Coil–Helix Transition

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Unravelling Secondary Structure Changes on Individual Anionic Polysaccharide Chains by Atomic Force Microscopy**

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***Abstract:** The structural conformations of the anionic carrageenan polysaccharides in the presence of monovalent salt close to physiological conditions are studied by atomic force microscopy. Iota-carrageenan undergoes a coil–helix transition at high ionic strength, whereas lambda-carrageenan remains in the coiled state. Polymer statistical analysis reveals an increase in persistence length from 22.6 ± 0.2 nm in the random coil, to 26.4 ± 0.2 nm in the ordered helical conformation, indicating an increased rigidity of the helical iota-carrageenan chains. The many decades-long debated issue on whether the ordered state can exist as single or double helix, is conclusively resolved by demonstrating the existence of a unimeric helix formed intramolecularly by a single polymer chain.*

Carrageenans are naturally occurring anionic polysaccharides extracted from red seaweed species and find application in food, cosmetic, and pharmaceutical product formulations where they serve as gelling agents, thickeners, stabilizers, and excipients.[1,2] Referring to a family of sulfated linear galactans, their principal disaccharide repeating unit consists of alternating β-(1→3)- and α-(1→4)-D-galactose or 3,6-anhydride residues.[1c] Following the traditional classification, iota- and lambda-carrageenan have two and three sulfate groups, respectively, with the former containing a 3,6-anhydride-bridge.[1a] These chemical and structural varieties combined with the ionic environment control their conformation.[3] The sequence motif of lambda-carrageenan has been suggested to prevent the formation of any secondary structure and thus gel formation, whereas iota-carrageenan has been reported to be capable of undergoing a coil–helix transition as a first event of gelation.[4] For their thermoreversible gelation in aqueous solution, a two-step process involving an initial ion-induced helix formation followed by association into networks is generally accepted. Nonetheless, the mechanisms governing the conformational transition, ion-specificity effects, and the mode of molecular association into a percolating gel are still highly debated.[2] An ordered dimeric double helix with 1.3 nm diameter was initially proposed for iota-carrageenan based on fiber X-ray diffraction results,[5a] which was further supported by osmometry,[5b] stopped-flow polarimetry,[5c] size-exclusion chromatography combined with light scattering,[5d,e] and differential scanning calorimetry.[5f] Strong indication for a single-stranded iota-carrageenan helix as the ordered conformation emerged, however, from other osmometry studies combined with measurements of optical rotation[4] and intrinsic viscosity,[6a] which were substantiated by light scattering data,[6b] leaving the nature of the ordered state a highly controversial issue.

This study focuses on resolving the disorder-order conformational transition in molecular solutions of these anionic polysaccharides by atomic force microscopy (AFM) imaging. The conformational change from disordered carrageenans in the random coil state[7] to ordered, helical chains, is expected to modulate the rigidity of individual polyelectrolyte chains[8] which will be assessed by applying polymer physics concepts to the AFM polymer traces. Commercial iota- and lambda-carrageenan (Figure 1 a,e) were transformed into the Na-form (Table S1) by combining dialysis and ion-exchange. Purified solutions, diluted to 1 μg mL^{-1} with either MilliQ water (0 mM) or an aqueous NaCl solution (100 mM), were adsorbed on different substrates (Figure S1). Using 0.05 % v/v (3-aminopropyl)triethoxysilane (APTES)-modified mica (AP-mica)[9] was found ideal for polymer adsorption and AFM imaging purposes. The negatively charged sulfate groups of the carrageenans ($\mathrm{p}K_\mathrm{a}=2$)[10] interact ionically with the positively charged surface aliphatic amino groups of the AP-mica ($\mathrm{p}K_\mathrm{a}=10.6$)[11] providing an efficient immobilization.

High resolution AFM imaging reveals individual, well separated polymer chains in condition of no added salt (0 mM) with an average chain height of about 0.3 nm for iota- as well as lambda-carrageenan (Figure 1 a,e and Figure S2 and S3). Analogous heights of about 0.3 to 0.5 nm were previously reported,[12,13] which is also in accordance with the estimated longitudinal axis of 0.3 nm for a hexose unit in its most stable $^4C_1$ chair conformation.[14] The polydispersity, inherent to polysaccharides,[15] is also reflected in the AFM images showing a broad range of contour lengths. Conditions of no added salt (0 mM) and ambient temperature imply a disordered carrageenan conformation.[4] The polymers appear to

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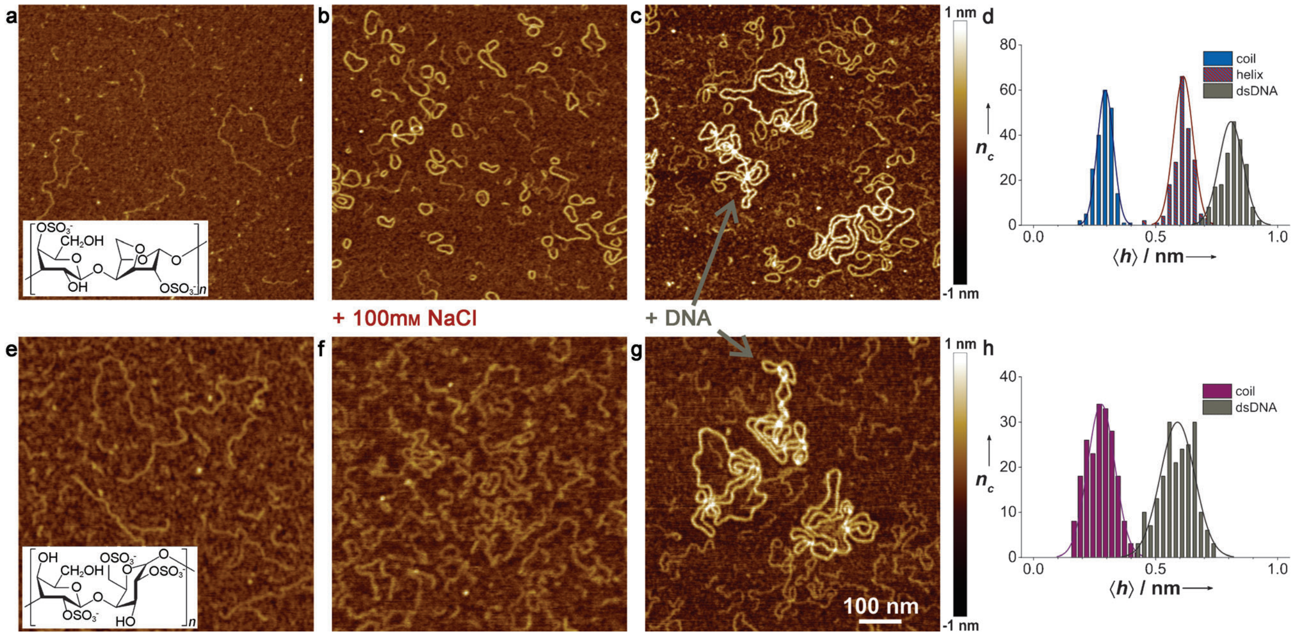

Figure 1. AFM height images of a–c) iota- and e–g) lambda-Na-carrageenan solutions, (a,e) as obtained after purification (0 mM) and with insets of the idealized disaccharide repeating units of the polymers, (b,f) upon addition of 100 mM NaCl, and (c,g) in the presence of cc dsDNA as internal height standard. The scale bars apply to all AFM images. d,h) Height histogram plots of averaged heights ⟨*h*⟩ of number of chains n_c of iota- and lambda-Na-carrageenan, respectively, extracted from the corresponding AFM images (c,g).

be flexible and randomly distributed when immobilized on AP-mica (Figure 1 a,e and Figure S2), different from KCl-modified mica, where carrageenans were reported to follow the substrate's crystallographic axes in the cleavage plane.[12] Owing to their polyelectrolytic nature, the intrinsically flexible chains bear, as expected from the Mark-Houwink exponent reported to be just below unity at low ionic strength,[7] a rather extended conformation. In conditions of 100 mM NaCl, the conformation of lambda-carrageenans is preserved, though a little less extended (Figure 1 f and Figure S2), possibly due to screened repulsive interactions among the sulfate groups. In sharp contrast, iota-carrageenans undergo dramatic changes upon exposure to 100 mM NaCl at RT (Figure 1 b and Figure S2), with the appearance of a second, thicker polymer population. This increase in thickness to an average chain height of about 0.6 nm (Figure 1 d and Figure S3) is proposed to arise from secondary structures, which indeed are expected[4] for iota- but not for lambda-carrageenan. Along with secondary structure formation, iota-carrageenans emerge as circular objects, coexisting with more extended, linear polymer chains. Unlike previous reports on cyclic and hairpin-like[13,16] iota-carrageenan morphologies, AFM images of the present study never reveal any ring-closing or overlapping ends (Figure S4).

To visualize the changes in dimension, closed circular double-stranded DNA (cc dsDNA) was added as an internal height standard just before deposition on AP-mica (Figure 1 c,g and Figure S2). Being another anionic biopolymer and furthermore in a circular, closed conformation, the absence of any interaction with the carrageenans is confirmed by AFM. Without salt added, cc dsDNA shows its characteristic breathing action[17] with portions of the polymer present as single-stranded DNA filaments (Figure S2), whereas at 100 mM NaCl, twisting, also known as supercoiling,[18] is observed (Figure 1 c,g and Figure S2). From the height histograms is inferred that cc dsDNA is significantly higher than both carrageenan types regardless of their characteristic conformation (Figure 1 d,h and Figure S3; Figure S5 for cross sections). From this series of experiments, a long-debated issue—whether the ordered helical conformation of carrageenans can exist only as a single[4,6,19] or double helix[5]—can be tackled. Starting from random coiled carrageenan polymers and equally thick single-stranded filaments of breathing cc dsDNA (Figure S3), a double helical conformation of carrageenan is expected to be thicker than dsDNA. Given that DNA bears specific base-pair interactions and carrageenan strands can only interact non-specifically, the result is a wider spacing between individual chains and thus a thicker double-stranded helical conformation. In reality, cc dsDNA is higher than the thick iota-carrageenan population (Figure 1 d and Figure S3). This ultimately implies that thicker strands represent polymers after the coil–helix transition with the ordered conformation consisting of a single polymer chain only.

To substantiate this statement, the coil–helix transition was imaged in situ on individual polymers by AFM in liquid conditions and as a function of time. Iota-carrageenan chains pre-immobilized on AP-mica are displaced only marginally during several scanning cycles and entire desorption of polymers is negligible (Figure S6). Replacement of the MilliQ water environment with 100 mM NaCl eventually demonstrates a secondary structure change on individually resolved chains in situ (Figure 2), whereas the polymers remain unaffected by exchanging the liquid with the same solution (Figure S7). In addition to an increased chain height, changes in secondary structure entail a chain contraction

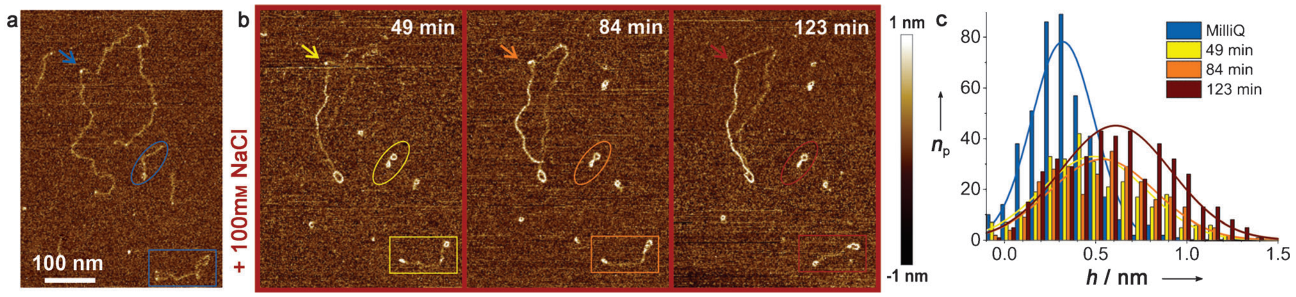

Figure 2. Coil–helix transition of iota-Na-carrageenan visualized in situ and versus time by AFM under liquid conditions. AFM height images of polymers pre-immobilized on AP-mica while being a) immersed in MilliQ water and b) after replacement of the liquid environment with 100 mм NaCl aqueous solution (images taken 49, 84, and 123 min after solvent exchange). Scale bars apply to all AFM images. c) Height histogram plot of the height *h* of data points n_p of the polymer highlighted with arrows in (a,b).

quantifiable by a reduced contour length (Figure S8). A double helix model can be ruled out unambiguously as secondary structure formation is resolved for individual chains in situ. The coiling of dangling polymer tails (Figure 2b and Figure S9) could be explained, in principle, by single chains folding into intramolecular double helices as suggested previously[13,16] for cyclic and hairpin-like morphologies. Such a model however cannot account for transitions along the contour of individual chains. Thus, the only possible mechanism remains the unimeric intramolecular single helix forming from chain portions loosely adsorbed on the substrate.

To attain higher image resolution, this experiment was also translated to AFM dry-state conditions. Briefly, pre-immobilized iota-carrageenans were incubated with either MilliQ water (0 mм) or an aqueous NaCl solution (100 mм), and imaged by AFM in the dry state. Whilst the polymers remain as disordered random coils upon surface incubation with MilliQ water (Figure 3a and Figure S10), at 100 mм NaCl, the formation of salt-mediated helix-stabilizing H-bonds[20] is enabled, thus outweighing the entropic barrier of the random coil state[21] and permitting the conformational transition to an ordered, helical state (Figure 3b). This secondary structure formation is virtually independent of the immobilization substrate and binding strength (Figure S11). Height profiles along the chain contours quantify the changes in height of thin parts of an average height of 0.3 to 0.5 nm for thick parts appearing either within the chain or as a coiling tail (Figure 3d). The absence of splitting at the chain ends in all incubation experiments once more conclusively confirms the model of an intramolecularly formed unimeric single helix.

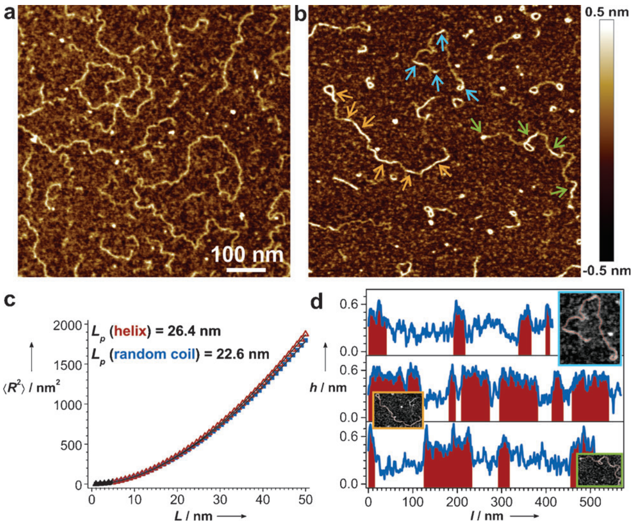

Figure 3. AFM height images of iota-Na-carrageenan after pre-immobilization on AP-mica and incubation with a) MilliQ water (0 mм) or b) aqueous 100 mм NaCl solution. The scale bars apply to both AFM images. c) Measured mean square end-to-end distance $\langle R^2 \rangle$ versus internal contour length *L* for separate helix and random coil conformation portions of the polymers. The 2D WLC fit is plotted in black. d) Height profiles along the contour of polymers (insets next to profiles) reveal thin and thick polymer portions corresponding to areas highlighted with arrows in (b).

As the coil–helix transition reduces the orientational degrees of freedom of the polymer to orient, secondary structure formation is expected to also affect the flexibility of the polymers. To test this hypothesis, a home-built tracking routine was applied on high resolution AFM images to extract the polymers' chain trajectories (Supporting Information). Using the 2D worm-like chain (WLC) model, the flexibility of a polymer of a certain length is defined by its persistence length (L_p) following $\langle R^2 \rangle = 4L_p(L-2L_p(1-\exp(-L/2L_p)))$, where $\langle R^2 \rangle$ is the average 2D mean square end-to-end distance and L the internal contour length between the two end points on the polymer for which $\langle R^2 \rangle$ is calculated.[22] Both iota-carrageenan conformations are well described by the WLC model as can be seen from the fitting in Figure 3c. The L_p extracted from 348 thin and 568 thick polymer contours demonstrates the stiffening of polymer chains as a result of the conformational transition, with an increase in L_p from 22.6 ± 0.2 nm to 26.4 ± 0.2 nm. Upon addition of an aqueous NaCl solution, iota-carrageenans are sequestering Na^+ counterions, thereby inducing a secondary structure formation. From the basic consideration of helical secondary structure formation, the potential of H-bonding sites along the polymer backbone is the driving force for the coil–helix transition.[20] Entropically, however, the helical structure restricts the configurational freedom of the polymer chain, as reflected by the changes in L_p. This loss in configurational entropy is thus thought to be the major factor preventing secondary structure formation in lambda-carrageenans, due to the kink in their primary structure.[23]

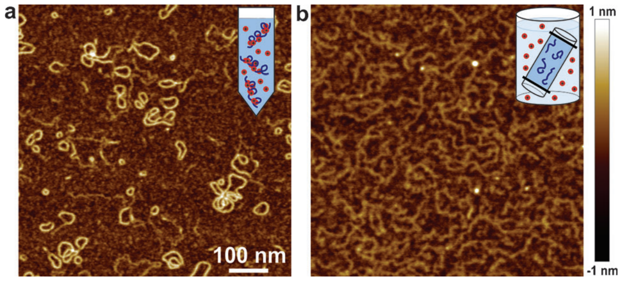

Figure 4. AFM height images of iota-Na-carrageenan a) in presence of 100 mM NaCl and b) after a subsequent dialysis against MilliQ water. The scale bars apply to both images.

To assess the reversibility of the coil–helix transition, iota-Na-carrageenan in 100 mM NaCl aqueous solution was again dialyzed against MilliQ water. While the polymers before purification showed the characteristic secondary structures with coexisting circular and linear polymer chains (Figure 4a), no internal structure was observed after an additional dialysis step (Figure 4b), thus confirming the reversibility of the conformational transition. Removal of the counterions that enable secondary structures leads to a reversible helix–coil transition.

In summary, iota- and lambda-Na-carrageenans were found to adopt a disordered random coil conformation at RT. Upon addition of NaCl, a coil–helix transition was induced in iota, but not in lambda polymers. The ordered state emerged as a unimeric intramolecular single-stranded helix, in agreement with considerations[19] of the thermodynamic stability. The molecular conformational transition of iota-carrageenan was furthermore quantified via the polymers' persistence length, although the chain rigidity was found to increase less dramatically than what has been proposed previously[8] based on rheological experiments on kappa-carrageenan. In the context of prior reports claiming a double helical ordered state, the present work corroborates the model of a single helix ordered state, although this does not preclude the possibility of superhelical formation or a mechanism changing from intramolecular single to intermolecular dimeric double helices when increasing the polymer concentration. Chain conformation and chemical structure of various natural polysaccharides are known to correlate with their biological activities.[24] Fundamental knowledge about control of the polymer conformational transitions might thus have an immediate implication on exploiting the bioactivity of carrageenans, reported to span from microbiocidal,[25a] to antioxidant,[25b] and immunomodulatory[25c] activity.

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