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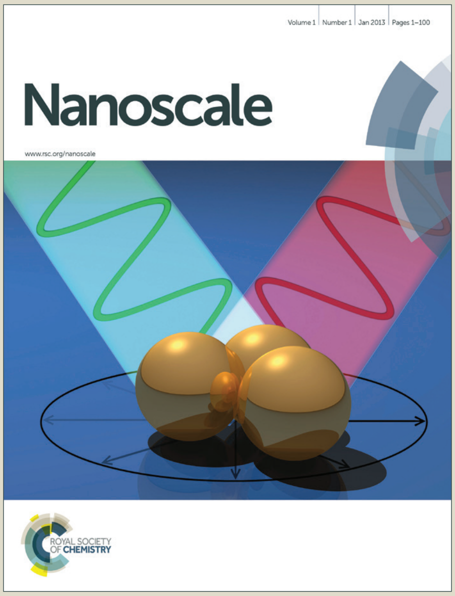

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Supramolecular Chiral Self-Assembly and Supercoiling Behavior of Carrageenans at Varying Salt Conditions†

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The self-assembly of anionic kappa and iota carrageenan polysaccharides in presence of NaCl, KCl and $CaCl_2$ is studied by high-resolution atomic force microscopy (AFM). A hierarchical supramolecular chirality amplification over various length scales is observed upon the addition of KCl, whereas in presence of NaCl and $CaCl_2$ the chains undergo solely a coil-helix transition with stiff kappa carrageenan and more flexible iota carrageenan helical conformations.

Self-assembling biopolymers have an immediate relevance for the understanding of biological processes and exploiting their structures and functions may enable the design of novel bio-inspired smart and optically active materials.[1] Supramolecular structure formation based on these systems can further be applied to control sol-gel transitions.[2] Belonging to a family of sulphated polyelectrolyte polysaccharides, carrageenans are an important class of food-grade gelling biopolymers.[3] The gel formation involves a transition of the polymers from a random coil to an ordered helical conformation[4] which is stabilized by salt-mediated H-bonding[5] along the primary carbohydrate structure of kappa and iota carrageenan. By following this coil-helix transition along individual polysaccharide chains, we were previously able to unambiguously demonstrate an intramolecular unimeric single helix[6] as the fundamentally ordered state. After the conformational transition, the helices may further associate to form a gel,[7] a process generally stimulated by the presence of salts combined with a reduction in temperature.[8-11] A multitude of studies have assessed the effects of the conformational transition and aggregation on scattering,[4e,8c,e,f,h] spectroscopic,[4d,8a,11b] optical,[4b,c,8b,c,e,h] thermal,[8i,9] rheological,[7a,c,d,8d,i,10] and structural[7c,d,8d,g,i,11a] properties. Despite these works, also due to the limited resolution of primarily electron microscopy experiments,[12] the molecular gelation model along with its transient states remains somewhat elusive, which motivates further insight brought by alternative methods, such as high-resolution atomic force microscopy (AFM).

In this study, the self-assembly of anionic polysaccharides – kappa and iota carrageenan – is studied upon their complexation with chloride salts of mono- and divalent cations at various concentrations. Conformational changes are expected to result in varying structural properties which are assessed by high-resolution AFM imaging. To understand the effect of individual counterions, the carrageenans were transformed to the Na-, K- and Ca-form, respectively (Table S1) and subjected to increasing concentrations of the corresponding chloride salts (Figure 1). As the added salt concentration is increased, the electrostatic repulsion among the negatively charged sulphate groups is screened by counterions, which together with specific interaction-driven ion condensation,[13] enables the formation of intramolecular secondary structures of increased height and stiffness at a concurrent reduction of length, as we have shown previously.[6]

In the case of NaCl (Figure 1(a)), this conformational transition from pure random coils is induced above 5 mM salt for kappa and 10 mM for iota, respectively, as demonstrated by the appearance of polymer populations with increased height. An earlier onset of this transition in kappa is expected as less salt is required to screen the negatively charged sulphate groups of the polyelectrolyte with lower linear charge density. By further increasing the salt concentration, the fraction of polymer portions in the helical conformation increases while the random coil fraction is reduced, with conversion predominantly reached at about 150 mM of added NaCl. By single-chain statistical analysis we quantified, via the persistence length, a 2-3-fold increase in chain rigidity upon the conformational transition,[13] with rather stiff kappa and more flexible, circularly-shaped iota single helical conformations. This analysis[13] also revealed an anomalous stiffening of the random coil chains, a phenomenon inferred from specific ion-polymer interactions and/or ion-ion correlations of nonlinear electrostatic origin.

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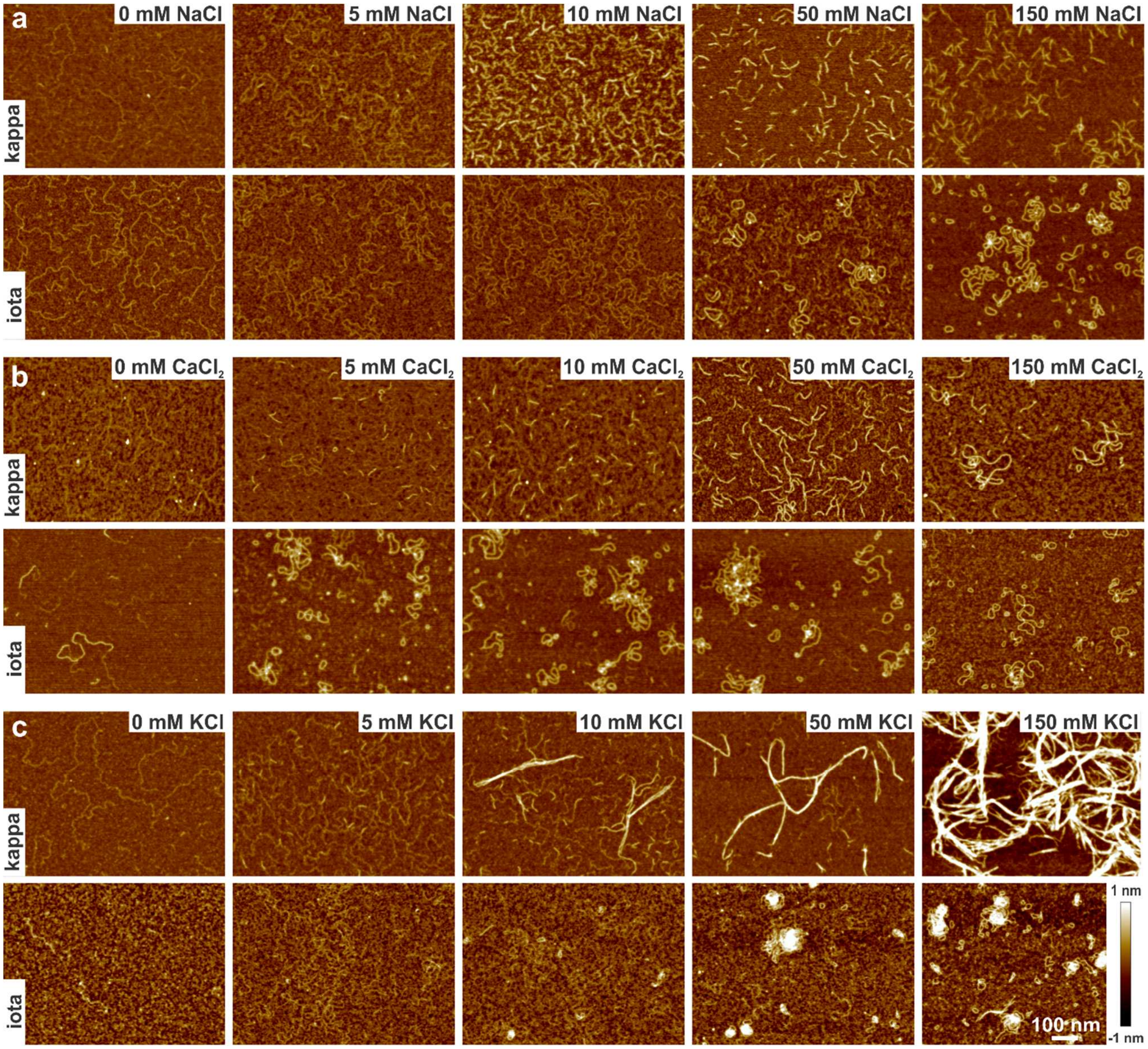

Fig. 1: Representative AFM height images of kappa and iota carrageenan as obtained after transformation to a specific ion-form and in the presence of (a) NaCl, (b) $CaCl_2$, and (c) KCl up to 150 mM added salt. NaCl and $CaCl_2$ lead to a progressive transition of the polymer chains from a random coil to a helical conformation with no further self-association, whereas KCl triggers the self-assembly of helical polymer strands to structures of higher complexity at high salt concentrations. The scale and color bars apply to all images.

The transformation of carrageenans to the Ca-form (Figure 1(b), see Figure S1 for kappa and S2 for iota for further AFM images at all salt concentrations) leads to partial helical conformations already during the purification process, i.e. without any added salt. The screening power of the divalent salt is higher compared to the monovalent NaCl and therefore for both, kappa and iota carrageenan, the onset of the conformational transition already occurs at very low salt concentrations. Further addition of $CaCl_2$ leads to a progressive transition of polymer strands from random coils to helical secondary structures with a conversion to single helices prevailing at around 7 mM $CaCl_2$ for iota (Figure S2), whereas kappa random coil conformations are still observable at 50 mM $CaCl_2$ (Figure S1). As for NaCl, kappa again forms rather stiff helical structures while iota forms flexible to circularly-shaped[11a] intramolecular secondary structures. In fact, the iota morphologies in increasing $CaCl_2$ concentrations resemble the condensation process of DNA[14] with an overall compaction of the condensed helical iota polymer chains. The very dilute conditions we apply to resolve individual polymer chains give rise to a stable, single helical state without further aggregation of the chains in presence of $CaCl_2$, in contrast to previous observations of precipitating aggregated kappa strands,[10] fine networks[7c] or coil-to-double helix transition and further dimerization[11b] of iota polymers.

Ion-exchange using KCl (Figure 1(c), see Figure S3 for kappa and S4 for iota for further AFM images at all salt concentrations) again results in carrageenan polymers in the random coil conformation exclusively. Unlike NaCl and $CaCl_2$ which lead to a progressive coil-helix transition without further association of the polymer chains, the presence of KCl triggers the formation of structures of higher hierarchical complexity, in agreement with previous works[4d,7a,8h]

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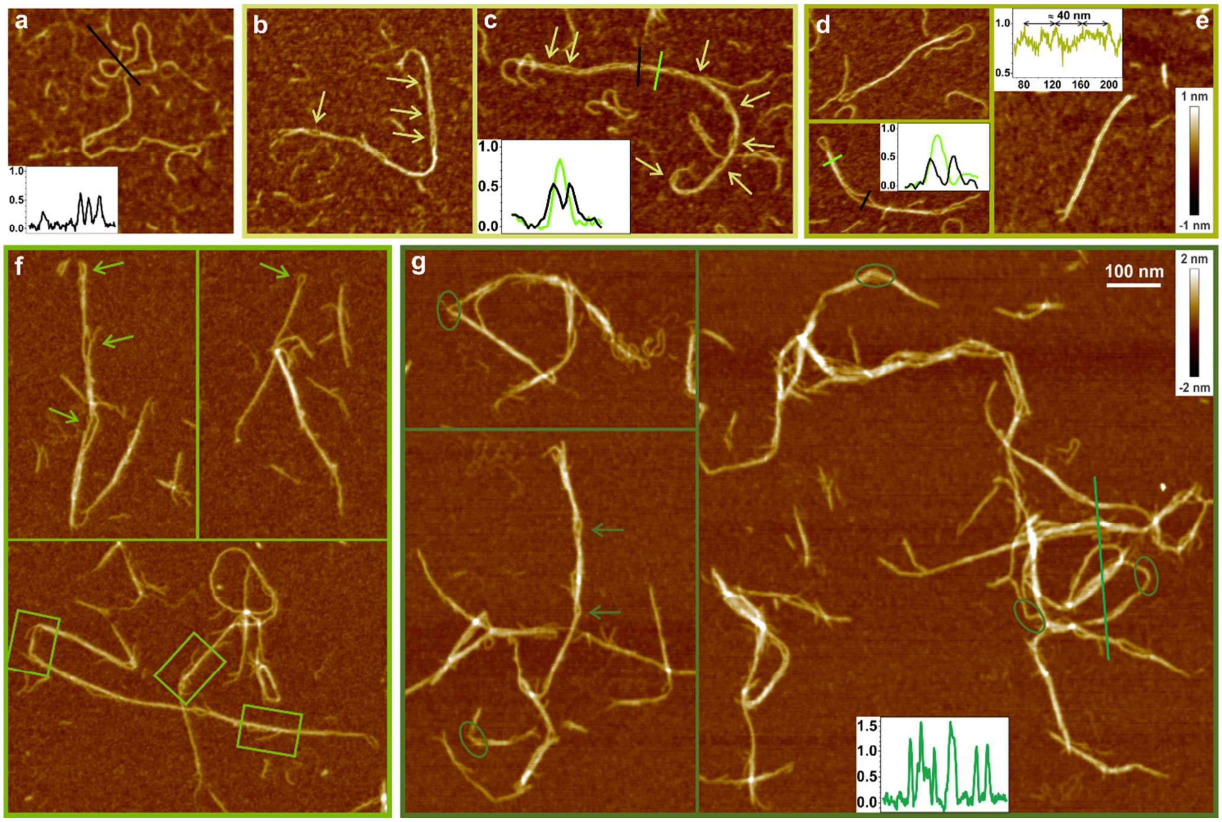

Fig. 2: High-resolution AFM height images depict the transient states of the supramolecular chiral self-assembly of kappa carrageenan in KCl environment starting from (a) single helix, to (b,c) loose twisting and (d,e) tight intertwining of two helical strands, which further assemble to (f,g) superstructures with structural features of bubbles (indicated by arrows), frayed ends (indicated by rectangles), and kinks (indicated by ellipses). The scale and color bars apply to all AFM images. The insets in (a,c,d,g) are cross-sectional profiles as indicated by the straight lines on the corresponding AFM images, whereas the inset in (e) shows the height profile along the contour length of the tight intertwined helical strands with a dominant periodic height fluctuation of 40 nm. The added salt concentrations are (a,b) 10, (c) 15, (d) 25, (e-f) 50, and (g) 100 mM KCl.

suggesting stronger effects on the structure for this salt. For kappa, parts of the polymer chains undergo coil-helix transition at added salt concentrations of as low as 0.1 mM KCl, with a transition prevailing above 50 mM KCl (Figure S3). The onset of the formation of strands occurs above 7 mM of KCl (Figure S3 and S5), which continue to self-assemble into multi-filament superstrands and network formation by increasing the salt concentration up to 150 mM of added KCl. It should be stressed at this point that while one structural feature is dominating at each ionic strength, mixed populations of intermediate states coexist. The assumption of a single equilibrium which previously led to the conclusion of a double helix as the fundamentally ordered state[4b] is therefore rebutted based on the present results. For iota carrageenan, individual chains begin to acquire secondary structures above 7 mM added KCl and concurrently self-assemble into flower-shaped clusters with single helical carrageenan loops emerging from a dense core (Figure 1(c) and Figure S4), a morphological feature which was also described for DNA[15] before. Initially these clusters are small and grow in number and size by assimilating additional chains in the helical conformation upon further addition of salt. These structures gradually dominate up to 150 mM of added KCl, when in addition also a few strand-like objects appear.

To gain insight into the structural features of the self-assembled superstructures triggered by the presence of KCl, we apply super high-resolution AFM imaging as an attempt to refine the aggregation mechanisms, which rule the sol-gel transitions.

Starting from kappa carrageenan in the random coil conformation of about 0.3 nm thickness, the addition of small quantities of KCl induces a conformational transition to intramolecular single helices of about 0.5 nm height (Figure 2(a)). The polymer chains appear to stiffen as a result of the coil-helix conformation, which is in agreement with estimates[8i] from intrinsic viscosity data. Above 7 mM added KCl, first strands of loosely twisted polymer chains in the helical conformation are observed (Figure S5), confirming the previously suggested[8c,e] coexistence of single helices and helical dimers at low K^+ contents. The presence of bubbles at one end of such strands, also called hairpin-loops,[11a] reflects an intramolecular dimerization process, in line with simulations[16] for semiflexible polyelectrolytes in ionic environments of higher valency, whereas splitting ends indicate an intermolecular assembly of two individual single helices (Figure S5-S8). These strands correspond to the previously reported[7c,8f] locally aggregated rigid bundles, however, instead of parallel alignment, a twisted configuration is resolved here. Sections of loose twisting span over

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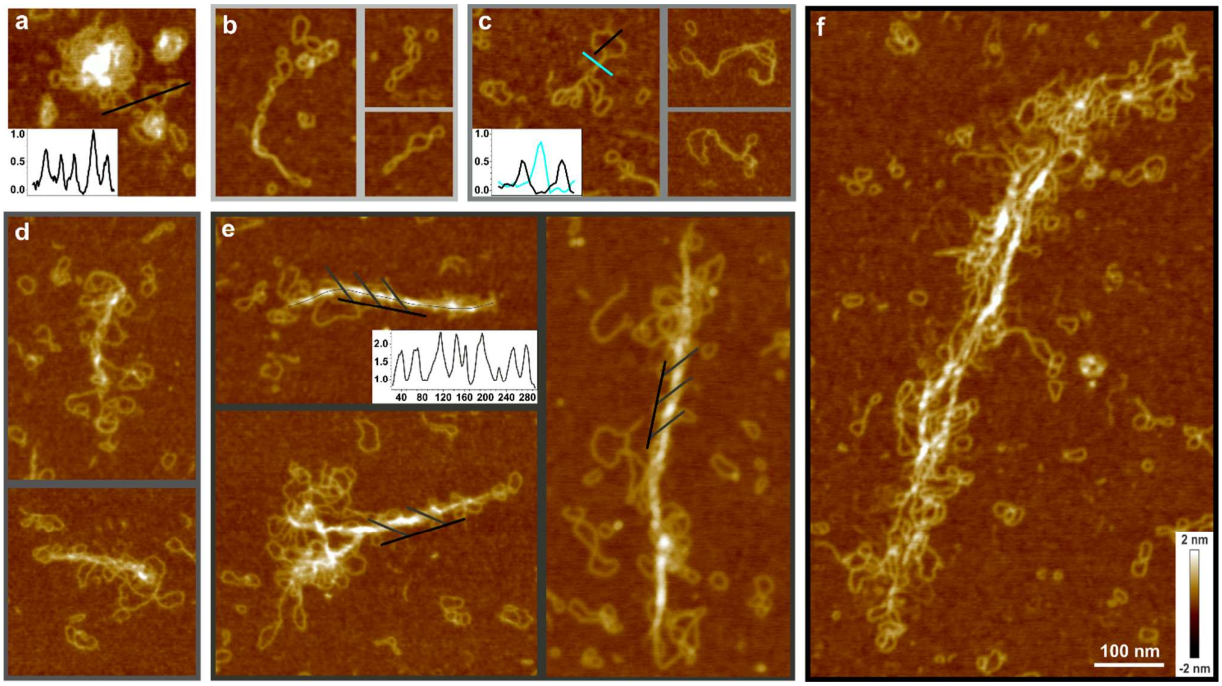

Fig. 3: High-resolution AFM height images illustrate the transient states of the supercoiling behavior of iota carrageenan in KCl environment starting from (a) polymer chains in the single helical conformation assembling in flower-shaped clusters, (b) loose and (c) tight intramolecular supercoiling, (d) intermediate pre-structures which further intertwine forming (e) supramolecular right-handed chiral (marked by tilt angles) superstrands, which (f) laterally expand toward network formation. The scale and color bars apply to all AFM images. The insets in (a,c) are cross-sectional profiles as indicated by the straight lines on the corresponding AFM images, whereas the inset in (e) shows the periodic height profile along the contour length of the right-handed chiral suprastructure. The added salt concentrations are (a) 150, (b) 250, (c-e) 500, and (f) 750 mM KCl.

several hundreds of nm, with alternating separated and crossed sections of about 0.5 and 0.8 nm thickness, respectively (Figure 2(b,c) and Figures S5-S7). The loose twisted helical strands intertwine tighter, forming longer and straighter sections of double helical configurations (Figure S7 and S8). Cross sectional analysis on bubbles (where the two single helices locally unwind) and on sections of tightly intertwined helices reveal again dimensions of about 0.5 nm for the single helices and 0.8 nm for the intertwined dimer (Figure 2(d)). Tracing[17] of height profiles along the tightly intertwined kappa dimers show periodic fluctuations with dominant signals at around 40 (Figure 2(e)) and 20 nm (Figure S9), respectively. Above 25 mM added KCl, the twisted strands assemble into superstrands consisting of several strands (Figure 2(f,g) and Figures S8-S11) and individual non-interacting polymer chains are no longer identified. This state reflects a transient state[8d] of strands presumably consisting of helical dimers, which further aggregate with increasing salt concentration. In contrast to these previously reported electron micrographs, however, the present high-resolution AFM images allow to identify structural features of hairpin-loops of intertwined dimers serving as building blocks for the superstructures, whereas bubbles occurring within a structure represent sections were the strands are locally separated (Figure 2(f,g) and Figures S8-S11 as indicated with arrows). In contrast to DNA where the double helix forms due to a perfect match of the respective base pairs, carrageenans do not feature such specific base pairing interactions between the two strands, hence sections of separated strands may be expected. The superstructures overall appear as rather stiff objects, in accordance with the aggregated superstrands[8d] on previously reported electron micrographs. The observation of kinks (Figure 2(f,g) and Figures S8-S11 as indicated with ellipses) supports a gelation mechanism via junction zones as suggested previously[8d] which would then stand as prerequisites for the formation of a percolating 3-dimensional gel. Frayed ends of helical tails (Figure 2(f) and Figures S8,S10,S11 as indicated with rectangles) which primarily appear together with the kinks, thereby serve as interaction sites for neighbouring superstructures, whose association eventually leads to gel network formation at physiological conditions of 150 mM added KCl (Figure S12). This points to network formation based on branching, in accordance with previous studies[7c,8g]. By analysing the nanomechanical properties of a kappa carrageenan network and thick film by peak force quantitative nanomechanical (PF-QNM) analysis in liquid conditions, a Young's modulus of about 26 MPa was estimated (Figure S13), which is similar to what was reported[18] for an iota carrageenan film by nanoindentation using force spectroscopy.

The self-assembly mechanism of iota carrageenan which differs from kappa in just an additional sulphate group per idealized disaccharide unit, is diverse when subjected to increasing concentrations of KCl. Initially, the chains undergo a coil-helix transition; however, the single helices of about 0.6 nm in thickness concurrently assemble into flower-shaped clusters (Figure 3(a) and Figure S14) similar to the folding pathway reported for DNA[15] in the

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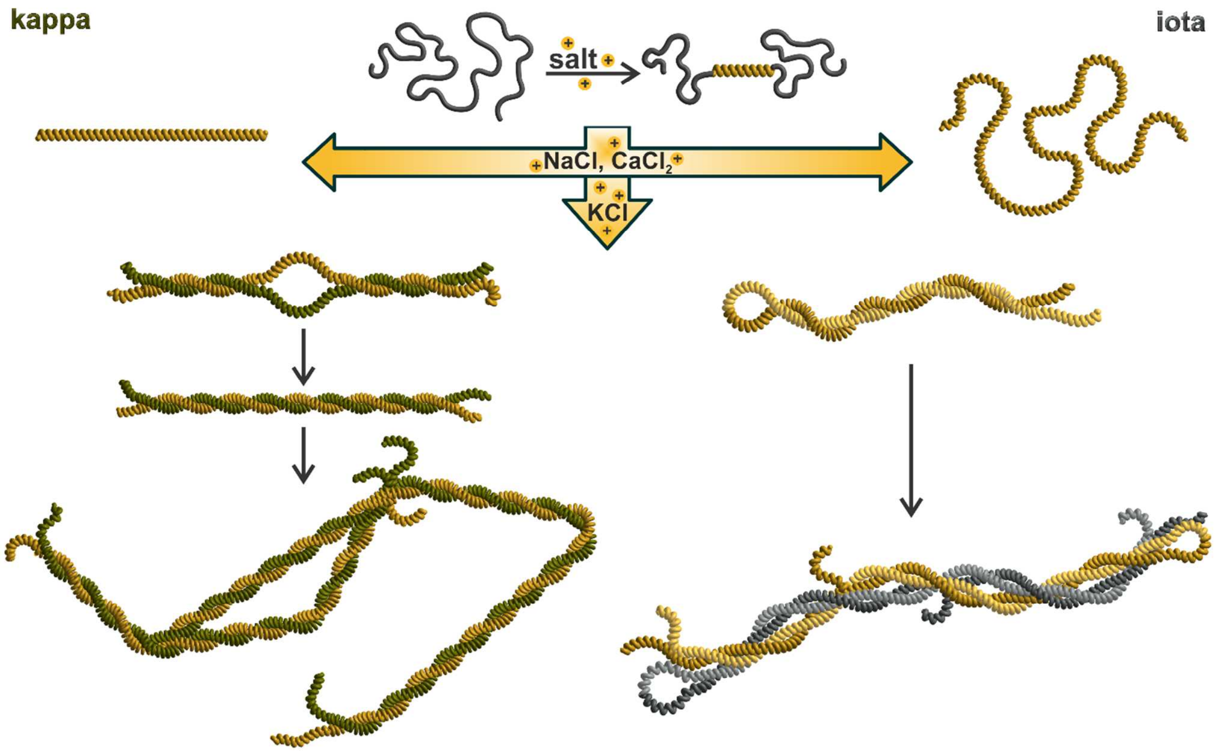

Fig. 4: Proposed ion-induced self-assembly mechanism of kappa and iota carrageenan in NaCl and CaCl$_2$ environment, where the polymers undergo a simple coil-helix transition with stiff and comparably flexible helical conformations for kappa and iota, respectively. In presence of KCl, the polymers in helical conformation intertwine or supercoil in the case of kappa and iota, respectively, resulting in supramolecular strands with a hierarchical chirality amplification.

presence of small molecules with multiple binding sites. Intramolecular supercoiling is observed upon further addition of KCl, which goes from loose- (Figure 3(b) and Figures S15 and S16) to tight-coiled (Figure 3(c) and Figures S15 and S16) single helical polymer chains at 250 and 500 mM added KCl, respectively. Supercoiling phenomena are generally known for their role in the packing of DNA[19] and were previously also reported for the schizophyllan polysaccharide[20]. Cross sectional analysis on locally unwound and tight coiled polymer strands reveal about 0.5 and 0.8 nm thicknesses for the single helices and the tight coiled sections involving two strands, respectively (Figure 3(c)). The observation of a single helical state before the chains supercoil is somewhat in contrast to thermal analysis data[9] where two consecutive cooperative conformational transitions from coil to double helices and further to dimers was reported. The fact that the first transition was observed only above 150 mM, the ionic strength at which we already observe a completed transition to single helices, might explain why the single helical state was missed before. The supercoiled iota helices, via intermediate pre-structures (Figure 3(d) and Figures S16 and S17), evolve to supramolecular multi-filament strands with periodic height fluctuations suggesting right-handed chirality (Figure 3(e) and Figures S16-S18). These iota superstrands, in contrast to the ones observed with kappa, are rather flexible and do not show any indication of kink formation. At even higher added KCl concentrations, these multi-filament structures widen by lateral aggregation above 750 mM KCl (Figure 3(f) and Figure S17), further driving the association into networks at 1000 mM KCl (Figure S19).

The diverse ion-induced self-assembly routes of kappa and iota carrageenan in NaCl, KCl and CaCl$_2$ are schematically summarized in Figure 4. NaCl and CaCl$_2$ induce a conformational transition from flexible random coils in the absence of external salt to rather stiff kappa and more flexible iota monomolecular single helices upon addition of salt. Previous simulations[21] have predicted a right-handed chirality and a pitch of 28 Å for these stable single helical conformations. With KCl, previously suggested as the salt having the strongest effect on carrageenan gelation among the ions studied,[4d,7a,8h] a refined self-assembly model is proposed with a fundamental building block of a single helix. These single helical structural units acquire higher ordered structures by undergoing transient states of loose twisting to tight intertwining of two structural units. Further association into superstructures possibly via highly selective K$^+$ binding sites[8a] reveals rather stiff kappa supramolecular objects, whereas iota features supercoiling (similar to the telephone cord) resulting in rather flexible chiral supramolecular multi-filament strands.

In summary, the ion-induced macromolecular conformational changes of anionic carrageenan polysaccharides in NaCl, KCl and CaCl$_2$ were visualized via high-resolution atomic force microscopy. The flexible polymers undergo a coil-helix transition with rather stiff kappa and comparably flexible iota intramolecular single helices presumably in a right-handed chirality[21], although this could not be resolved here at the single chain level. The onset of this transition

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occurs at lower salt concentrations for kappa compared to iota and $CaCl_2$ is more efficient than NaCl in inducing this transition. For the individual polymers, the transition for kappa follows $K^+ \approx Ca^{2+} > Na^+$, whereas for iota $Ca^{2+} > K^+ > Na^+$ is followed. In NaCl and $CaCl_2$ environments the polymers do not further associate, whereas in KCl the singles helices serve as the fundamental units which further self-assemble to supramolecular architectures with hierarchical complexity and amplification of chirality. The diverse self-assembly mechanisms ruling what becomes the sol-gel transition at higher concentrations, are refined by resolving transient states of twisting and supercoiling single helices forming helical dimers, which further associate through cation-mediated aggregation to supramolecular multi-filament structures of overall right-handed chirality, as resolved by super high resolution AFM imaging. These superstructures remain rather flexible for iota and stiff for kappa and are suggested as the underlying structural features resulting in elastic and brittle gel networks[7d], respectively.

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