Reexamining the Egg-Box Model in Calcium−Alginate Gels with X-ray Diffraction

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The structure of the Ca−alginate junction zones was investigated with X-ray scattering on gels prepared with different methods. Fiber diffraction reveals the popular egg-box model may not be the only possible structure for the junction zones. The (001) reflection, which should be extinguished due to 2/1 helical conformation in the egg-box model, was observed. This was further confirmed by the measurements on Ca−alginate gel beads prepared at different pH where large pieces were formed through a relatively slow process, which leads to a higher crystallinity and a more perfect ordering. The results suggest a 3/1 helical conformation is more proper for Ca−alginate gels formed slowly. This does not exclude the possibility for the 2/1 helical conformation in fast gelatinized Ca−alginate in which the 2/1 helix is a metastable form. Comparing the X-ray scattering results of the fresh, dehydrated, and rehydrated gels, a reversible aggregation of junction zones is found during dehydration and rehydration. The different stabilities of initial bonds and bonds formed during drying are speculated to be the contribution of MG block or short G blocks in the junction zones.

Introduction

Alginate is a linear copolysaccharide isolated from brown seaweeds and certain bacteria. Chemically it is a (1−4)-linked block copolymer of β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G), with residues arranged in homopolymeric sequences of both types and in regions which approximate to the disaccharide repeating structure (MG).[1,2] Commercially alginate is widely used as a gelling agent not only in foods but also in other industries such as pharmaceutical, biomedical, and personal care.[3] As it is easy to prepare alginate ionotropic gels at mild conditions, it is possible to entrap living cells in alginate gels, which allow a wide application of alginate as scaffolds for tissue engineering, drug delivery systems, and cell encapsulation and transplantation.[4,5] All these properties and applications are ultimately dependent on the molecular architecture and gelling mechanism.

Though a general picture for the gelation of alginate was identified for more than 30 years,[6,7] a continuous revision and improvement has been carried out based on new experimental evidence. Numerous physicochemical studies have been performed in order to obtain information regarding the mechanisms and the structural features involved in the gelation process. Morris et al.[6,7] have shown that calcium ions induce chain−chain association and proposed a model for the junction zone, popularly known as the "egg-box model" (Figure 1). The egg-box model is based on X-ray fiber diffraction and dialysis of Ca^{2+} ion.[6−10] However because calcium alginate fibers give a very poor diffraction pattern, it is impossible to deduce the crystal structure.[8−10] The 2/1 zigzag conformation in the egg-box model was deduced from the similarity of acid gel, which gives a clear scattering pattern corresponding to 2/1 helical conformation.[9,10] The dialysis experiment performed by Morris and Rees showed that a ratio of 4:1 between G units and Ca^{2+} ions exist in Ca−alginate gel, which fits well with the egg-box model (dimer). Nevertheless, the dialysis result is an indirect evidence for the structure model, and the egg-box model may not be the only possible interpretation. Though the egg-box model is popularly used by many researchers in this field, it has been questioned by many authors. On the basis of calculated free energy, a parallel and an antiparallel model seem more stable than the egg-box model.[11,12] Recently Donati et al.[13] suggested that not only G blocks but also random GM blocks contribute to the junction zone. The egg-box model with a dimer structure is not supported by the in situ observation of small-angle X-ray scattering during gelation of Ca−alginate.[14,15] Even at the beginning of gelling, the width of the junction zone is already much larger than that for the egg-box dimer.

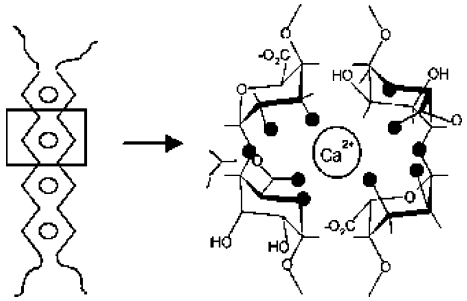

Figure 1. Schematic picture illustrating the egg-box model for Ca−alginate, as proposed by Morris et al.[6,7]

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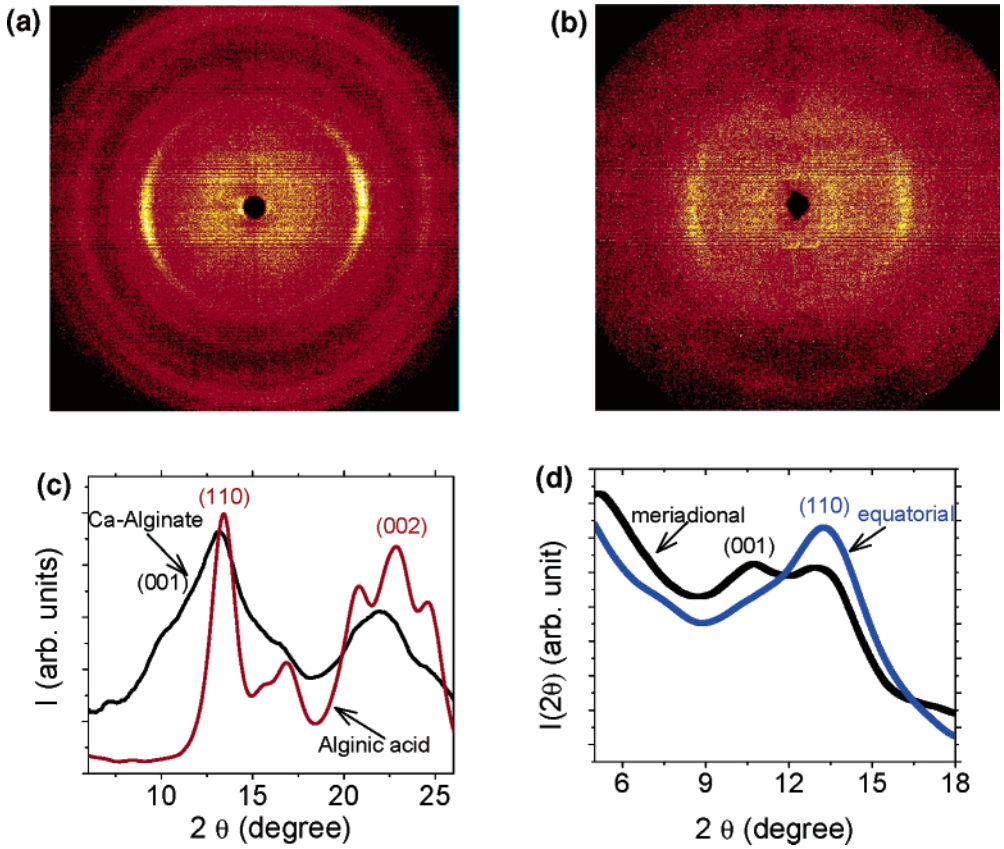

Figure 2. Two-dimensional X-ray fiber diffraction patterns for dried alginic acid (a) and Ca−alginate gels (b). Fiber axes are in the vertical direction. (c) One-dimensional X-ray diffraction patterns obtained through integration of the patterns in panels a and b, respectively. (d) Integrated one-dimensional intensity curves from the pattern in panel b in meridional and equatorial sections, respectively.

In this work, we revisited the conformational structure of the junction zone with X-ray scattering. New developed X-ray instruments and slow gelling processes are expected to provide better structural information about the junction zones of Ca−alginate gels. Fiber diffraction showed that the junction zones may have a 3/1 helix conformation rather than the 2/1 zigzag structure described in the egg-box model. This structure is further confirmed by the X-ray measurements on fresh, dehydrated, and rehydrated Ca−alginate gels.

Experimental Section

Sodium alginate with ~70% guluronic residues was purchased from FMC. A total of 1 wt % of sodium alginate was dissolved in Millipore filtered water at 80 °C. Calcium alginate and alginate acid fibers were prepared through extruding sodium alginate solution into 1 wt % $CaCl_2$ (pH = 7) and HCl (pH = 2) solutions, respectively. The fibers were immediately taken out and dried under tension in a humidity controlled cabinet. In order to obtain high crystallinity, the fibers were annealed in high humidity at 75 °C for 48 h.

Ca−alginate fibers have a low crystallinity and microcrystals due to the fast gelation process, although an annealing process is added. In order to obtain junction zones with higher perfection and crystallinity, a Ca^{2+} ion diffusion controlled gelation process, as developed by Skjak Braek et al.,[16] was adopted in this work. Briefly, sodium alginate solution was filled in a cylindrical plastic bottle capped with a membrane. The bottle was placed in a 1 wt % $CaCl_2$ aqueous solution (pH = 7) to allow Ca^{2+} ions to diffuse through the membrane and induce the formation of Ca−alginate gel.

Ca−alginate beads with a diameter of ~5 mm were prepared through dropping 1 wt % sodium alginate solution in 1 wt % $CaCl_2$ aqueous solutions at different pH values. After keeping them in the $CaCl_2$ solutions for more than 5 h to reach equilibrium, the Ca−alginate beads were fished out and dried in a humidity controlled cabinet (RH = 33% and 25 °C).

The structure of the junction zone was investigated with X-ray diffraction on a Bruker D8 Discovery equipped with a Cu Kα source with wavelength of 0.154 nm and a two-dimensional gas-filled multiwire detector. The two-dimensional scattering patterns were integrated along the azimuthal angle into one-dimensional intensity curves, which were displayed as function of 2θ. Here θ is the scattering angle. All measurements were carried out in a transmission geometry. In order to obtain enough signals, tissue papers were used to remove excess water from fresh and rehydrated Ca−alginate gels and the concentration of polymer was increased from 1% to about 10%.

Results

X-ray Diffraction on Dried Fibers. Panels a and b of Figure 2show two-dimensional X-ray diffraction patterns from alginic (Guluronatic) acid and Ca−alginate dried fibers, respectively. Both patterns are very similar to those reported by Mackie et al.[10] Alginic acid fibers give sharp diffraction peaks, which indicate a high crystallinity and a more perfect ordering. On the other hand, Ca−alginate fibers only have broad diffraction peaks, suggesting defective crystals and a low crystallinity. In order to have a close examination of the structure, the two diffraction patterns were integrated along the azimuthal angle to obtain one-dimensional patterns, which is displayed in Figure 2c. Following Atkins's[9] assignment, we labeled the Miller index for the diffraction peaks of alginate acid. Note here the fiber axis is defined as the *c*-axis of the lattice, which is also applied to Ca−alginate gels. The two main peaks (110) and (002) are at 2θ of ~13.4° and ~22.9°, which correspond to *d*-spacing of

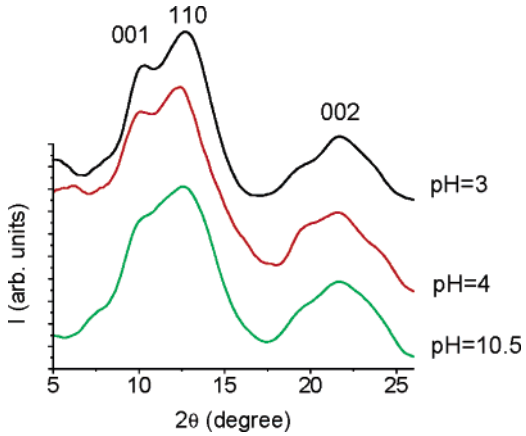

Figure 3. One-dimensional X-ray diffraction intensity curves of dried Ca−alginate beads prepared in 1 wt % $CaCl_2$ aqueous solution with different pH values.

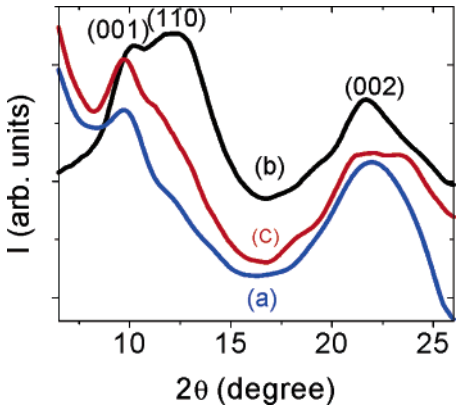

Figure 4. One-dimensional X-ray diffraction intensity curves for Ca−alginate pieces: (a) fresh, (b) dried, and (c) rehydrated.

0.69 and 0.39 nm, respectively. The *d*-spacing of the (002) plane is much smaller than the value (0.43 nm) reported from Atkins et al.[9] and Mackie et al.[10] This difference is greater than normal experimental error. Indeed we repeated the measurements on another setup and with different alginates, and the results are the same. Though the difference in the *d*-space of the (002) plane is observed, the 2/1 helix of alginic acid is confirmed by the absence of the (001) reflection.

Compared with alginic acid, all reflections of Ca−alginate fibers are rather diffusive, which makes it difficult to elucidate the structure. Nevertheless, even without any detailed structure interpretation, the result shows that the scattering peak at ∼13° corresponds to the lateral packing among molecular chains, and the peak at ∼21.5° is from the layer spacing along the molecular chain direction. Using the initial egg box model, we index the peak at ∼13° as (110) and the peak at ∼21.5° as (002). Additionally, a small shoulder is observed at the 2θ of about 10.6°. Taking the value of the (002) peak in Figure 2c, the shoulder at the 2θ of about 10.6° corresponds to the (001) position. This cannot fit into the egg-box model due to the extinction of the (001) reflection from the 2/1 helical conformation. To check whether the shoulder is on the meridional direction or not, we integrated the scattering pattern (Figure 2b) in equatorial (azimuthal angle from 45° to 135°) and meridional (azimuthal angle from 135° to 215°) sections separately, which is displayed in Figure 2d. Evidently, the peak at 10.6° locates in the meridional direction in the fiber diffraction pattern, which confirms the assignment to the (001) plane. This assignment leads to a 3/1 helical conformation for the cross-link of Ca−alginate rather than the egg-box model with the 2/1 helical conformation.

Effect of pH. Figure 3 shows one-dimensional X-ray diffraction patterns of dried Ca−alginate beads prepared in solutions with different pH values. Different pH values do not affect the junction zones significantly and result in similar diffraction patterns. Compared to fibers, Ca−alginate beads give sharper diffraction peaks due to larger junction zones, which give (001) and (110) reflections well separated from each other. This further confirms the fiber diffraction result and strongly suggests a 3/1 helix is more plausible for the junction zones of Ca−alginate gels.

Effect of Dehydration and Rehydration. As the X-ray diffraction was performed on dry Ca−alginate fibers and beads, the 3/1 helix might form during drying. In order to check this, X-ray scattering was carried out on fresh wet samples, which were gelled through slow diffusion to form a more perfectly ordered junction zone. Figure 4a shows the wide-angle X-ray diffraction pattern of the fresh alginate gel, which as mentioned before had excess water removed to increase the concentration of polymer. For comparison, X-ray scattering measurements were also carried out on dried (b) and rehydrated gels (c), which are presented in Figure 4. The (001) reflection clearly appears on the scattering pattern of the fresh Ca−alginate gels and supports that the 3/1 helical conformation forms during initial gelation rather than during drying. Interestingly, the (110) reflection in the fresh gels is very weak, which suggests that the number and the lateral size of the junction zones are small.

The scattering measurements on the dried gels revealed that the 3/1 helical conformation is preserved after drying. Additionally there is evidence that the junction zones grow during drying, which is indicated by the increase in the intensity of the (110) reflection (Figure 4b). The dried Ca−alginate gels were rehydrated by placing them in an aqueous NaCl (1 wt %) solution. Surprisingly, the rehydrated Ca−alginate gels gave a scattering pattern almost identical to that of the fresh one (Figure 4c), suggesting that the dehydration and rehydration is a reversible process. The (001) and (002) reflections again survive after rehydration, while the intensity of the (110) peak reduces to a very low level, indicating junction zones reducing back in size.

Discussion

On the basis of our X-ray scattering measurements, clearly the (001) reflections are not extinguished, which supports a 3/1 helix conformation of alginate chains rather than the 2/1 helix promoted in the egg-box model. It is surprising to observe such a big deviation from the initial model, as the egg-box model has been widely used in the past 30 years.[6,7] Let us first look at what are the evidences of the egg-box model. As stated in the introduction, the fiber diffraction of Ca−alginate should be essential evidence. However, due to a low crystallinity, small lateral size, and defective junction zones, the diffraction patterns from dried Ca−alginate fibers are always rather poor, which makes it rather difficult to give a conclusive interpretation.[6,10] In fact, Mackie et al.[10] initially made a very cautious statement: "it is *unlikely* that the conformation of Ca−alginate deviated from the 2//1 helical conformation in acid and potassium form". This suggests that perhaps there should be another explanation for the data. To support the egg-box model, especially the dimer, Morris et al.[7] did the dialysis with different Ca^{2+} and Na^{+} concentrations. A transition was observed at a ratio of 4:1 between guluronic resides in G blocks and Ca^{2+} ions, which fits well with the dimer egg-box model. However, this did not take into account the MG blocks and the different

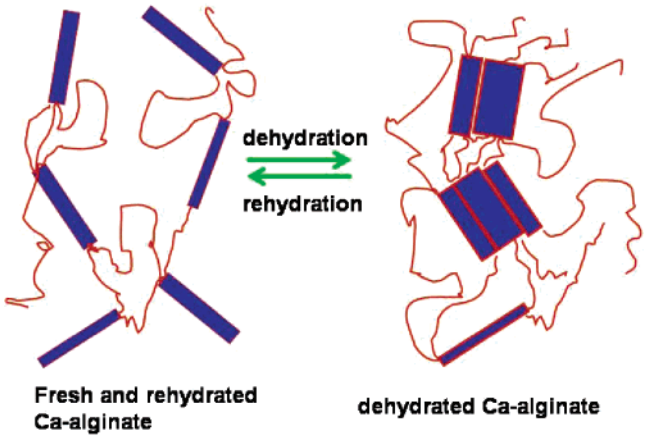

Figure 5. Schematic model illustrating the evolution of the junction zones in Ca−alginate gels during dehydration and rehydration.

lengths of G blocks,[13] indicating the dimer egg-box model is not the only solution of their observation. Recent SAXS measurements showed that a rather large width of the junction zones formed even at initial gelation of Ca−alginate.[14,15] The contribution of the MG block on the gel strength suggests MG blocks may also bind with Ca^{2+} ions and contribute to the junction zone. Moreover, even if a dimer does exist, it is not necessary that it is the 2/1 helical form constructed in the zigzag pattern. Simulations have revealed that the egg-box is energetically metastable.[11,12]

In fact, the fiber diffraction from Mackie et al.[10] already showed that a peak existed at the (001) position (see Figure 5e in reference 10). However, the authors did not analyze the data in detail at that time, which may be hindered by the techniques at that moment. Without good resolution and the separate integration in the meridional and the equatorial directions by modern detection and analysis techniques, it may have easily been accepted that the reflection at the (001) position is due to the scattering from the (110) plane, as these two reflections are close to each other and the intensity of (001) is relatively low. It is perhaps not so unnatural to accept the 3/1 helical conformation for Ca−alginate, since Ca−pectin, a close cousin in the ionic polysaccharide family, has a 3/1 helix conformation.[17]

Comparing the intensities of (001) and (002) reflections from fiber diffraction, in some cases the first-order peak (001) is weak. This suggests that the 2/1 helical conformation may not be completely ruled out in Ca−alginate gels, and the 3/1 and 2/1 helical conformation may coexist. The rate of Ca−alginate gelation has significant influence on the relative intensity between the (001) and the (002) reflections. A slow gelation process such as the diffusion controlled process gives a higher intensity of the (001) reflection, which fits better to a 3/1 helical conformation rather than the egg-box model. A fast gelation like spinning thin fibers in $CaCl_2$ solution leads to a weak (001) peak, which seems close to the 2/1 helical conformation. This difference coincides with the metastable nature of the egg-box model, as shown by simulation.[11,12] A fast gelation process creates small and defective junction zones, which are kinetically trapped in the metastable phase. Once the gelation takes place, rearrangement in and among Ca−alginate junction zones through annealing is much more difficult than alginic acid and other alginate salts (see Figure 2c and Figure 4).[10] The egg-box model has primarily been derived from X-ray measurements made on dried Ca−alginate fibers prepared through fast gelation. However, as indicated earlier, this model may not be applicable to alginate gels that have been made via slow gelation through either diffusion control or other methods to prepare homogeneous gels.[16]

The structural evolution during dehydration and rehydration is extremely important for controlled release and other encapsulation involving this process.[3] Unlike normal chemically cross-linked synthetic polymer gels, where no structural change occurs to the cross-links during dehydration and rehydration (or swelling), the junction zone of Ca−alginate are physical cross-links of nanocrystals. As such structural evolution of the junction zones is expected to happen through crystallization and dissolution during dehydration and rehydration, respectively. This process can be revealed through comparing the diffraction patterns of the fresh, dehydrated, and rehydrated gels in NaCl solution, as shown in Figure 4. The low reflection intensity of the (110) plane for the fresh Ca−alginate gels indicates that the junction zones are small in the lateral direction, while relatively high intensities of the (001) and (002) reflections correspond to a large size in the molecular chain direction. This picture is in agreement with the general model for the junction zone[18] and the broken-rod model from small-angle X-ray measurements[14,15] (see Figure 5). Upon dehydrating, the (110) reflection increases more significantly than the reflections from the planes in the molecular chain direction (Figure 4a,b). The intensity increase of the (110) reflection can originate either from the number and/or the lateral size of the junction zones. If the number of junction zones increase and the sizes remain constant, we would expect the intensities of all reflections to increase proportionally, which is not supported by the experimental observation. The disproportional increase of the reflection intensities can be attributed to the aggregation of junction zones (see Figure 5b). This process allows a large increase of the lateral size of the junction zones ((110) reflection) and keeps the size in the molecular direction unchanged (the (001) and (002) reflections).

The similar scattering pattern of the fresh and the rehydrated Ca−alginate gels suggests that the aggregation of junction zones is mostly reversible during dehydration and rehydration (Figure 4). After rehydration, the reduction of the (110) and the surviving of the (001) and (002) reflections indicate that the new interactions in the junction zones formed during dehydration are weaker than that created during the initial gelation. This process is surprisingly similar to that proposed for the aggregation of the dimers in the egg-box model, though the actual process may involve larger units rather than the dimers. Though the exact origins for the different stabilities of the bonds formed initially and the one formed during dry are unclear at this moment, we speculate two possible mechanisms. One may be due to the distribution of the G block length. Long blocks may form junctions in the initial gelation process, while with increasing Ca^{2+} ion concentration during drying, short G blocks may join in later. The second mechanism may involve the GM blocks. As showed by Skjak-Braek et al.,[13] GM blocks do contribute to the junction zones, which are assumed to bind at the edge of the junction zones due to a defective structure.[13] If aggregation occurs during drying, the association of the defective boundaries of the junction zones will certainly lead to a less stable bonding, which will be naturally disassembled during rehydation.

Conclusion

Based on the X-ray diffraction results on Ca−alginate gels prepared with different methods to control the gelation kinetics, a 3/1 helical conformation is proposed for the junction zones.

The existence of the (001) reflection, which should be absent due to 2/1 helix in the egg-box model, provides direct evidence for the 3/1 helical conformation. For large gel pieces or beads, the slow gelation process results in junction zones with higher perfection, which leads to sharp reflections. This observation gives an unambiguous support to the 3/1 helical conformation in the junction zone. However, the relative intensities of (001) and (002) reflections do not match well for Ca−alginate fibers, which were formed through a fast gelation process, suggesting the junction zones could be trapped in a metastable state. Comparing the X-ray scattering results of the fresh, dehydrated, and rehydrated gels, a reversible aggregation of junction zones is found during dehydration and rehydration. The different stabilities of the initial bonds and the bonds formed during drying are speculated to be the contribution of MG block segments or short G blocks associating in the junction zones.

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