. Bakker. Single chain force spectroscopy experiments were performed by F. Bano and analyzed together with R.P. Richter. For more details, see Author contributions.
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Figure 3.1: a) Molecular structure of a disaccharide unit of HA containing amide (red) and carboxylate (green) groups on adjacent saccharide units. b) Linear FTIR infrared spectra for a solution of HA at 20 mg/ml in water containing 0, 25, 50, 150 or 300 mM of calcium ions (as indicated). We observe the absorption peaks of the anti-symmetric stretching mode of the carboxylate anion group ($\nu_{\text{ant}}$), and of the amide I vibration ($\nu_{\text{AM,I}}$). All the spectra are background subtracted. c) Differential FTIR infrared spectra obtained by subtracting the infrared spectrum in pure water (0 mM Ca$^{2+}$) from the other spectra shown in b). The error bars represent experimental error due to background fluctuations and were obtained by comparing two independent measurements of the same solution.

A part of the amide groups. Computational studies have shown that in simple model systems containing a single amide group, calcium ions have a significant probability to be located close to the carbonyl oxygen ($<2.5\,\text{Å}$), and thus to be in direct contact with the amide group [33–35]. The creation of such a cation-amide pair induces a red-shift in the amide I vibration with respect to the frequency of this mode in the gas phase, but this red-shift is smaller than the red-shift that results from the formation of hydrogen bonds with water molecules. In a previous study, calcium was found to bind to the carbonyl oxygen in a collinear fashion, displacing both water molecules [33], thus corroborating that the observed blue-shift of the amide vibration results from the binding of calcium ions.

At calcium concentrations below 150 mM, there is no significant difference between the linear absorption spectra measured with and without added salt. Nevertheless, based on previous reports, we do expect a significant effect of calcium...
ions on the HA structure already in this low-concentration regime. We studied the molecular-scale effect of calcium at low concentrations with 2DIR spectroscopy. 2DIR is a nonlinear technique in which molecular vibrations are excited from the ground state \((n = 0)\) to the first excited level \((n = 1)\) with an intense femtosecond mid-infrared light pulse (pump pulse). This excitation leads to a change of the absorption of the excited and other vibrations that we probe with a second, weaker, broadband, femtosecond mid-infrared light pulse (probe pulse). The absorption change \(\Delta \alpha\) measured with 2DIR is proportional to the square of the vibrational cross-section, \(\sigma(\Delta \alpha \sim \sigma^2)\), while in linear infrared spectroscopy the signal is linearly proportional to the vibrational cross-section \((\alpha \sim \sigma)\). Therefore, 2DIR is ideally suited to distinguish species with high cross-sections and low concentration (e.g., molecular vibrations of amide and carboxylate groups) from a background of species with low cross-sections and high concentrations (e.g., molecular vibrations of water). In Fig. 3.2a, we present 2DIR spectra measured for a HA solution with 0 and 25 mM calcium ions (Fig. S1 in [31] shows additional data spanning from 0 to 300 mM). In both spectra, we observe a strong signal when exciting at a pump frequency of 1607 cm\(^{-1}\), which extends to higher probe frequencies and shows a shoulder at 1630 cm\(^{-1}\). The peak and the shoulder colored in blue represent a decrease in absorption due to bleaching of the fundamental \(n = 0\) to \(n = 1\) transitions of the \(\nu_{\text{ant}}\) and \(\nu_{\text{AM.1}}\) vibrations, respectively. The signals at lower probe frequencies colored in red represent the induced absorption of the \(n = 1\) to \(n = 2\) transition. Upon addition of calcium ions, we observe an enhanced absorption at higher frequencies (indicated by the arrow in Fig. 3.2b around 1660 cm\(^{-1}\)), which can be seen more clearly in the lower part of Fig. 3.2b where we show the difference between the two slices taken along the diagonal of the bleach (also shown in Fig. S1 in [31]), illustrating the enhanced absorption.

We observe a significantly increased absorption on the blue side of the amide vibration peak already at a calcium concentration of 25 mM. As with the linear absorption spectra, we assign this enhanced absorption to the complexation of the amide carbonyl to the calcium ion, which we will indicate as \(\nu_{\text{AM.1-Ca}^{2+}}\). We fit the 2DIR data with three Gaussian-shaped peaks to extract the relative area of the \(\nu_{\text{AM.1-Ca}^{2+}}\) amide band at different concentrations of calcium ions. In this fit, we used two Gaussian-shaped peaks to describe the \(\nu_{\text{ant}}\) and \(\nu_{\text{AM.1}}\) vibrational bands in the absence of calcium, and a third Gaussian-shaped peak to describe \(\nu_{\text{AM.1-Ca}^{2+}}\) (see Supplementary Methods in [31] for details). The central frequencies and the widths of the three bands were global parameters in the fit, meaning they were fixed at all studied calcium concentrations, and only the amplitudes of the three bands were allowed to be different at different calcium concentrations. Examples of the fits are reported in Figs. S2-3 in [31]. We assume that \(\nu_{\text{AM.1-Ca}^{2+}}\) and \(\nu_{\text{AM.1}}\) have the same cross-section, and thus the fraction of amide groups bonded to calcium ions follows directly from the areas of the \(\nu_{\text{AM.1}}\) and \(\nu_{\text{AM.1-Ca}^{2+}}\) bands (Fig. 3.2c). We observe that at a low calcium concentration of 10 mM, a significant fraction of amide groups is already bonded to calcium ions. Due to limited experimental sensitivity, measurements at physiological calcium conditions (1-2 mM) were not possible. The fraction of amide bonded groups rises quickly with increasing calcium concentration.
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Figure 3.2: a) 2DIR spectra of HA at a concentration of 20 mg/ml in water containing 0 (left) and 25 mM (right) calcium ions. The waiting time between the pump and probe pulse was 0.3 ps. The yellow rectangles indicate the regions with the largest changes in absorption. b) Top: transient absorption 2DIR signals taken along the diagonal slice (dashed line: guideline for the eye set at 2DIR signal equal to 0) of the bleach in (a). Bottom: Differential spectrum obtained by subtracting the spectrum measured for a solution without calcium (0 mM) from the spectrum of a solution with 25 mM calcium ions. c) Fraction of complexed amide groups as a function of calcium concentration. The data (symbols with error bars) are fitted with a model that includes an increasing energy penalty with increasing occupation of the amide groups (see main text and Supplementary Methods in [31] for details). The mean values and error bars (which represent the standard deviation) were obtained by averaging over three different experiments. d) Illustration of the complex formed. The grey dashed lines show the bidentate binding of the carboxylate anion group to Ca\(^{2+}\).

but effectively saturates at a fraction of 10-15 mol % of N-acetylglucosamine.

The observed saturation implies that the binding between Ca\(^{2+}\) and HA is best described with a model that accounts for a high affinity for calcium ions at low concentrations, but includes an energetic penalty for further binding upon complex formation. This energy penalty depends on the fraction of formed complexes, and can be accounted for by using an expression for the association equilibrium constant that contains an exponential term with a constant energy term \(E_p\) (normalized by the thermal energy, \(k_B T\)) weighted by the fraction of occupied binding sites \(f\):

\[
K_{a,H} (f) = K_{a,H_0} e^{-\frac{E_p}{k_B T} f} = \frac{[Ca^{2+}HA]}{[Ca^{2+}][HA]} \quad (3.1)
\]
with

\[ f = \frac{[\text{Ca}^{2+}\text{HA}]}{[\text{HA}]+[\text{Ca}^{2+}\text{HA}]} \]  

(3.2)

Here, \([\text{Ca}^{2+}]\) is the concentration of free calcium ions, and \([\text{HA}]\) and \([\text{Ca}^{2+}\text{HA}]\) are the concentrations of unoccupied and occupied \(\text{Ca}^{2+}\) binding sites on HA (assuming one binding site per HA disaccharide), respectively. Using \([\text{Ca}^{2+}]+[\text{Ca}^{2+}\text{HA}] = [\text{Ca}^{2+}]_i\), where \([\text{Ca}^{2+}]_i\) is the total concentration of calcium ions in the solution and the index \(i\) runs over all concentrations investigated, and \([\text{HA}]+[\text{Ca}^{2+}\text{HA}] = [\text{HA}]_0\), where \([\text{HA}]_0\) is the total concentration of binding sites, we can re-write the equilibrium expression 3.1 as:

\[ K_{a,H} = \frac{x}{([\text{Ca}^{2+}]_i - x)([\text{HA}]_0 - x)} \]  

(3.3)

where \(x = [\text{Ca}^{2+}\text{HA}]\). Solving Eq. 3.3 for \(x\) and using expression 3.2 we obtain:

\[ f = \frac{1}{2} + \frac{1}{K_{a,H} + [\text{Ca}^{2+}]_i} \]

\[ - \sqrt{\left(\frac{1}{K_{a,H} + [\text{Ca}^{2+}]_i + [\text{HA}]_0}\right)^2 - 4 [\text{Ca}^{2+}]_i [\text{HA}]_0} \]  

(3.4)

To extract the zero-concentration binding constant \(K_{a,H_0}\), and the penalty energy \(E_p\), we globally minimize:

\[ \sum_i \left(f_{\text{exp}}([\text{Ca}^{2+}]_i) - f([\text{Ca}^{2+}]_i)\right)^2 \]  

(3.5)

where the \(f_{\text{exp}}([\text{Ca}^{2+}]_i)\) values are obtained from the 2DIR experiments. The \(f([\text{Ca}^{2+}]_i)\) values follow from solving the coupled equations 3.1 and 3.4 (see Supplementary Methods in [31] for details), where we fit the fraction of bonded amide. We obtain \(K_{a,H_0} = 7 \pm 2 \text{ M}^{-1}\) and \(E_p = 30 \pm 5 k_B T\). The result of the fit is shown in Figure 3.2c. This binding constant is significantly higher that the binding constants of the separate amide or carboxylate anion groups to calcium. As reported in the Supplementary Methods in [31], we find that for N-acetylglucosamine, \(K_b = 0.013 \pm 0.05 \text{ M}^{-1}\), and for glucuronic acid, \(K_b = 1.2 \pm 0.2 \text{ M}^{-1}\) (Fig. S9 and S10 in [31]).

3.2.2. Effect of Ca\(^{2+}\) ions on the persistence length of hyaluronan

To assess the effect of ions on the persistence length of HA at the level of individual chains, we devised an atomic force microscopy (AFM) assay to probe the stretching of individual HA chains under tensile force, as schematically illustrated in Fig. 3.3a (see Supplementary Methods in [31] for details). Force spectroscopy with polymer chains benefits from a uniform population of molecules with well-defined characteristics. Here we made quasi-monodisperse (i.e., size distribution approaching the
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Figure 3.3: a) Illustration of the setup to measure the persistence length of HA by single molecule force spectroscopy: HA polysaccharide chains (red; MW = 647 kDa) were grafted via their non-reducing end to an AFM tip; extracellular domains of the HA receptor CD44 were anchored via their C-terminal end to a planar support, and served as baits to capture HA chains. b) Representative force curves obtained for stretching a single HA chain in CaCl\(_2\) (red curve) and NaCl (black curve; offset by 100 pN along the y-axis for clarity). Except for a region of non-specific binding at small separations (< 50 nm), the data are well-fit by the worm-like chain (WLC) model (blue curves). c) Histograms of persistence lengths \(L_p\) determined from WLC model fits for CaCl\(_2\) (red bars) and NaCl (black bars). These are well-fit by Gaussian-shaped curves (lines in matching colors), giving mean and standard deviations of \(L_p = 3.2 \pm 1.0\) nm in NaCl and \(1.7 \pm 0.7\) nm in CaCl\(_2\). Conditions: retract velocity of 1 \(\mu\)m/s, 150 mM NaCl or 50 mM CaCl\(_2\).

ideal) HA polymers with a “handle” only at a single specified point. In the polysaccharide realm, our HA probe is much more homogeneous than previous naturally occurring and semi-synthetic preparations [36].

For the single chain force spectroscopy experiments, briefly, HA polysaccharide chains were anchored through a thiol handle (SH-HA); a sulfhydryl group was site-specifically introduced at the non-reducing end (a difficult or impossible location for all previous syntheses with HA polysaccharides due to the terminus’ relative lack of unique chemical reactivity, in contrast to the reducing end), to a gold-coated AFM probe. The AFM probe was brought into contact with a planar support displaying the HA receptor CD44, which acted as a bait to capture an HA chain dangling from the AFM tip. Pulling the AFM probe away from the planar support then generated a tensile force that was monitored as a function of the probe-support distance, as exemplified in Fig. 3.3b. Care was taken to adjust the surface densities of HA and CD44 such that the rupture of individual HA-CD44 bonds, and thus the stretching of individual HA chains, could be resolved (Fig. S4 in [31]). The force vs. extension curves could be well-fitted with the worm-like chain (WLC) model (Fig. 3.3b; see also Supplementary Methods in [31] for details), as expected for flexible and sufficiently long polymer chains. Histograms of the persistence lengths \(L_p\) extracted from these fits (Fig. 3.3c) show that calcium ions decrease the persistence length of HA. At physiological concentrations of monovalent salt (150 mM NaCl), we found \(L_p = 3.2 \pm 1.0\) nm, in reasonable agreement with previous work [37]. In the presence of 50 mM CaCl\(_2\), the persistence length was reduced almost two-fold, to \(1.7 \pm 0.7\)
nm. Such a marked decrease in persistence length, or equivalently, increase in chain flexibility, indicates that calcium ions strongly affect the molecular conformation of HA.

3.3. Computational results

3.3.1. Effect of Ca$^{2+}$ ions on the atomic conformation of hyaluronan

To understand why calcium condensation causes an increased chain flexibility, we performed force-field MD simulations with atomistic resolution and an explicit description of the solvent molecules. We performed MD simulations of aqueous solvated HA at a concentration of 50 mM CaCl$_2$, as used in the AFM experiments. We used specialized and previously tested force-field parameters for a short HA oligomer dissolved in NaCl and CaCl$_2$ solvent environments. We employed two different sets of parameters for Ca$^{2+}$, referred to as CaCl$_2$-Deublein and CaCl$_2$-OPLS (see Appendix 3.A for details), since the treatment of divalent cations is known to be challenging at the force-field level of theory [33, 38]. We ran 200 ns of unbiased MD simulations of HA oligomers with a length of 8 disaccharides, starting from a straight-chain state, i.e. without chain bending, in the presence of either calcium or sodium ions. The last 50 ns were used for analysis, in particular to probe the effect of the cations on the structure and dynamics of the hydrogen-bonds within the HA oligosaccharide.

In Fig. 3.4a, we show the structure of the oligomer in which we also indicate the five studied hydrogen bonds (labeled A to E). In addition, we also studied the geometry of the complex formed with the cation (labeled F). In Fig. 3.4b, we show 2D histograms of the donor-acceptor distances and angles of the hydrogen bonds A to E and the amide/carboxylate/cation complex in a NaCl environment. Strong hydrogen bonds are characterized by donor-acceptor distances shorter than 3 Å and angles larger than 2.5 rad [39], the latter implying a high degree of alignment between the acceptor, the hydrogen and the donor.

In Fig. 3.5, we show the changes in the 2D histograms (blue for increasing, red for decreasing) when NaCl is replaced by CaCl$_2$. For contacts A to E, we observe positive peaks at longer distances and smaller angles, indicating a weakening of these hydrogen bonds. An exception is hydrogen bond D, which, in addition to some shift to longer distances and smaller angles, also shows a small positive peak at a distance of 3 Å and an angle of 2.8 rad. The largest difference is observed for the complex F. The Ca$^{2+}$ cations form close contacts with the carboxylate (< 3 Å) and both close (< 3 Å) and far (> 3 Å) contacts with the amide. The presence of close contacts between the cation and the amide group agrees with the IR and 2DIR experimental observation that, upon addition of calcium, amide groups experience dehydration because of the formation of a direct bond between amide oxygen and the divalent ions (Figs. 3.1 and 3.2). The positive change at a distance of 10 Å observed for both the amide and the carboxylate groups can be explained from cations located on neighboring monomers. The MD results thus confirm that Ca$^{2+}$ ions have a high propensity to bind to the carboxylate and amide groups, and show that this binding results in a weakening of the intramolecular hydrogen bonds of HA.
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Table 3.1 shows the probabilities of the formation of close contacts between the cation and the amide or the carboxylate group, or with both groups. For both force fields of Ca\(^{2+}\), we find that the CaCl\(_2\) environment yields much greater probabilities of close contacts than the NaCl environment. Both CaCl\(_2\)-OPLS and CaCl\(_2\)-Deublein yield similar values for the probability of close contacts with the amide (O2N; 17 and 18%, respectively), which is larger than the \(\sim\)10\% extracted from the 2DIR experiments (Fig. 3.2c). Such discrepancy may arise because of the force-field choice and/or because the cross-section of the amide vibrational band may change upon formation of a bond with the calcium ion. In Table 3.1 we also observe that the probabilities of close contacts between the cation and the carboxylate (O6A/B)
3.3. Computational results

Figure 3.5: Differential 2D histograms for contacts A to F (cf. Fig. 3.4a), generated by subtracting the histograms for HA in the NaCl environment (Fig. 3.4b) from histograms for HA in the CaCl$_2$-OPLS environment (Fig. 3.A.1a). Blue indicates an increase in Ca$^{2+}$ compared to Na$^+$ and red indicates a decrease. The differential 2D histograms obtained for the CaCl$_2$-Deublein environment are presented in Fig. 3.A.2a.

Table 3.1: Probability (± one standard deviation) of close contact (< 3 Å) of Na$^+$ or Ca$^{2+}$ cations with the carboxyl (O6A/B) or amide (O2N) oxygens, or with both oxygens simultaneously (O6A/B & O2N).

<table>
<thead>
<tr>
<th>Probability of close contact with cation</th>
<th>O2N</th>
<th>O6A/B</th>
<th>O2N &amp; O6A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA in NaCl</td>
<td>1.8 ± 0.7%</td>
<td>1.1 ± 0.7%</td>
<td>0.3 ± 0.3%</td>
</tr>
<tr>
<td>HA in CaCl$_2$-OPLS</td>
<td>17.2 ± 1.9%</td>
<td>42.9 ± 0.0%</td>
<td>17.2 ± 1.9%</td>
</tr>
<tr>
<td>HA in CaCl$_2$-Deublein</td>
<td>18.1 ± 3.8%</td>
<td>25.6 ± 10.1%</td>
<td>9.9 ± 8.5%</td>
</tr>
</tbody>
</table>

differ between the two Ca$^{2+}$ force fields. Previous literature reports that OPLS overestimates the Ca$^{2+}$ carboxylate affinity [40] and points to CaCl$_2$-Deublein being more realistic. In Fig. 3.A.1, we show 2D histograms of the different hydrogen bond lengths and angles obtained with CaCl$_2$-Deublein and CaCl$_2$-OPLS.

The time-averaged HA end-to-end chain length remained around 70 Å for all unbiased MD trajectories starting with a straight chain configuration (Fig. 3.A.3). This is only slightly shorter than the contour length of the HA oligosaccharide (80 Å), and suggests that large deformation events do not occur within the computational timescale and for this relatively small polymer size. In order to estimate the effect of Ca$^{2+}$ on the flexibility of HA, we calculated the bending free energy of the same HA oligosaccharide in NaCl, CaCl$_2$-Deublein and CaCl$_2$-OPLS environments. This was achieved by means of a variation of the constrained MD method [41] (see Supplementary Methods in [31] for details). The resulting bending free-
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energy profiles, spanning end-to-end lengths from a slightly stretched (compared to the time-average length) HA oligosaccharide (75 Å) to a half-bent U-shaped one (35 Å), are shown in Fig. 3.6a. The two environments with Ca\textsuperscript{2+} are consistent with each other and present significantly lower free energies of flexed configurations than the Na\textsuperscript{+} environment, indicating a higher flexibility of HA in the presence of Ca\textsuperscript{2+}. For flexible polymers, the bending free energy is linearly related to the persistence length [42]. This proportionality enables a quantitative comparison of the MD simulation results with the experimental force spectroscopy results. The free energy of the U-shaped bent HA decreases by approximately 40% from 4.96±0.73 kcal/mol in NaCl, to 3.04 ± 0.63 kcal/mol in CaCl\textsubscript{2}-OPLS (or by 35% to 3.24 ± 0.87 kcal/mol in CaCl\textsubscript{2}-Deublein). This reduction agrees with the approximately twofold decrease in persistence length observed by AFM (Fig. 3.3c).

In Fig. 3.6c, the differences in the five key hydrogen bonds are analyzed for the bent configuration with an end-to-end length of 35 Å for both CaCl\textsubscript{2}-OPLS and NaCl. There is a general weakening of all contacts, even more considerable than the weakening observed for the unconstrained chain in Fig. 3.5. Contacts B to E shift to either longer distances (> 4 Å) or smaller angles (< 2 rad), showing a clear weakening of these hydrogen bonds. Contact A is the only hydrogen bond with a small positive change at close distances. The bending is thus accompanied by a weakening of most intramolecular hydrogen bonds.

### 3.4. Discussion

The 2DIR and MD results show that the HA polymer chains bind calcium ions at millimolar concentrations, leading to the formation of specific calcium complexes with amide and carboxylate groups of adjacent saccharide units. The force spectroscopy measurement and MD simulations consistently show an increase in HA flexibility in the presence of calcium. The combination of the three techniques provides a direct link between the molecular mechanism of calcium binding and its effect on the HA chain mechanics.

The 2DIR results show that the association constant of Ca\textsuperscript{2+} and HA has a relatively high value of 7 M\textsuperscript{-1} at low Ca\textsuperscript{2+} concentrations. This association constant is much higher than the association constant for the binding of Ca\textsuperscript{2+} to amide groups, for which an association constant of ∼0.1 M\textsuperscript{-1} has been reported [34]. We observed a similar association constant for Ca\textsuperscript{2+} and N-acetylglucosamine (0.013 ± 0.05 M\textsuperscript{-1}, see Supplementary Methods and Fig. S9 in [31]). This difference can be explained from the fact that in case of HA, the Ca\textsuperscript{2+} not only binds to an amide group but at the same time to a nearby carboxylate anion group. The binding with the latter group will be rather strong, thus explaining the much larger association constant of Ca\textsuperscript{2+} to HA compared to isolated amide groups.

It is also interesting to compare the association constant of Ca\textsuperscript{2+} and HA with the second saccharide unit that constitutes the building block of HA, glucuronic acid. For glucuronic acid, we found an association constant of 1.2 ± 0.2 M\textsuperscript{-1} (Supplementary Methods and Fig. S10 in [31]), which is similar to the association constants found for complexes of simple acids and calcium [43]. The association constant of glucuronic acid and Ca\textsuperscript{2+} is thus approximately six times smaller than that of HA.
3.4. Discussion

Figure 3.6: a) Bending free-energy profile of HA for the three different simulation environments described in the text. The shaded regions refer to one standard deviation, as determined from a blocking analysis (see Appendix 3.A and Fig. 3.A.4, as well as Table 3.A.1). b) Visualization of the amide/carboxylate/cation complex rendered by averaging atomic positions during the last 25 ns of the CaCl$_2$-OPLS run constrained at an end-to-end length of 35 Å. The picture is centered at the middle of the chain. The calcium is represented according to its van der Waals radius. c) Differential 2D histograms of the distances and angles in key HA inter-monosaccharide hydrogen-bonds (labeled A to E; cf. Fig. 3.4a) and in the cation-amide (O2N)-carboxylate (O6A/B) complex (labeled F) generated by subtracting histograms obtained in the NaCl simulation environment from histograms obtained in the CaCl$_2$-OPLS simulation environment, with both systems constrained at an end-to-end length of 35 Å. The differential 2D histogram obtained in the CaCl$_2$-Deublein environment is presented in Fig. 3.A.2b.

and Ca$^{2+}$. This finding indicates that in HA the relative position and orientation of the amide group of N-acetylglucosamine and the carboxylate anion of glucuronic acid lead to a highly favorable binding of Ca$^{2+}$. 
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The strong affinity of HA for calcium may also be due to the size-charge requirements of the calcium ions. Due to its larger diameter compared to other cations, such as magnesium, the calcium ion binds well to less polar oxygens than water oxygens. Hence, the favorable inner chelation with other less polar groups, such as the amide oxygen in this case, greatly increases the stability of the calcium complex \[44-47\]. It is likely that the restrained conformational fluctuations and the size-charge requirements add up synergistically, explaining the high affinity of HA to calcium. Interestingly, we find that the high affinity of HA for Ca\(^{2+}\) rapidly drops when the concentration of Ca\(^{2+}\) increases, i.e. we observe that the occupation of the binding sites saturates at an occupied fraction of 10-15%. This strong saturation is not observed for glucuronic acid. It thus appears that the binding of a calcium ion at a particular binding site of HA hinders the binding of other Ca\(^{2+}\) ions at nearby binding sites. This hindrance may be due to a conformational change of the HA induced by calcium binding, with the result that the neighboring binding sites no longer possess the highly favorable conformation of the carboxylate and amide groups that caused the initial high association constant of 7 M\(^{-1}\). This explanation is supported by MD simulations that show that the binding of Ca\(^{2+}\) induces a weakening of the hydrogen bonds and increases the flexibility of the polymer chain. The increase of the flexibility, which is also borne out by the force measurements, implies that the conformational fluctuations increase in amplitude. As a result, the time fraction in which the conformation of the binding site is favorable for binding Ca\(^{2+}\) is reduced, thereby decreasing the association constant. An additional contribution to the decrease of the affinity for Ca\(^{2+}\) may originate from electrostatic repulsion between Ca\(^{2+}\) ions at neighboring units.

### 3.5. Conclusion

In this work, we have studied the interaction between HA polymers and Ca\(^{2+}\) ions with a combination of linear infrared spectroscopy, 2DIR spectroscopy, molecular-scale force measurements and MD simulations. We find that HA binds Ca\(^{2+}\) with an affinity that is unusually high for inorganic ions, with an association constant of 7 ± 2 M\(^{-1}\) in the limit of millimolar Ca\(^{2+}\) concentrations. This association constant is ~6 times higher than that of glucuronic acid, which contains the same carboxylate anion motif as HA. This finding, as well as our spectroscopic data, indicates that the relative position and orientation of the amide group and the carboxylate anion groups of HA are highly favorable for binding of Ca\(^{2+}\). The MD simulations confirm that HA has a high affinity for Ca\(^{2+}\) ions. We also observe a strong saturation of the binding of Ca\(^{2+}\) to HA at higher Ca\(^{2+}\) concentrations. This saturation effect can be well modeled with a free-energy penalty that scales with the fraction of bound Ca\(^{2+}\). The decreased binding affinity can be explained from the increase of the flexibility of the HA polymers upon the binding of Ca\(^{2+}\), as shown by the MD simulations. An additional contribution to the saturation effect may come from electrostatic repulsion, i.e., the positive charge of Ca\(^{2+}\) repels the binding of other positive charges at nearby binding locations.

The force measurements show that the binding of Ca\(^{2+}\) leads to a large decrease
of the persistence length of the HA polymers which amounts to 50% at a calcium concentration of 50 mM. The MD simulations explain this decrease of the persistence length in terms of the weakening of several of the intramolecular hydrogen bonds, induced by the formation of the complex of Ca$^{2+}$ with the carboxylate and amide groups.

In summary, by using a multi-technique approach, we show that a selective and localized cation binding process takes place between calcium and HA polymers, leading to the formation of specific complexes. Here we provide a detailed molecular picture of ion condensation on a polymer highlighting the severe effect of few, selective and confined electrostatic interactions on the rigidity of a polyelectrolyte chain. As the extracellular matrix contains calcium, this ion’s effect on the structure of HA chains and thus their binding to hyaladherin proteins and receptors should be considered. Moreover, given the vast employment of glycosaminoglycans to devise hydrogels with tailored applications, such as drug delivery, our findings may lead to novel ideas for creating smart materials by exploiting the unique structural properties that can be tuned by the addition of specific ions.

3.A. Appendix
3.A.1. Molecular dynamics
The system preparation was done with GROMACS 5.1.4. [48] The starting topology of HA with 8 disaccharides (where N-acetylglucosamine was the monosaccharide at the reducing end) was taken from the Supporting Data of [49]. The interatomic interactions of the chain were modeled with the GLYCAM06h force field [50], specifically designed for polysaccharides. The oligosaccharide was solvated in a $10 \times 10 \times 10$ nm$^3$ cubic box containing 1000 g/l water molecules, which were modeled by the SPC/E force field [51]. The force-field parameters of water (SPC/E) were chosen for their compatibility and have been used successfully before in systems with Ca$^{2+}$ ions [33, 38]. We prepared two different simulation systems. In the first, random water molecules were replaced with Na$^+$ and Cl$^-$ until a concentration of 50 mM NaCl was reached. The Na$^+$ and Cl$^-$ atoms were treated with the AMBER99SB-ILDN force field [52], which is widely accepted in force-field MD. In the second system, random water molecules were replaced with Ca$^{2+}$ and Cl$^-$ until a concentration of 50 mM CaCl$_2$ was reached. The divalent calcium ions are known to be difficult to parameterize with force fields [38]. We therefore used two different models for parameterization. The parameters established by Deublein and coworkers [38] (here called Deublein) were purposely designed for cations in aqueous solutions, while the general purpose OPLS-AA force field [53] (here called OPLS) has been reported to show a good match of the amide-Ca$^{2+}$ dissociation free energy with the free energy obtained with quantum mechanical density functional theory (DFT) based MD [33]. Both Ca$^{2+}$ force fields have been used before in combination with the SPC/E water model, whose lack of polarizability effects does not seem to affect the quality of the simulated structures and free-energy profiles. All systems were simulated in the NPT (i.e. constant number of particles, pressure and temperature) ensemble with the canonical sampling through velocity rescaling (CSVR) thermostat [54], which
was set to 310 K with a time constant of 0.1 ps, and the Parrinello-Rahman barostat \[55\], which was set to 1.0 bar with a time constant of 1.0 ps. Unbiased MD simulations were run for 200 ns, and data for analysis were taken from the last 50 ns of each run. The MD time step size was 2.0 fs. All systems were energy minimized before starting equilibration and production runs.

Figure 3.A.1: Histograms of the key hydrogen-bond distances and angles (labeled A to E) and of the cation-amide(O2N)-carboxylate(O6A/B) complex distances (labeled F) for HA (see Fig. 3.4a) in the unbiased straight-chain state in the CaCl$_2$-OPLS (a) and CaCl$_2$-Deublein (b) simulation environments. All histograms have a color bar range from 0 to 0.4 normalized units.
3.A.2. Constrained molecular dynamics

We employed a variation of the constrained MD method with stiff restraints instead of holonomic, or absolute, constraints [41]. In this scheme, several simulations are performed, each of them with a stiff harmonic restraint centered on a particular value along a chosen collective variable, \( z(r) \), which is a function of the system’s atomic coordinates. If the restraints are stiff enough, and the oscillations around the centers of the restraints are negligible, after sufficient sampling the average force exerted by each restraint is an estimate of the negative of the underlying free-energy gradient at that \( z \) value, \( \langle f(z) \rangle \approx -\partial F / \partial z \). Subsequently, we used numerical integration to recover a free-energy profile along \( z \). We choose the end-to-end length as collective variable and set the stiffness restraints (with force constants of \( k = 1000 \text{ kcal mol}^{-1} \text{ Å}^{-2} \)) every 5.0 Å for a range of 75 Å to 35 Å, which spans configurations from an almost fully stretched polymer, to a half-bent (U-shaped) chain.

The restraints were set using PLUMED 2.3.0 [56]. We used the biasing technique of steered MD [41] to prepare the initial configurations for the constrained MD free-energy calculations. We used a harmonic upper and lower wall with a force constant of \( k = 1000 \text{ kcal mol}^{-1} \text{ Å}^{-2} \) and steered it in 200 ps to the desired value for the free-energy calculations. We allowed for 5 ns of equilibration to allow the conformation to relax before sampling the forces that we use for the free-energy estimation. The total sampling time was 50 ns. For the free-energy estimation, the last 45 ns of sampling were divided into 9 blocks of 5 ns each, to obtain 9 free-energy profiles. Fig. 3.6a shows the average of these profiles (solid line) and one standard deviation above and below. The increased standard deviation in the direction of the shorter end-to-end lengths is due to the fact that the integration starts from the longest distance.

To ensure that 9 blocks of 5 ns provide stable force averages, we performed a blocking analysis. We started by subdividing the last 45 ns of sampling into 90 blocks of 0.5 ns each, and then gradually increased the block size by 0.5 ns until 9 blocks of 5 ns were obtained. Fig. 3.A.4 shows the averages and standard deviations of the forces exerted by the stiff restraint in each simulation. The average force, which is the estimator of the free-energy gradient, is stable independent of the block size. The standard deviation decreases significantly and appears to be stabilized at a block size of 5 ns for most cases. The only samples in which the standard deviation does not decrease are the stretched configurations with a 75 Å end-to-end length, however, these do not impact the bending free energy as they correspond to the stretched, not the bent, chain.

To further test the free-energy calculations, we also extended the simulation time for the runs constrained at end-to-end length of 35 Å in CaCl₂ (see Table 3.A.1). After performing an additional 50 ns of MD, the average force exerted by the constraint did not change beyond the error bars shown in Fig. 3.A.4, confirming that the sampling time is sufficient to robustly estimate the free-energy gradient.
Table 3.A.1: Consistency of the free-energy gradient estimation.

<table>
<thead>
<tr>
<th>Force exerted by the constraint (kcal mol(^{-1}) Å(^{-2}))</th>
<th>Initial 50 ns</th>
<th>Additional 50 ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA in CaCl(_2)-OPLS</td>
<td>0.05 ± 0.05</td>
<td>0.05 ± 0.06</td>
</tr>
<tr>
<td>HA in CaCl(_2)-Deublein</td>
<td>0.09 ± 0.03</td>
<td>0.09 ± 0.05</td>
</tr>
</tbody>
</table>

Figure 3.A.2: Histograms of the differences in the key hydrogen-bond distances and angles (labeled A to E) and in the cation-amide(O2N)-carboxylate(O6A/B) complex distances (labeled F) for HA (see Fig. 3.4a) in the CaCl\(_2\)-Deublein simulation environment compared to NaCl with both systems in the unbiased straight-chain state (a) and with both systems in the biased bent state with an end-to-end distance of 35 Å (b).
Figure 3.A.3: Time series of end-to-end distances (left panels), and histograms of end-to-end distances (right panels) derived from unbiased molecular dynamics simulations of HA made of 8 disaccharides in the NaCl environment (a), the CaCl$_2$-OPLS environment (b), and the CaCl$_2$-Deublein environment (c). Time-averaged mean values are also indicated in the left panels (green solid lines).
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Figure 3.A.4: Blocking analysis of the forces exerted by each restraint (from 35 Å to 75 Å) used for the free-energy calculations in the three distinct solvent environments. As a function of the block size, the solid color line shows the average force and the shaded regions show one standard deviation.
References


