ELSEVIER

Carbohydrate Polymers **23** (1994) 261–264

0144-8617/94/$07.00

Direct imaging of polysaccharide aggregates in frozen aqueous dilute systems

J. Sugiyama,* C. Rochas,‡ T. Turquois,§ F. Taravel & H. Chanzy¶

Centre de Recherches sur les Macromolécules Végétales, CNRS, B.P. 53, 38041 Grenoble Cedex 9, affiliated with the Joseph Fourier University of Grenoble, France

(Received 21 September 1993; accepted 22 September 1993)

The frozen-hydrated/cryo-transmission electron microscopy technique has been used to observe polysaccharide aggregates in vitreous ice. Images of kappa-carrageenan in the helical state showed fairly large aggregates of a loosely intertwined network of thin rigid rods. Aggregates of agarose in the helical conformation were also visualised. In comparison with those of kappa-carrageenan, the strands of agarose were shorter and less rigid. This difference is discussed in terms of the current knowledge of the gelation of these polysaccharides.

INTRODUCTION

Over the years, a number of reports have been presented where individual polysaccharide molecules have been visualised by transmission electron microscopy (TEM). To achieve such observations, the molecules need to be adsorbed on an appropriate substrate on which they are subsequently dried. They are then either positively (Hanke & Northcote, 1975) or negatively (Snoeren *et al.*, 1976; Harada *et al.*, 1991) stained or shadowed with a metallic deposit (Snoeren *et al.*, 1976; Holzwarth & Prestridge, 1977; Milas *et al.*, 1988). The critical step in these techniques is to ensure that the molecules, once dried, present a contour that corresponds to an actual minimum energy conformation similar to those adopted in solution. Various preparative methods have been proposed and evaluated. In some of them, the sample is only left to evaporate (Snoeren *et al.*, 1976). Other authors have successfully used a freeze-etching method for their preparations (Hermansson, 1989; Hermansson *et al.*, 1991). In a recent series of papers, Stokke and co-workers (Stokke *et al.*, 1989, 1993; Stokke & Brant, 1990) have shown that the drying of dilute solutions of polysaccharide in aqueous glycerol gave images where individual polysaccharide molecules could be observed with reliable contours.

All the methods described so far depend on the observation of more or less granular heavy atom deposits aligned along the polysaccharide molecules. It is tempting to find other techniques where individual molecules would be visualised directly. The frozen-hydrated/cryo-TEM method pioneered by Dubochet and co-workers (Dubochet *et al.*, 1982, 1988; Lepault *et al.*, 1983) seems appropriate. The technique has the advantage of not only avoiding the drying artefacts but also of yielding images of the objects themselves instead of those of their replica, shadow-cast or embedding stain. The frozen-hydrated/cryo-TEM method is a powerful tool of observation that has provided a wealth of outstanding electron micrographs showing small biological objects in aqueous suspensions without stain or shadow (Adrian *et al.*, 1984; Rachel *et al.*, 1986; Stewart & Vigers, 1986; Talmon, 1986; Booy *et al.*, 1989; Vinson *et al.*, 1989). To our knowledge, this technique has not been applied to the observation of dilute preparations of polysaccharide. In this report, we have used this method to try to observe directly the aggregated state of aqueous solutions of agarose at ambient temperature as well as the conformational change of kappa-carrageenan when the helix–coil transition is induced.

*Permanent address: Wood Research Institute, Kyoto University, Uji, 611, Japan.
‡Present address: Laboratoire de Spectrométrie Physique (Associated Laboratory of CNRS), Joseph Fourier University of Grenoble, B.P. 67, 38042 Saint Martin d'Hères, Cedex, France.
§Present address: NESTEC SA, Vers-chez-les-Blanc, P.O. Box 44-1000 Lausanne 26, Switzerland.
¶To whom correspondence should be addressed.

EXPERIMENTAL

Kappa-carrageenan was obtained from ELF BIO SANOFI (France). It had a $\overline{Mw}$ of 510 000 (Rochas *et al.*, 1990). A 0·1 g/litre solution was prepared at 95°C. For kappa-carrageenan in the helix state, the solution was diluted under stirring by adding hot KCl aqueous solution to reach a polymer concentration of 0·01 g/litre and a salt concentration of 0·05 M. The solution was then filtered through a 1·2-μm Millipore filter, cooled to ambient temperature and kept overnight before sampling. For kappa-carrageenan in the coil state, a preparation similar to that of the helix state was used, except that KCl was replaced by NaCl.

Agarose was obtained from SOBIGEL (France). It had a $\overline{Mw}$ of 112 000 (Rochas & Lahaye, 1989). A 0·1 g/litre solution was prepared at 95°C. It was diluted under stirring by adding hot water to reach a final concentration of 0·01 g/litre. The solution was then filtered on a 1·2 μm Millipore filter, cooled to ambient temperature and kept overnight before sampling.

Electron microscopy

Micronet electron microscopy carbon grids were prepared following the method of Fukami & Adachi (1965) and sputtered with gold. As their surface was hydrophobic, they were glow-discharged prior to each experiment. A drop of solution was then deposited on top of a grid that was clamped on a pair of tweezers mounted on a guillotine apparatus. After 30 s, the grid was squeezed twice inside a folded filter paper in order to leave only a thin film of solution within the holes of the micronet grid. The grid was then quenched at once into a liquid propane bath kept at a temperature slightly above that of liquid nitrogen. The grids were then transferred into liquid nitrogen and positioned on a precooled Gatan cryo sample holder mounted in its cryo-transfer unit. The holder was then inserted quickly into the column of a Philips EM400 T electron microscope fitted with a Gatan anticontaminator and a Lhesa image intensifier. All observations were achieved at liquid nitrogen temperatures under low-dose conditions. Images were obtained at a magnification of 13 000×, with an accelerating voltage of 120 kV and an underfocus of several micrometres. For the recording, we used Mitsubishi electron microscopy films MEM, developed in a PQ Ilford developer (diluted to 150 : 2000 v/v).

RESULTS AND DISCUSSION

When a solution of kappa-carrageenan in the coil state was examined by cryo-TEM, it was not possible with our technique to observe any detail in the solidified solution. In contrast, when kappa-carrageenan was in the helical state, most of the specimen grid was also devoid of any structure but a few large aggregates were clearly observed (Fig. 1). These aggregates have lateral dimensions reaching up to 10 μm in size. They consist of a loosely intertwined network of rigid thin rods. All the rods have similar width, estimated at around 5 nm. The measurement of their lengths shows more dispersity, with average values around 1 μm.

If dilute agarose solutions in which the coil–helix transition has been induced by cooling are observed, images that are substantially different from those of kappa-carrageenan are shown. A typical preparation of frozen-hydrated agarose is illustrated in Fig. 2. As in the case of kappa-carrageenan, the aggregates are localised only in a few areas of the specimen. The agarose aggregates, which

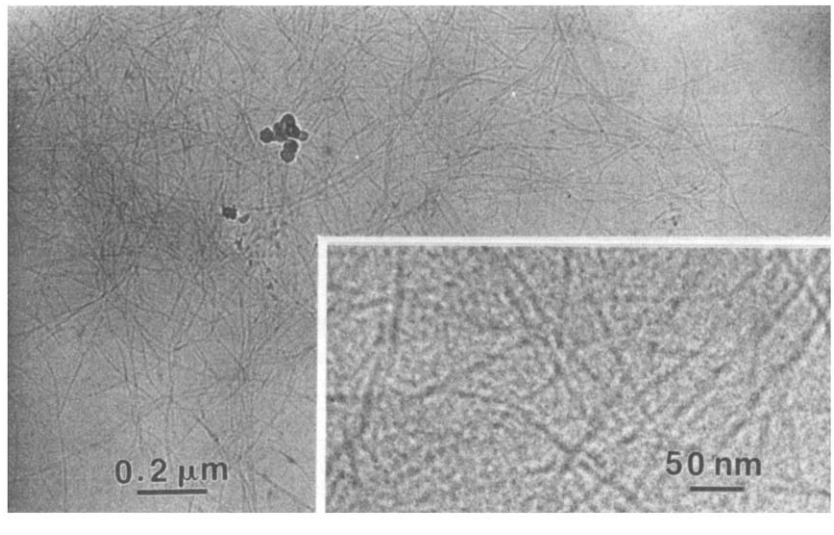

Fig. 1. Image of a kappa-carrageenan aggregate in vitreous ice. *Insert*: higher magnification of another area of the specimen. The sample which was unstained and unshadowed corresponds to a thin film of an aqueous solution of 0·01 g/litre of kappa-carrageenan in the presence of 0·05 M KCl at ambient temperature.

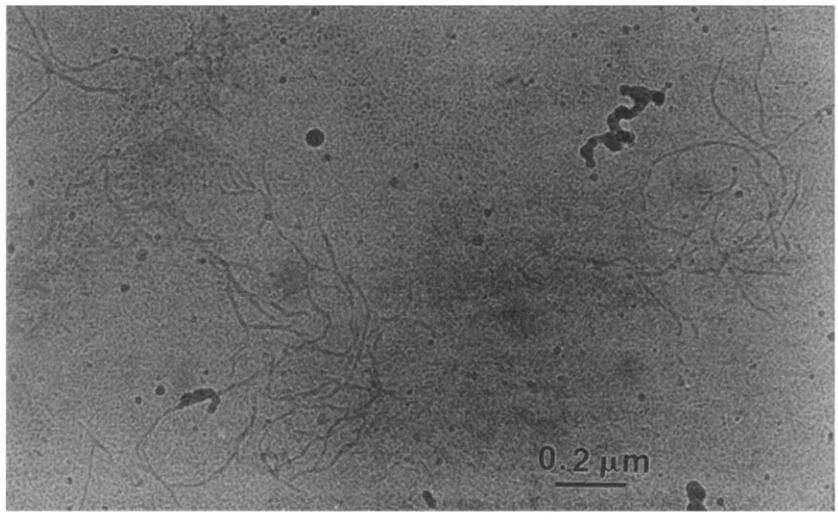

Fig. 2. Image of agarose aggregates in vitreous ice. The sample which was unstained and unshadowed corresponds to a thin film of an aqueous solution of 0·01 g/litre of agarose at ambient temperature.

have lateral dimensions of only 1–2 μm, consist of a number of interconnecting strands that are smaller than in the case of kappa-carrageenan. The individual strands have longitudinal dimensions ranging from 0·2 to 0·4 μm and widths of the order of 10 nm or smaller. As opposed to the rigid rods that compose the aggregates of kappa-carrageenan, the strands of agarose appear less rigid and are frequently seen with curly contours. In addition, their thickness is much less uniform than in the case of kappa-carrageenan. Frequent overlaps are observed where two or more strands merge to one another to give a thickening of the corresponding strand. A number of branch points are also observed where the strands separate from one another at their ends.

The images in Figs 1 and 2 can be compared with the expected molecular lengths for both samples. In the case of kappa-carrageenan in the helix conformation, a pitch of 2·5 nm is expected for three disaccharide residues (Millane *et al.*, 1988). As each disaccharide has a molecular weight of 408, one expects an average length of the fully extended helices of 1 μm for a sample of $\overline{Mw}$ 510 000. This is precisely the range of length that is observed in Fig. 1. For agarose, the disaccharide has a molecular weight of 306 and a projected axial advance of 0·88/0·97 nm (Foord & Atkins, 1989). From these figures, one expects an average length of 0·3 μm for the fully extended helices of the present sample. This calculated length matches closely the value of the observed length in Fig. 2.

When comparing the morphology of the aggregates in Figs 1 and 2, one is struck by the difference in the rigidity of the strands that constitute the aggregates. For kappa-carrageenan, the strands are fairly extended. Their morphology is quite consistent with the well-established duplex (double helix or side-by-side) character adopted by carrageenan in the presence of KCl (Millane *et al.*, 1988; Cairns *et al.*, 1991; Nerdal *et al.*, 1993). In the case of agarose, despite earlier reports of a double helix structure (Arnott *et al.*, 1974), recent results are more in favour of a single helical conformation (Foord & Atkins, 1989; Guenet *et al.*, 1993*a*, *b*). Despite a fairly high rigidity, such single helices are expected to be somewhat more flexible than the kappa-carrageenan duplexes. As the strands in Fig. 2 are much more wavy than those in Fig. 1, our images are consistent with a robust duplex for kappa-carrageenan and a more flexible single-chain helix for agarose.

The observations in Fig. 1 can be correlated with the TEM images of kappa-carrageenan already in the literature. A picture such as that in Fig. 1 closely matches the images of kappa-carrageenan published by Snoeren *et al.* (1976), Hermansson (1989), Harada *et al.* (1991) and Hermansson *et al.* (1991). Several of these authors, however, have reported the existence of two families of rod-like structures in their gels: namely, the basic duplex together with superstrands that correspond to the association of these duplexes. In Fig. 1, we see only one type of strand, which seems to correspond to the basic duplexes. Thus, our experimental conditions seem to exclude the occurrence of superstrands. In a recent scanning tunnelling microscopy (STM) study, Lee *et al.* (1992) showed a series of high-resolution images of kappa-carrageenan adsorbed on graphite and recorded under hydrated conditions. Their images are consistent with ours in that they show exclusively individual kappa-carrageenan duplexes. However, their STM pictures seem to be better resolved as they tend to show the double helicity of the duplexes.

To our knowledge, there are only two papers where TEM visualisation of individual agarose gel elements has been reported (Hickson & Polson, 1968; Harada *et al.*, 1991). In their work, Hickson & Polson (1968)

present two images of agarose 'molecules' of $\overline{Mw}$ 120 000. Their pictures have striking similarities with Fig. 2. Both the lengths and the curly contours of the agarose strands, together with the overlap and branching, can be observed in their micrographs as well as in ours. Therefore, we believe that an electron micrograph such as that in Fig. 2 is a fairly reliable image of the state of aggregation of agarose in dilute solution at ambient temperature.

The recording of the frozen-hydrated images of unshadowed and unstained polysaccharide aggregates in vitreous ice requires the use of strong defocussing to reveal the images by phase contrast (Stewart & Vigers, 1986). Therefore, the resulting molecular thicknesses that are observed are enlarged by a factor that depends on the degree of defocussing. As a consequence, our measured thicknesses of 5 nm for the rods of kappa-carrageenan and 10 nm for the strands of agarose are artificially enlarged under our operating conditions. In order to correct for this, one would need not only to know exactly the defocussing of the corresponding images but also to record several electron micrographs of the same areas at various degrees of defocussing. Such a series of images, in combination with computer processing, should even allow the sugar repeat along the strands of the aggregates to be visualised.

The main result obtained in this study is the observation of the polysaccharide aggregates as they occur in dilute aqueous systems. Indeed, we believe that the shape and the inner morphology of these elements in our images are reliable since they have no time to reorganise during the quench-freezing step used in the technique. The only deformation resulting from the method corresponds to the squeezing step, during which the three-dimensional aggregates are compressed into a thin solution of fluid that is no more than 0·1 μm in thickness. This must create some distortion when the aggregates are several micrometres in size, as in the case of kappa-carrageenan. Less distortion is expected with the smaller agarose aggregates.

ACKNOWLEDGEMENTS

J.S. was the recipient of a fellowship from the Centre National de la Recherche Scientifique (CNRS). T.T. was supported by the Institut National de la Recherche Agronomique (INRA).

REFERENCES

Adrian, M., Dubochet, J., Lepault, J. & McDowall, A.W. (1984). *Nature*, **308**, 32–6.
Arnott, S., Fulmer, A., Scott, W.E., Dea, I.C.M., Moorhouse, R. & Rees, D.A. (1974). *J. Mol. Biol.*, **90**, 269–84.
Booy, F.P., Newcomb, W.W., Trus, B.L., Brown, J.C., Baker, T.S. & Steven, A.C. (1989). *Cell*, **64**, 1007–15.
Cairns, P., Atkins, E.D.T., Miles, M.J. & Morris, V.J. (1991). *Int. J. Biol. Macromol.*, **13**, 65–8.
Dubochet, J., Chang, J.J., Freeman, R., Lepault, J. & McDowall, A.W. (1982). *Ultramicroscopy*, **10**, 55–62.
Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., McDowall, A.W. & Schultz, P. (1988). *Quart. Rev. Biophys.*, **21**, 129–228.
Foord, S.A. & Atkins, E.D.T. (1989). *Biopolymers*, **28**, 1345–65.
Fukami, A. & Adachi, K. (1965). *J. Electron Microsc.* **14**, 112–8.
Guenet, J-M., Brûlet, A. & Rochas, C. (1993*a*). *Int. J. Biol. Macromol.*, **15**, 131–2.
Guenet, J-M., Brûlet, A. & Rochas, C. (1993*b*). *J. Phys. IV*, **3**, 99-102.
Hanke, D.E. & Northcote, D.H. (1975). *Biopolymers*, **14**, 1–17.
Harada, T., Kanzawa, Y., Kanenaga, K., Koreeda, A. & Harada, A. (1991). *Food Struct.*, **10**, 1–18.
Hermansson, A.M. (1989). *Carbohydr. Polym.*, **10**, 163–81.
Hermansson, A.M., Eriksson, E. & Jordansson, E. (1991). *Carbohydr. Polym.*, **16**, 297–320.
Hickson, T.G.L. & Polson, A. (1968). *Biochim. Biophys. Acta*, **165**, 43–58.
Holzwarth, G. & Prestridge, E.B. (1977). *Science*, **197**, 757–9.
Lee, I., Atkins, E.D.T. & Miles, M.J. (1992). *Ultramicroscopy*, **42–44**, 1107–12.
Lepault, J., Booy, F.P. & Dubochet, J. (1983). *J. Microsc.*, **129**, 89–102.
Milas, M., Rinaudo, M., Tinland, B. & de Murcia, G. (1988). *Polym. Bull.*, **19**, 567–72.
Millane, R.P., Chandrasekaran, R., Arnott, S. & Dea, I.C.M. (1988). *Carbohydr. Res.*, **182**, 1–17.
Nerdal, W., Haugen, F., Knutsen, S. & Grasdalen, H. (1993). *J. Biomol. Struct. & Dynam.*, **10**, 785–91.
Rachel, R., Jakubowski, U., Tietz, H., Hergel, R. & Baumeister, W. (1986). *Ultramicroscopy*, **20**, 305–16.
Rochas, C. & Lahaye, M. (1989). *Carbohydr. Polym.*, **10**, 289–98.
Rochas, C., Rinaudo, M. & Landry, S. (1990). *Carbohydr. Polym.*, **12**, 255–66.
Snoeren, T.H.M., Both, P. & Schmidt, D.G. (1976). *Neth. Milk Dairy*, **30**, 132–41.
Stewart, M. & Vigers, G. (1986). *Nature*, **319**, 631–6.
Stokke, B.T. & Brant, D.A. (1990). *Biopolymers*, **30**, 1161–81.
Stokke, B.T., Smidsrød, O. & Elgsaeter, A. (1989). *Biopolymers*, **28**, 617–37.
Stokke, B.T., Elgsaeter, A. & Kitamura, S. (1993). *Int. J. Biol. Macromol.*, **15**, 63–8.
Talmon, Y. (1986). *Colloids Surf.*, **19**, 237–48.
Vinson, P.K., Talmon, Y. & Walter, A. (1989). *Biophys. J.*, **56**, 669–80.