Sodium ι-Carrageenan: A Paradigm of Polymorphism and Pseudopolymorphism

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ABSTRACT: X-ray fiber diffraction patterns of the sodium salt of ι-carrageenan correspond to three (I, II, and III) distinct packing arrangements of half-staggered, 3-fold, double helices having nearly the same helical repeat (*c*-axis 13.0−13.2 Å). While two of the three forms crystallize in trigonal nets with $a = 24.02$ for I and 21.8 Å for III, II favors an orthogonal net with $a = 13.70$ and $b = 20.08$ Å. Detailed structure analysis of I has shown that three helices in the unit cell, each pair at 13.9 Å apart, interact only through their 2-sulfate and 4-sulfate groups with the aid of sodium ions and ordered water molecules [Janaswamy, S.; Chandrasekaran, R. *Carbohydr. Res.* **2001**, *335*, 181−194]. The ability of sodium to induce two other crystalline structures, in which the helix−helix separations are considerably shorter (12.2 in II and 12.6 Å in III), suggests that interhelical interactions vary significantly among the polymorphs. These observations have ramifications to the practical applications of ι-carrageenan in food and pharmaceutical industries.

Introduction

Carrageenans belong to a special class among the extensively studied biopolymer systems. They represent a family of sulfated polysaccharides extracted from marine algae and have the ability to complex with other hydrocolloids and proteins. Some of them are used in the food industry as gelling agents. The basic structure of the polymer is a linear galactan backbone having a disaccharide repeat of →3)-β-D-galactopyranose-(1→4)-α−D-galactopyranose-(1→ along with variable amount of sulfation at different hydroxyl positions (O-2H, O-4H, and O-6H). Fifteen carrageenans, namely, κ, ι, λ, θ, μ, ν, ξ, α, β, ω, ο, π, γ, δ, and ψ, have been identified so far; κ, ι, θ, α, β, and ω contain a 3,6-anhydro bridge in the α-D-galactose residue. Five of them, κ, ι, λ, μ, and ν, are commonly found in most of the seaweed specimens; the rest are either rare or restricted to just one species or produced by chemical modifications. All the saccharides are in the $^4C_1$ conformation with the exception of $^1C_4$ when anhydro bridges are encountered. The latter induce kinks and greater flexibility to the polymer chain. Only κ-, ι-, and λ-carrageenans have been subjected to considerable rheological studies for understanding their physical behavior. They contain one, two, and three sulfate groups, respectively, per disaccharide repeat. Both κ- and ι-carrageenans form thermally reversible gels in aqueous solution upon heating and cooling, but λ-carrageenan is better known for its viscous nature.[1−3] These polymers have promising applications in the pharmaceutical industry due to their anticoagulant,[4] antitherapeutic,[5] antitumor,[6] and anti-HIV[7−9] activities. Also, reports suggest that regular inclusion of carrageenan in the human diet reduces blood cholesterol and lipid levels.[10]

Within the family, ι-carrageenan [disaccharide repeat →3)-β-D-Gal-4-SO_3^--(1→4)-3,6-anhydro-α-D-Gal-2-SO_3^--(1→] has been widely studied with regard to its gelation behavior in the presence of cations, and literature contains a plethora of important information. Being a polyanion, its charge density is fairly large (1.39) in the extended conformation.[11] There is a reversible transformation in solution from an ordered to a disordered state depending on temperature, polymer, and salt concentrations.[12−21] These results support double-helix formation by ι-carrageenan irrespective of cation type; however, the helix−cation binding nature is not the same; e.g., K^+ and Na^+ ions favor pair formation among the helices while NMe_4^+ has passive nonspecific binding.[13] The shear modulus (G') increases more quickly with divalent than monovalent ions, at the same polymer concentration.[22] Dynamic viscoelasticity measurements indicate a plastic behavior with Ca^{2+} ions in contrast to Newtonian and pseudoplastic nature with K^+ ions.[16,23] Nuclear magnetic resonance (NMR) data lean toward a selective binding for K^+ and Rb^+ cations.[24] But, it was concluded that cations alone are not the sole contributors for gelation based on subsequent multinuclear NMR data collected over a range of temperatures.[25] Interestingly, stress−strain plots of the gels as a function of temperature for K^+ and Na^+ have a linear behavior, but at low temperatures Ca^{2+} exhibits two regions with different slopes.[26] Thus, monovalent and divalent cations have distinct effects on the gelation properties of ι-carrageenan. This is further reinforced by differential scanning calorimetry studies that showed two consecutive cooperative conformational transitions upon heating of K^+ ι-carrageenan: the dissociation of dimers of double helices first and then the double helix-to-coil transition.[27,28] Similarly, photon transmission experiments point out an extra dimer-to-dimer transition in the presence of Ca^{2+} ions.[29,30] All these observations are congruent with the available small-angle X-ray scattering reports.[31−34]

The above physicochemical results connote that cations play a key role in the ι-carrageenan gelation mechanism, albeit a clear picture at the molecular level is yet to emerge. In this regard, we have undertaken a detailed X-ray structural investigation of the carrageenan structure and interactions in the presence of various cations. Our findings on the Na^+ and Ca^{2+} cationic forms of hydrated ι-carrageenan fibers in the solid state have revealed the quintessential participation of both 2- and 4-sulfate groups in the double helix−double helix association leading to junction zones. The two free hydroxyl groups (O-2H and O-6H) of β-D-galactosyl units are required for the

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double-helix stabilization through interchain O-6H···O-2 and O-2H···O-5 hydrogen bonds.[35,36] The fiber repeat is almost the same (~13 Å) in the sodium and calcium forms. This is equally true for the potassium, rubidium, and another calcium polymorph despite having larger basal nets.[37] Thus, in the crystalline state, ι-carrageenan exhibits diverse packing modes depending on the nature and concentration of the surrounding cations. In fact, there are three distinct crystalline arrangements in the case of sodium ions. The novel findings are described here.

Results

X-ray diffraction patterns recorded soon after stretching fibers corresponded to polymorph I. Equilibration of fibers for nearly 4 months in desiccator (75% RH) yielded polymorph III. Our experience with nucleic acids[38] and polysaccharides[39] illustrates that adding drops of salt solution while stretching the fiber sometimes helps to improve crystallinity. Polymorph II was detected through such trials. Representative patterns are shown in Figure 1a−c. The Bragg reflections on each layer line confirm that the fibers are uniaxially oriented and polycrystalline. The sharper reflections in polymorph I (Figure 1a) are qualitatively diagnostic of larger crystallites than in the other two. The meridional reflection on the third layer line assigns 3-fold helical symmetry to the polymer chain.[35] Nevertheless, there are intensity variations among the three patterns. For example, the profiles (generated using FiberFix[40]) on the equator (Figure 2) readily help to infer the contrasts. Further, the intensity distribution and peak positions confirm that the basal nets are not the same between the polymorphs.

For polymorph I, the foremost reflection observed at ξ (cylindrical radius in reciprocal space) = 0.0829 Å^{-1} is the strongest followed by a medium intense peak at 0.0976 Å^{-1}. However, the innermost reflection at 0.0475 Å^{-1} on the first layer line gives the clue about the actual unit cell dimensions. All the 44 reflections in the pattern (Figure 1a) index on a trigonal unit cell with $a = 24.02$ and $c = 12.96$ Å.

On the other hand, the first reflection observed at $\xi = 0.0817$ Å^{-1} for polymorph II is slightly broader than the rest and the second is medium intense at 0.0958 Å^{-1}. The reflections in Figure 1b could be initially indexed on a trigonal cell except for the one at 0.159 Å^{-1} (medium intensity, second arrow in Figure 2, middle panel). Upon further scrutiny, the first broader reflection (near the first arrow in Figure 2) was found to be a composite of two. When this was taken into account, all the 37 reflections are indexable on an orthorhombic (or monoclinic with $\gamma = 90°$) unit cell with $a = 13.70$, $b = 20.08$, and $c = 13.16$ Å.

Finally, the (100) reflection at $\xi = 0.0529$ Å^{-1} (first peak in Figure 2, top panel) is seen only in polymorph III. All the 37 reflections also index on a trigonal unit cell as in I, but with smaller dimensions: $a = 21.8$ and $c = 13.1$ Å. Thus, the basal net area of III has contracted by ~10% relative to I.

The unit cell volumes of I, II, and III are 6475.6, 3620.3, and 5391.6 Å^3, respectively. Structure analysis of polymorph I[35] shows that the unit cell accommodates three double helices (half a turn each), and in the basal net projection the helix looks like a triangle with sulfate groups on the edges. Neighboring helices are too far (13.9 Å apart) to have any direct attractive interactions, but strategically located sodium ions and water molecules snugly connect them via their peripheral 2-sulfate and 4-sulfate groups. Along similar lines, three double helices can pass through the unit cell of III, but only two in the case of II. However, the helices in II and III are drawn closer by 1.7 and 1.3 Å, respectively, relative to I. The nominal change (~0.1 Å) in the *c*-repeat and significantly different basal nets suggest that the core structure is virtually the same, but not the packing mode. While polymorph III has the same space group ($P3_1$) as I, the packing of II is compatible with $P2_1$, the screw along the *a*- or *b*-axis. Even though any systematic study as a function of relative humidity is yet to be conducted, humidity could as well impact the ι-carrageenan assembly.

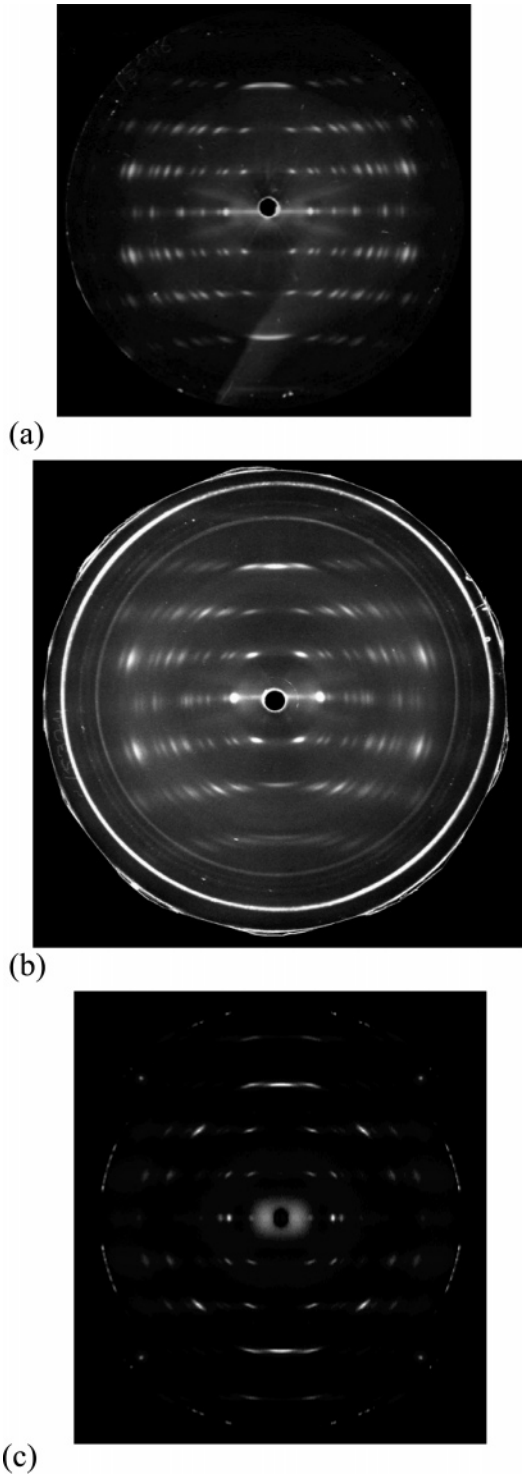

Figure 1. X-ray diffraction patterns from well-oriented and polycrystalline fibers of sodium salt of ι-carrageenan: (a, b) from in-house generator and (c) using synchrotron radiation.

Thus, the two salient features (i) occurrence of (100) reflection only in polymorph III and (ii) change from a higher 3-fold trigonal to a lower 2-fold orthorhombic (or monoclinic with $\gamma = 90°$) lattice symmetry in II imply that the ways in which the

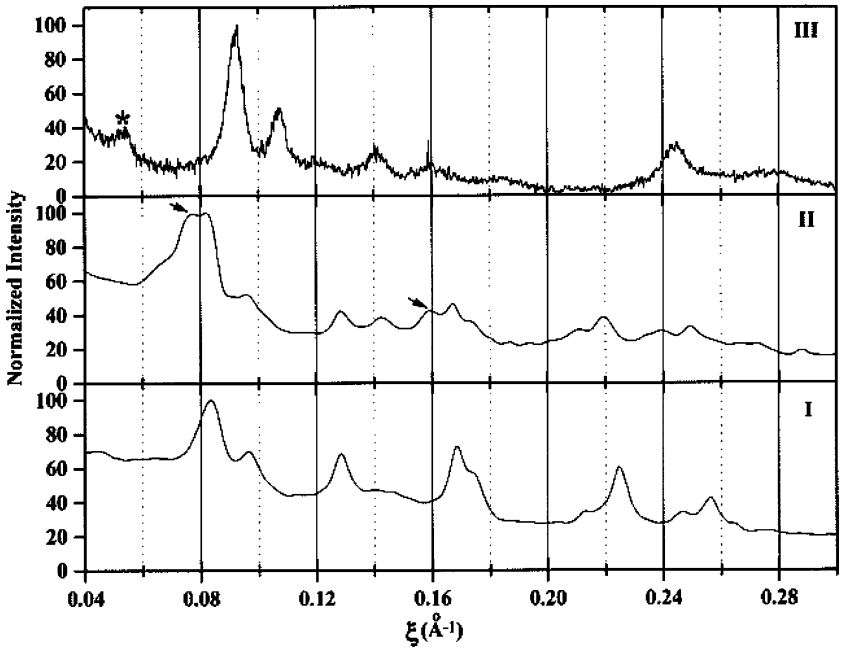

Figure 2. Intensity distribution on the equator in polymorphs I, II, and III. The arrows in II indicate the two reflections violating trigonal lattice symmetry. The star in III represents a new reflection observed only in this polymorph.

helices associate in these polymorphs are not the same. Although the complete structural details of II and III are not available at this stage (work in progress), it can be inferred from the above observations that the packing strengths would increase from I to III to II because of progressive decrease in the interhelical separation from 13.9 to 12.6 to 12.2 Å, in that sequence.

Discussion

Polymorphism and Pseudopolymorphism. Polymorphism is a solid-state phenomenon of a compound that adopts two or more molecular structures and/or packing arrangements.[41−44] This behavior has been amply documented in many organic, inorganic, and drug molecules.[45−59] The details aid to develop reliable computational techniques for predicting crystal structures. This is particularly important in the pharmaceutical industry wherein the reproducibility of a given polymorph is essential due to structurally contingent physical and related properties. Incidentally, biopolymers are not exceptions and a variety of possible packings are known for proteins,[60,61] nucleic acids,[62,63] and carbohydrates.[64] Dermatan sulfate,[65] chondroitin 4-sulfate,[66,67] hyaluronan,[64] cellulose,[68,69] chitin,[70,71] starch,[72] resistant starch type III,[73] and ι-carrageenan[37] are proven examples of polymorphic polysaccharides. In contrast, pseudopolymorphs are solvated forms of compounds that take up dissimilar crystalline structures and differ merely in the nature and amount of included solute and solvent.[74,75] In recent years, pseudopolymorphic nature has been repeatedly noticed in several small molecules.[76−82] Now we have evidence that a complex polysaccharide such as ι-carrageenan is not any exception. Despite its familiarity in the chemical literature for a very long time, the usage of the term pseudopolymorph or pseudopolymorphism is still under debate.[83−86]

Why ι-Carrageenan Is Vulnerable to Polymorphism and Pseudopolymorphism? The clues can be inferred from the molecular morphology of polymorph I, a robust half-staggered double-helix core with peripheral charged moieties.[35] In each chain, across the (1→3)-linkage, the 4-sulfate group (4-S) is rotated by −36° around the helix axis and translated by 3.6 Å from the 2-sulfate group (2-S); similarly, across the (1→4)-linkage, the 2-S group is rotated by 156° and translated by 5.0 Å from the 4-S group. Thus, the successive charged groups are unevenly positioned (5.0 and 11.4 Å apart) on the surface (Figure 3a). Nevertheless, half-staggering between the two chains in the double helix brings the 2-S group of one chain to almost the same *z*-level as the 4-S group of the other. This interdigitation leads to a contiguous arrangement of 2-S (chain 1), 4-S (chain 2), 2-S (chain 2), and 4-S (chain 1) along the helix surface (Figure 3b). Consequently, the sulfate groups are arranged in a left-handed helical array with distances alternating between 7.1 and 5.0 Å. Thus, the ι-carrageenan core is wrapped by negative charges. Hence, helix−helix association is hindered by the strong electrostatic repulsion but made possible by

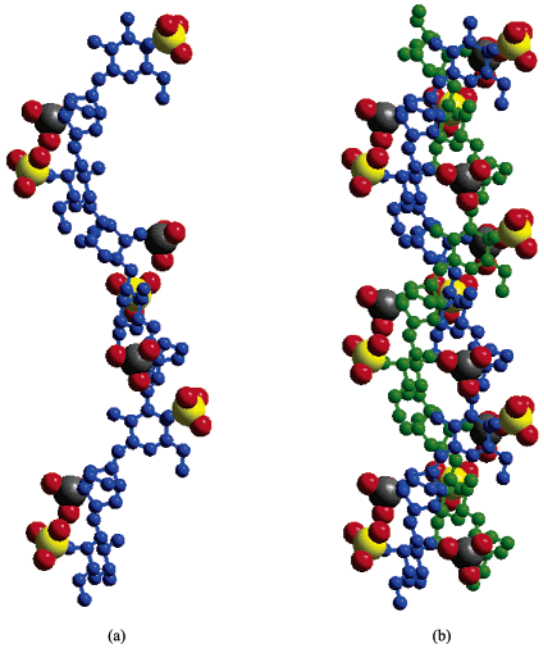

Figure 3. Nearly one and a half turns of ι-carrageenan: (a) one chain and (b) double helix. The yellow, charcoal, and red atoms represent the 4-S, 2-S, and sulfate group oxygen atoms, respectively. Hydrogen atoms are not shown for clarity.

neutralizing the charges with cations often aided by ordered water molecules. In the case of polymorph I,[35] two sodium ions (Na) and four water molecules (*W*) per disaccharide repeat are responsible for as many as 10 types of interhelical interactions: 4-S···*W*/Na···4-S, 2-S···*W*···*W*···*W*···4-S, 2-S···Na···2-S, 2-S···Na···4-S, 2-S···Na···*W*···2-S, 4-S···*W*···*W*···4-S, 4-S···Na···*W*···*W*···4-S, 2-S···*W*···Na···4-S, 4-S···Na···*W*···Na···4-S, and 2-S···*W*···*W*···*W*···4-S. Thus, the guest molecules are acting as space fillers in the gaps between helices through ionic interactions and hydrogen bonds (···) promoting junction zones. The bridge size is anywhere from one to four spacers; the shorter the bridge, the stronger the association. Any change in the number of ordered water molecules or sodium content relentlessly perturbs the bridges, leading to alternate packing sequences. It is equally possible that even a subtle translation along, and rotation about, the helix axis of the double helices could influence and revise the inter-helix−helix association. Such factors are the major contributors for the formation of polymorphs II and III.

Above all, the oxygen atoms in the sulfate group are two bonds away from the helix core pyranosyl unit, giving rise to two single-bond rotations ($C_{n-1}-C_n-O_n-S_n-O_S$). As a result, the charged groups can swing around and thus are extended farther away from the helix surface. This extravagancy is a major drive for the sulfated polysaccharides to adopt a variety of stable tertiary structures.[37,65−67] In the case of ι-carrageenan, as each monosaccharide carries one sulfate moiety, this effect will be quite pronounced. Nonetheless, it has the unique capacity to propagate the variable conformations to a substantial extent laterally, enabling it to crystallize in dissimilar unit cells while retaining the core structure. All these facts establish a new paradigm for understanding the causative effect of polymorphism and pseudopolymorphism in ι-carrageenan.

Quoting McCrone[87] "every compound has different polymorphic forms and that, in general, the number of forms known for a given compound is proportional to the time and money spent in research on that compound". It is proven for small drug molecules that polymorphism/pseudopolymorphism can be controlled and desired phases obtained by using cocrystals.[88] Now, ι-carrageenan is a perfect example to be explored further since polymorphic drugs mixed with carrageenans have shown promising results.[89] We strongly believe that by using suitable cosolutes the artifacts of pseudopolymorphism and polymorphism can be efficiently avoided for better practical applications of ι-carrageenan, importantly as an anti-HIV carrier.

Experimental Section

Fiber Preparation. A pure sample of ι-carrageenan (MW ∼ 600 kDa) was provided by FMC Corp.. Its composition was 87% carbohydrate, 8% moisture, and 5% salt. It was converted into the sodium salt form as follows: 40 mg of the sample and 60 mg of NaCl were dissolved in 10 mL of distilled water (about 10 sodium ions per disaccharide). The solution was heated to and kept at 100 °C with constant stirring for about 1 h. The polysaccharide was then precipitated with two volumes of 2-propanol at 0 °C. The precipitate was rinsed successively in 80 and 100% 2-propanol to remove any unbound sodium ions and then washed thoroughly with 100% acetone and vacuum-dried. A concentrated aqueous solution of this material was subsequently prepared, and in the first set of experiments, a few drops were placed in between the beaded ends of the two glass rods in a fiber puller kept at 75% RH. Oriented fibers 3−5 mm long were obtained after stretching the semidried solution for about 2 h at regular intervals. In a second procedure, a few NaCl (0.01 M) droplets were also added during the drying stage. Each polymorph was revalidated in at least 2−3 fibers.

X-ray Patterns. In-house diffraction patterns were recorded on flat photographic films in pinhole cameras using Ni-filtered Cu Kα radiation ($\lambda = 1.5418$ Å). The exposure lasted up to 2 days, and the generator was operated at 40 kV and 6 mA. A steady stream of helium gas, previously bubbled through a saturated salt solution, was continuously flushed through the specimen chamber to minimize air scattering and maintain 75% RH. Calcite powder (characteristic spacing 3.035 Å) was used for internal calibration. Positions of reflections were obtained by using a Stoe measuring device. Synchrotron data were collected at the Argonne National Laboratory (ANL), Chicago, using 1 Å radiation from fibers previously stored at 75% RH. The data were recorded on a CCD with 8 s exposure, and no appreciable radiation damage was detected. The FiberFix[40] version 1.2 from the CCP13 suite was utilized for processing the data and obtaining the reflection positions. All these measurements were employed in calculating the pattern center, fiber rotation and tilt, and finally the distance of each reflection from the origin of the reciprocal lattice (ρ). The relationship involving the lateral ξ and vertical ζ components of ρ is given by $\rho^2 = \xi^2 + \zeta^2$, where $\zeta = lc^*$; $\xi = a^*(h^2 + k^2 + hk)^{1/2}$ for the trigonal system, and $\xi = (a^{*2}h^2 + b^{*2}k^2)^{1/2}$ for orthorhombic system. The Miller indices (h, k, l) for each reflection and reciprocal unit cell (a^*, b^*, and c^*) dimensions were estimated using in-house programs.

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