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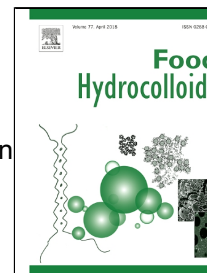
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# Accepted Manuscript

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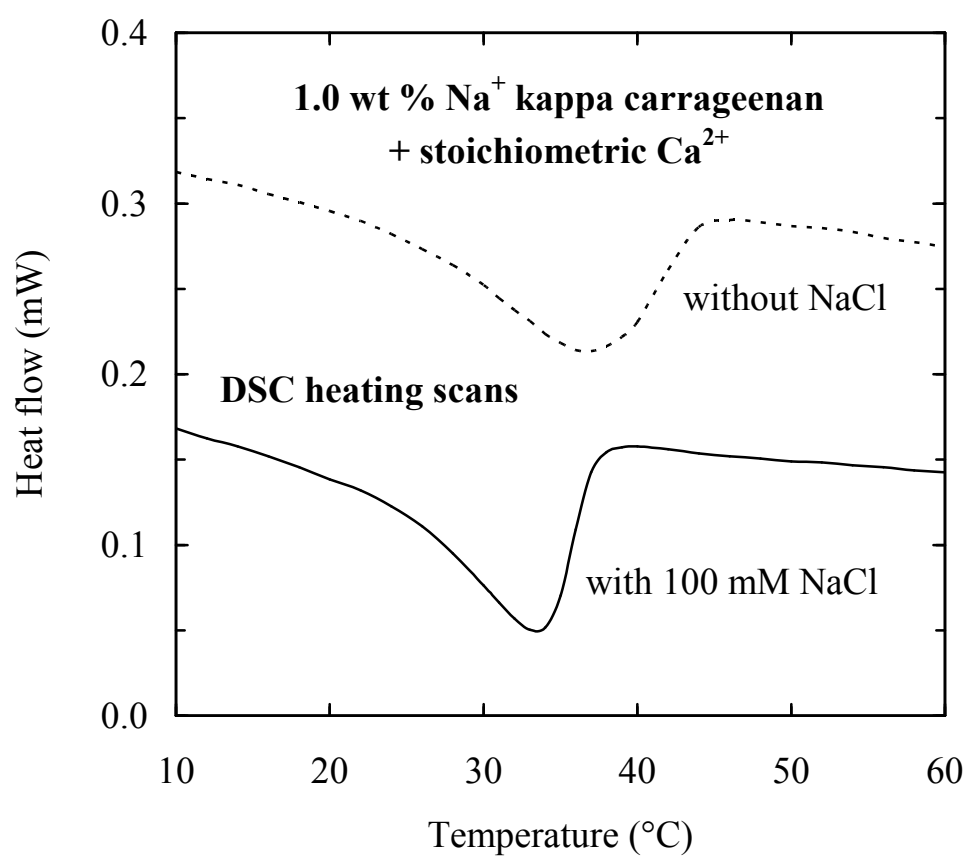


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## Graphical abstract



## **Effect of monovalent cations on calcium-induced assemblies of kappa carrageenan**

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## Abstract

The effect of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  cations on the thermal stability and aggregation of kappa carrageenan double helices has been explored by differential scanning calorimetry (DSC). Previous studies have shown that kappa carrageenan helices bind  $\text{K}^+$  cations, but not  $\text{Na}^+$ . The kappa carrageenan used in this work was therefore in the  $\text{Na}^+$  salt form, to avoid complications from site-bound counterions to the polymer, and was studied at a fixed concentration of 1.0 wt % (~25 mN w.r.t. sulfate groups).  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  cations were added as chloride salts. Values of peak-maximum temperature ( $T_{\text{max}}$ ) in DSC cooling and heating scans ( $0.5^\circ\text{C}/\text{min}$ ) increased progressively with increasing salt concentration, following the order  $\text{Na}^+ < \text{Ca}^{2+} < \text{K}^+$ , but greatest thermal hysteresis was seen with  $\text{Ca}^{2+}$ . Our proposed interpretation is that  $\text{Ca}^{2+}$  cations "cement" the carrageenan helices together by binding directly between them, giving greater thermal stability, and thus greater hysteresis, than  $\text{K}^+$  cations which act indirectly by suppressing charge. On progressive addition of NaCl or KCl to solutions incorporating  $\text{Ca}^{2+}$  at concentrations of 5 mM or 12.5 mM (stoichiometric equivalence) the values of  $T_{\text{max}}$  moved asymptotically towards those seen for the same concentrations of the monovalent cations in the absence of calcium, suggesting progressive displacement of site-bound  $\text{Ca}^{2+}$ . Thus  $T_{\text{max}}$  for the order-disorder transition was increased by KCl but reduced by NaCl, with the strange consequence that addition of NaCl *lowered* the transition temperature rather than raising it.

## Keywords

Kappa carrageenan; Differential scanning calorimetry; Calcium ions; Cation binding

## 1. Introduction

Kappa carrageenan is a structural polysaccharide from numerous species of red seaweed (Rhodophyta) and is used extensively as a gelling agent, particularly in food products (Glickman, 1983; Stanley, 1990; Therkelsen, 1993).

It is one of a family of sulfated galactans (Painter, 1983) with primary structures based on a linear disaccharide repeating sequence of alternating 1,3 and 1,4-linked residues. The other gelling carrageenan used commercially is iota. In both iota and kappa carrageenan the 1,4-linked residues occur predominantly as the 3,6-anhydride (Fig. 1), and the 1,3-linked residues have a sulfate group at C(4). In nature, the anhydride bridge is absent in a significant proportion of the 1,4-linked residues, but in commercial production structural regularity is normally enhanced by treatment with alkali (Smidsrød, Larsen, Penas & Haug, 1967; Stanley, 1963), giving structures close to the idealised repeating sequence in Fig. 1. Unlike kappa carrageenan, iota has an additional sulfate substituent at C(2) of the 1,4-linked residue (Anderson, Dolan & Rees, 1973). Thus iota carrageenan has two negative charges per disaccharide, whereas kappa has only one.

This difference in pattern of sulfation causes profound differences in gelation behaviour. (Picullel, 1995). Iota carrageenan gels are formed on cooling by conversion of disordered coils to 3-fold double helices (Arnott, Scott, Rees & McNab, 1974) which constitute the junction zones of the gel network (Rees, 1970). The coil-helix and accompanying sol-gel transitions are reversed on heating, with little, if any, thermal hysteresis.

Kappa carrageenan has a double helix structure similar to that of iota (Anderson, Campbell, Harding, Rees & Samuel, 1969; Millane, Chandrasekaran, Arnott & Dea, 1988). Again, gels form on cooling and melt on heating, but with substantial thermal hysteresis (in marked contrast to the sol-gel and gel-sol transitions of iota). Hysteresis is attributed to cation-induced aggregation of the kappa carrageenan double helices, with the aggregates surviving to higher temperatures than those at which individual helices are formed on cooling (Morris & Norton, 1983; Morris, Rees & Robinson, 1980). Some limited aggregation of helices, with associated slight thermal hysteresis, may also occur for iota carrageenan in the presence of high concentrations of  $\text{Ca}^{2+}$  cations (Goycoolea, Morris & Gidley, 1995; Tari, Kara & Pekcan, 2010).

It has been demonstrated by NMR (Belton, Morris & Tanner, 1986; Grasdalen & Smidsrød, 1981; Piculell, Nilsson & Ström, 1989) that, unlike  $\text{Li}^+$  and  $\text{Na}^+$ ,  $\text{K}^+$  (and larger Group I cations) bind to kappa carrageenan double helices. This reduces the net negative charge on the helices, and promotes helix–helix aggregation by suppressing electrostatic repulsion. Aggregation increases gel strength by giving an additional mechanism of crosslinking (Viebkke, Piculell & Nilsson, 1994), and commercial kappa carrageenan is therefore normally produced and supplied with  $\text{K}^+$  as the predominant cation.

The interaction of kappa carrageenan with  $\text{Ca}^{2+}$  is less well understood. On addition of increasing concentrations of  $\text{K}^+$  to 1.0 wt % solutions of kappa carrageenan in the  $\text{Na}^+$  salt form (to avoid complications from site-binding of counterions to the polymer), Doyle, Giannouli, Philp & Morris (2002) observed a large, monotonic increase in gel strength. With  $\text{Ca}^{2+}$  cations, by contrast, gel strength passed through a maximum at stoichiometric equivalence to the sulfate groups of the carrageenan. Maximum gel strength at stoichiometric equivalence of  $\text{Ca}^{2+}$  to the negative charge of the polymer has been observed subsequently (Doyle, Giannouli, Richardson & Morris, 2018) for other concentrations of kappa carrageenan (in the range 0.5 - 3.0 wt %), suggesting that  $\text{Ca}^{2+}$  cations promote aggregation of the double helices by site-binding between them (rather than by binding to individual helices, as happens with  $\text{K}^+$ ).

In the present work, we have used differential scanning calorimetry to explore the effect of monovalent cations ( $\text{K}^+$  and  $\text{Na}^+$ ) on the stability of  $\text{Ca}^{2+}$ -induced aggregates of kappa carrageenan.

## 2. Materials and methods

Kappa carrageenan in the  $\text{Na}^+$  salt form was kindly supplied by Quest International Ireland Ltd., Carrigaline, Co. Cork, Ireland. It is an alkali-modified extract from *Euchema cottonii*, identical to the material used by Doyle et al. (2002) and Doyle et al. (2018).  $\text{NaCl}$ ,  $\text{KCl}$  and  $\text{CaCl}_2$  were AnalaR grade from BDH. Distilled deionised water was used throughout.

Differential scanning calorimetry (DSC) measurements were made at a fixed carrageenan concentration of 1.0 wt % (~25 mM w.r.t. sulfate groups) using a Setaram DSC III scanning microcalorimeter at heating and cooling rates of  $0.5^\circ\text{C}/\text{min}$ . Water was used as thermal reference, and sample and reference pans were balanced to within 0.05 mg (typical loading ~850 mg).

Carrageenan was dissolved at 2.0 wt % by mechanical stirring at 80°C. Solutions of NaCl, KCl and/or CaCl<sub>2</sub> were also prepared at twice the required concentration and mixed in equal volumes with 2.0 wt % kappa carrageenan at 80°C, to give mixtures containing 1.0 wt % polymer in the presence of the appropriate concentration of salt(s).

To take account of non-specific screening of electrostatic repulsion between carrageenan helices, comparisons were made at equivalent values of ionic strength (with 1 mM CaCl<sub>2</sub> having the same ionic strength as 3 mM NaCl or KCl).

### 3. Results

Thermal hysteresis between the DSC exotherm arising from the disorder–order transition on cooling and the endotherm from the order–disorder transition on heating is illustrated in Fig. 2 for 1.0 wt % Na<sup>+</sup> kappa carrageenan in the presence of 100 mM KCl. The same general pattern of a sharp exotherm and somewhat broader endotherm was observed for all carrageenan–salt mixtures studied, but with large differences in the position of the DSC peaks on varying concentration and type of salt.

Disorder–order and order–disorder transition temperatures can be conveniently characterised by the temperature of maximum (absolute) heat flow in DSC (i.e. the temperature,  $T_{\max}$ , at the top of the exotherms and bottom of the endotherms). As shown in Fig. 3, the effectiveness of increasing concentrations of the three salts studied in raising  $T_{\max}$  values for 1.0 wt % Na<sup>+</sup> kappa carrageenan followed the order NaCl < CaCl<sub>2</sub> < KCl for both the disorder–order transition on cooling (Fig. 3a) and the order–disorder transition on heating (Fig. 3b).

However, the extent of thermal hysteresis (Fig. 4) between the ordering and disordering processes followed a different order: NaCl < KCl < CaCl<sub>2</sub>. Greater thermal hysteresis with CaCl<sub>2</sub> than with KCl is consistent with the proposal (Doyle et al., 2002) that Ca<sup>2+</sup> cations "cement" kappa carrageenan double helices together by binding between them in an arrangement analogous to the "egg box" binding (Grant, Morris, Rees, Smith & Thom, 1973) of Ca<sup>2+</sup> between polyuronate sequences in calcium alginate and calcium pectinate gels, whereas K<sup>+</sup> acts indirectly by binding to individual helices and thus suppressing electrostatic repulsion.

To explore the stability of Ca<sup>2+</sup>-induced aggregates of kappa carrageenan double helices the effect of adding monovalent cations (Na<sup>+</sup> and K<sup>+</sup>) was investigated. Two different concentrations of Ca<sup>2+</sup> were used in mixtures with 1.0 wt % Na<sup>+</sup> kappa carrageenan: 12.5 mM (stoichiometric) and 5 mM, and increasing concentrations of NaCl or KCl were added.



As shown in Fig. 5, as the concentration of KCl was raised the observed values of  $T_{\max}$  for the disorder–order transition on cooling (Fig. 5a) and the order–disorder transition on heating (Fig. 5b) increased from those observed with 12.5 or 5 mM  $\text{Ca}^{2+}$  alone towards the higher values found (Fig. 3) with KCl, suggesting displacement of bound  $\text{Ca}^{2+}$  cations.

When the same experiment was repeated (Fig. 6) using NaCl instead of KCl, addition of high concentrations of NaCl was found to cause a slight increase (Fig. 7a) in the temperature of the disorder–order transition on cooling (Fig. 6a), which can be readily explained by non-specific screening of electrostatic repulsion between the charged polymer coils, thus facilitating intermolecular association into double helices.

In the heating direction (Fig. 6b), however, increasing concentrations of NaCl caused a progressive reduction in  $T_{\max}$  for the disorder–disorder transition, towards the lower values observed (Fig. 3) for NaCl alone. In consequence, addition of high concentrations of NaCl had the surprising effect of *lowering* the transition temperature (Fig. 7b), rather than giving the increase in  $T_{\max}$  that would be expected from the general behaviour of charged polysaccharides.

#### 4. Discussion and conclusions

The obvious interpretation of the results presented in Figs. 5 - 7 is that progressive addition of KCl or NaCl causes progressive displacement of  $\text{Ca}^{2+}$  cations bound between kappa carrageenan double helices, with the overall behaviour then approaching that observed (Fig. 3) for the same concentrations of the monovalent cations in the absence of  $\text{Ca}^{2+}$ . The outcome is an increase in thermal stability of the helix–helix aggregates on addition of KCl and a reduction with NaCl, which leads to the unusual (perhaps unprecedented) observation that addition of NaCl lowers the temperature of the order–disorder transition (Fig. 7b).

The ability of monovalent cations to displace  $\text{Ca}^{2+}$  is, in itself, further evidence of site-binding of calcium ions to kappa carrageenan double helices (if they weren't bound they could not be displaced), and reinforces the conclusion from greater thermal hysteresis with  $\text{Ca}^{2+}$  than with  $\text{K}^+$  (Fig. 4) and maximum in gel strength at stoichiometric equivalence of  $\text{Ca}^{2+}$  to sulfate groups of the polymer (Doyle et al., 2002) that calcium ions bind *between* the double helices, rather than to individual helices.

The ability of monovalent salts to displace  $\text{Ca}^{2+}$  cations bound to anionic polysaccharides has been demonstrated previously for calcium alginate (Morris, Rees, Thom & Boyd, 1978) and calcium pectinate (Morris, Powell, Gidley & Rees, 1982). Both of these form "egg box" structures (Grant et al., 1973) in which calcium ions are sandwiched in site-bound arrays between 2-fold (zig-zag) sequences of polyguluronate (from alginate) or polygalacturonate from pectin. On addition of increasing concentrations of monovalent cations ( $\text{Na}^+$  or  $\text{Me}_4\text{N}^+$ ), the amount of bound  $\text{Ca}^{2+}$  dropped from around stoichiometric equivalence to the carboxylate groups of the polyuronate sequences to 50% stoichiometric, indicating a highly stable dimeric structure with a single array of calcium ions bound between two polysaccharide chains, with only the inner faces of the 2-fold ordered structures participating in binding.

Displacement of  $\text{Ca}^{2+}$  cations bound between (but not within) "egg box" dimers suggests a parallel with similar displacement from between the dimers (double helices) of kappa carrageenan.

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## Legends to figures

**Fig. 1.** Idealised disaccharide repeating structure of gelling carrageenans. R = H in kappa and SO<sub>3</sub><sup>-</sup> in iota.

**Fig. 2.** DSC traces recorded on cooling and heating at 0.5°C/min for 1.0 wt % Na<sup>+</sup> kappa carrageenan with 100 mM added KCl.

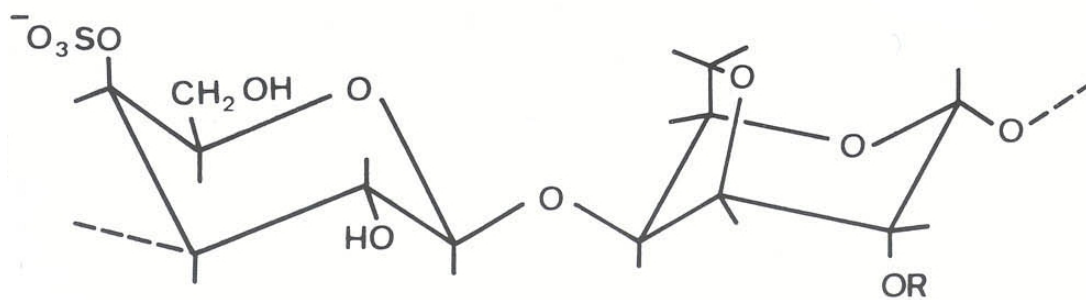
**Fig. 3.** Effect of ionic strength on peak-maximum temperature (T<sub>max</sub>) for 1.0 wt % Na<sup>+</sup> kappa carrageenan on (a) cooling and (b) heating in the presence of increasing concentrations of NaCl (▲), KCl (■) or CaCl<sub>2</sub> (●).

**Fig. 4.** Effect of ionic strength on the extent of thermal hysteresis between DSC cooling and heating scans (Fig. 3) for 1.0 wt % Na<sup>+</sup> kappa carrageenan in the presence of increasing concentrations of NaCl (▲), KCl (■) or CaCl<sub>2</sub> (●).

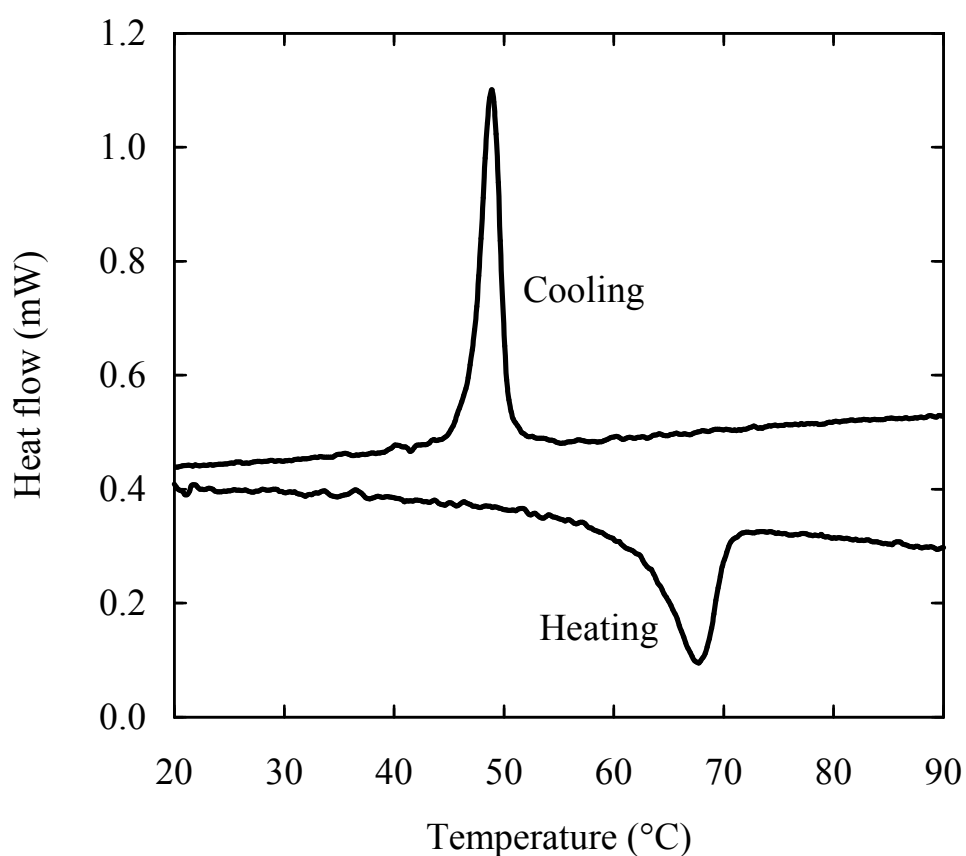
**Fig. 5.** Variation of peak-maximum temperature with ionic strength on addition of increasing concentrations of KCl to 1.0 wt % Na<sup>+</sup> kappa carrageenan in mixtures with 5 mM (●) or 12.5 mM (▲) CaCl<sub>2</sub> on (a) cooling and (b) heating. The corresponding values from addition of KCl (□) or CaCl<sub>2</sub> (○) alone (Fig. 3) are shown for comparison.

**Fig. 6.** Variation of peak-maximum temperature with ionic strength on addition of increasing concentrations of NaCl to 1.0 wt % Na<sup>+</sup> kappa carrageenan in mixtures with 5 mM (●) or 12.5 mM (▲) CaCl<sub>2</sub> on (a) cooling and (b) heating. The corresponding values from addition of NaCl (Δ) or CaCl<sub>2</sub> (○) alone (Fig. 3) are shown for comparison.

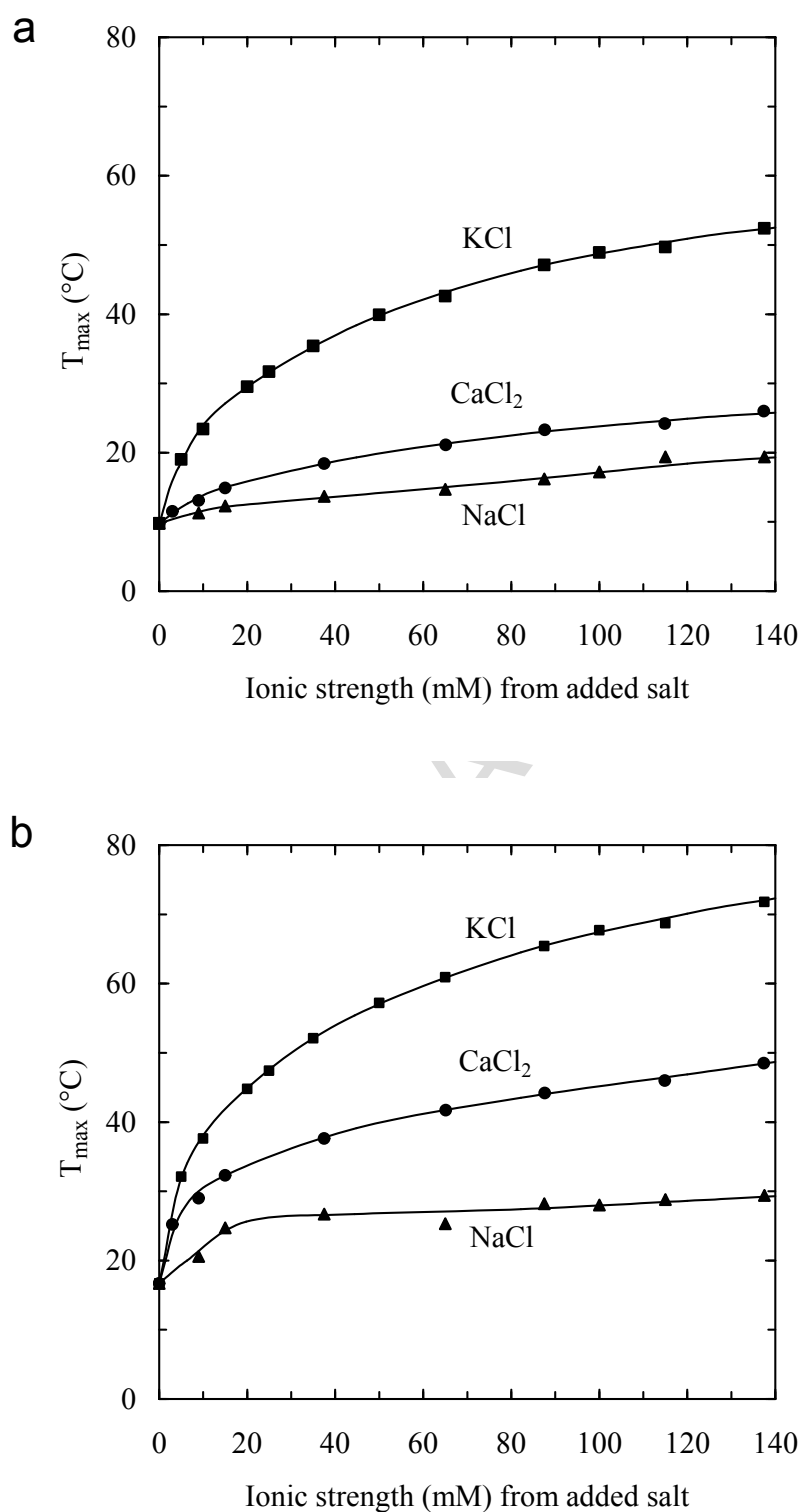
**Fig. 7.** DSC traces recorded on (a) cooling and (b) heating for 1.0 wt % Na<sup>+</sup> kappa carrageenan with 12.5 mM CaCl<sub>2</sub> in the presence (solid line) or absence (dashed line) of 100 mM NaCl.



**Fig. 1.** Idealised disaccharide repeating structure of gelling carrageenans. R = H in kappa and  $\text{SO}_3^-$  in iota.

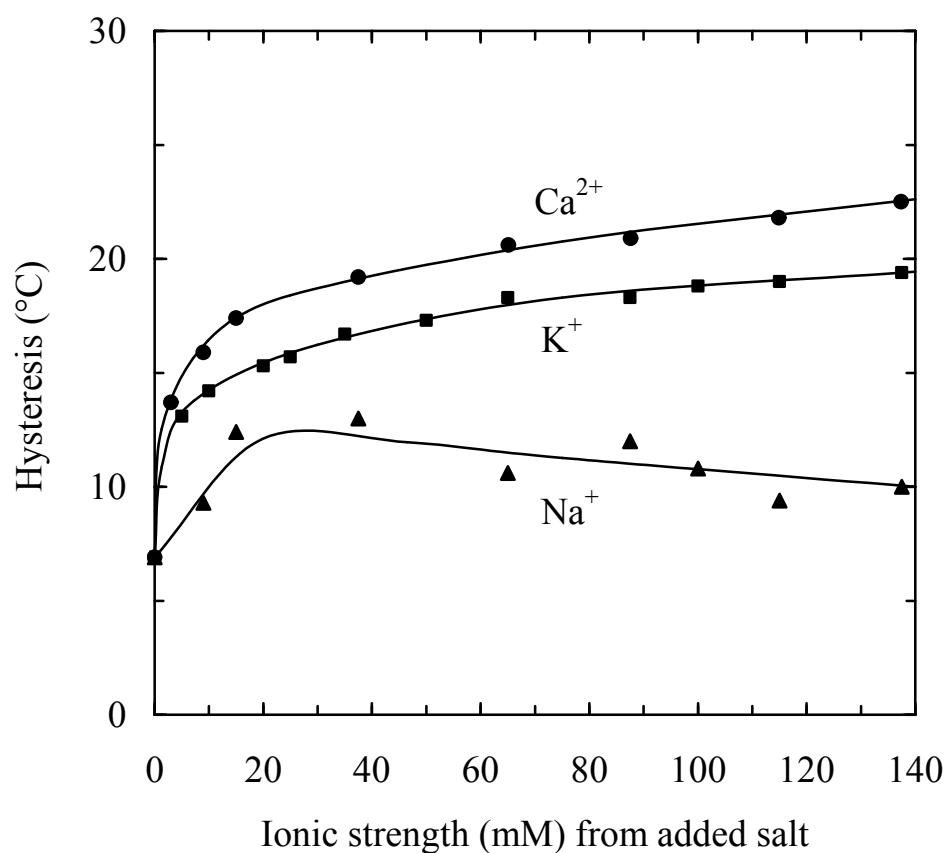


**Fig. 2.** DSC traces recorded on cooling and heating at  $0.5^\circ\text{C}/\text{min}$  for 1.0 wt %  $\text{Na}^+$  kappa carrageenan with 100 mM added KCl.

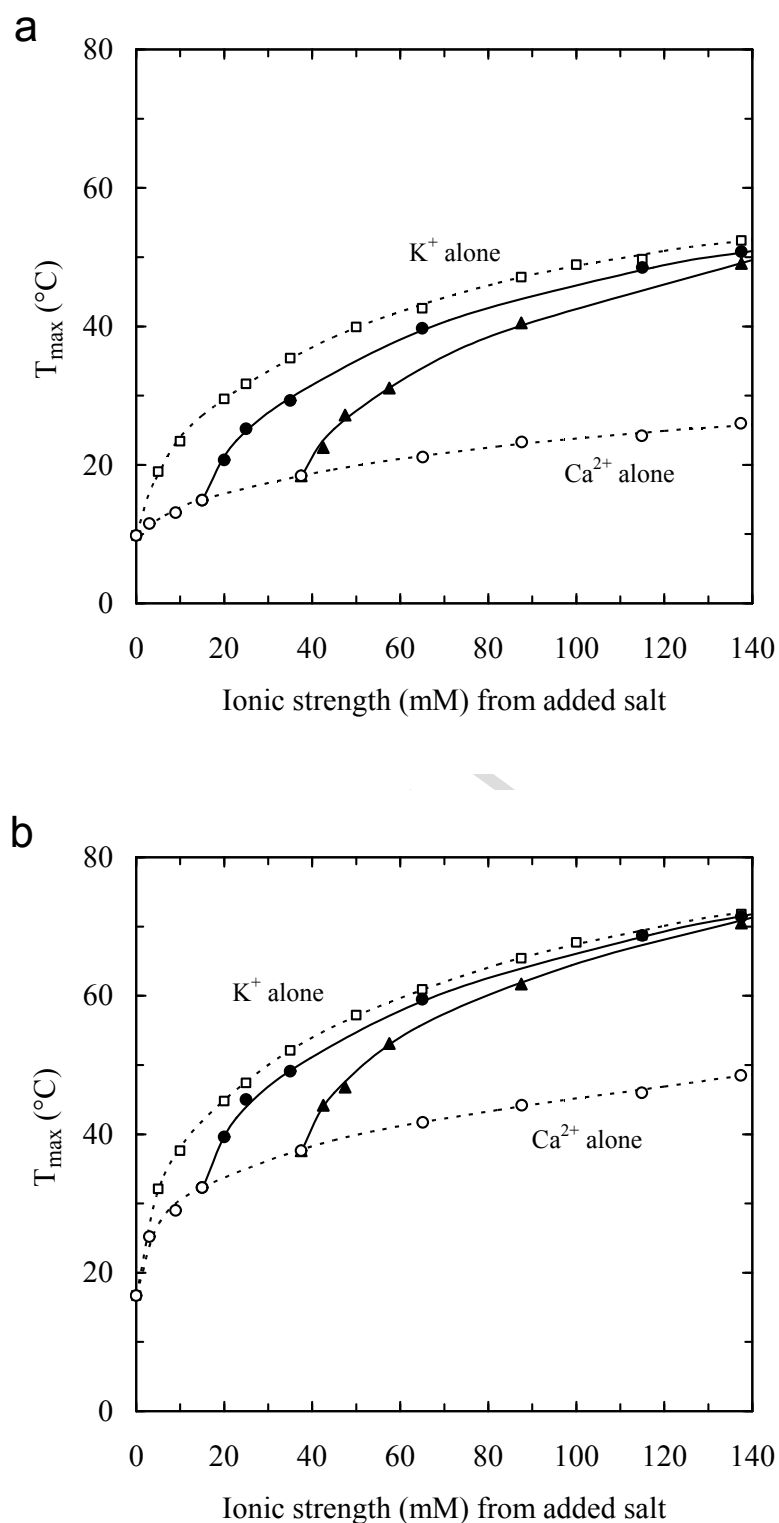


**Fig. 3.** Effect of ionic strength on peak-maximum temperature ( $T_{\max}$ ) for 1.0 wt %  $\text{Na}^+$  kappa carrageenan on (a) cooling and (b) heating in the presence of increasing concentrations of NaCl (▲), KCl (■) or  $\text{CaCl}_2$  (●).

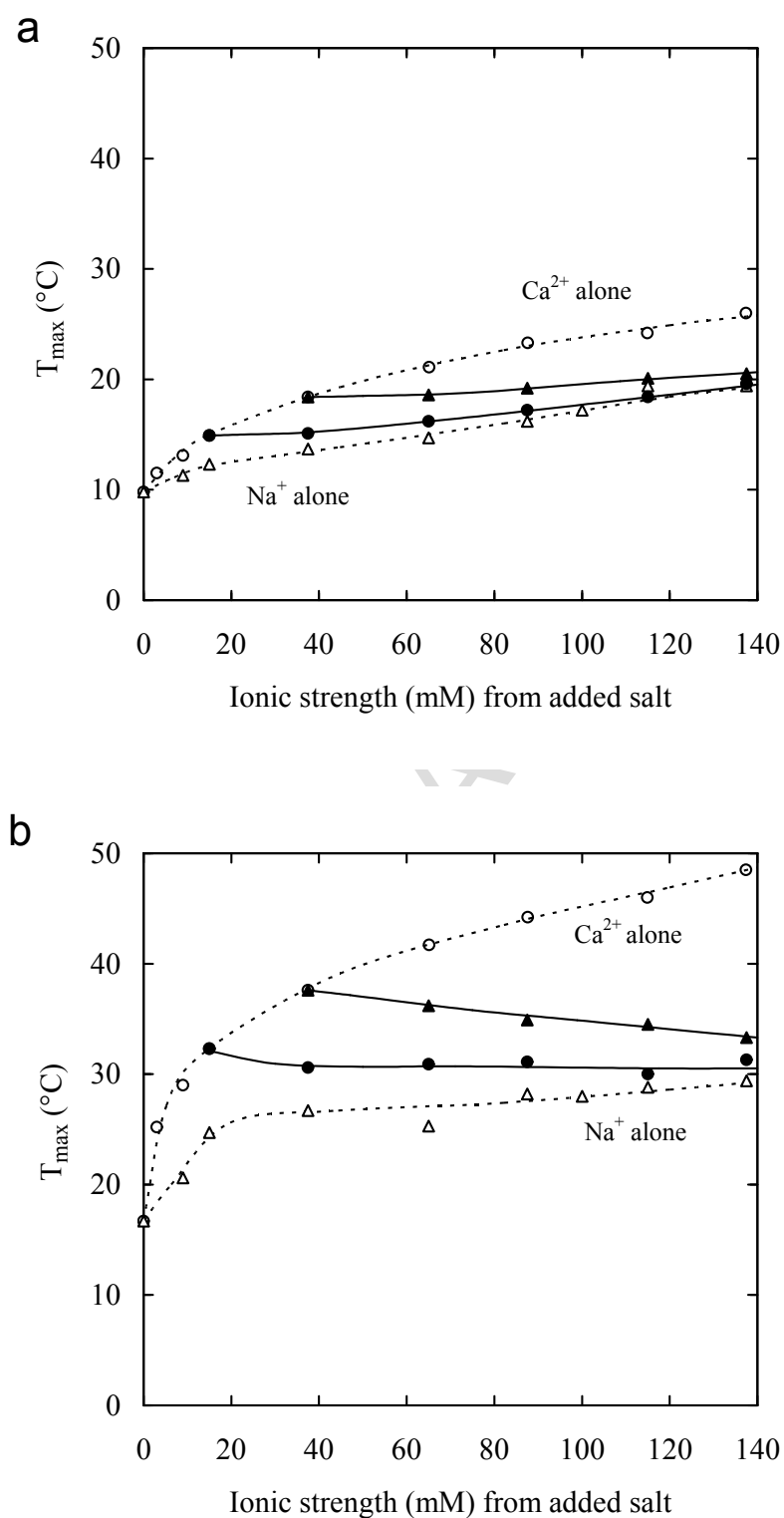




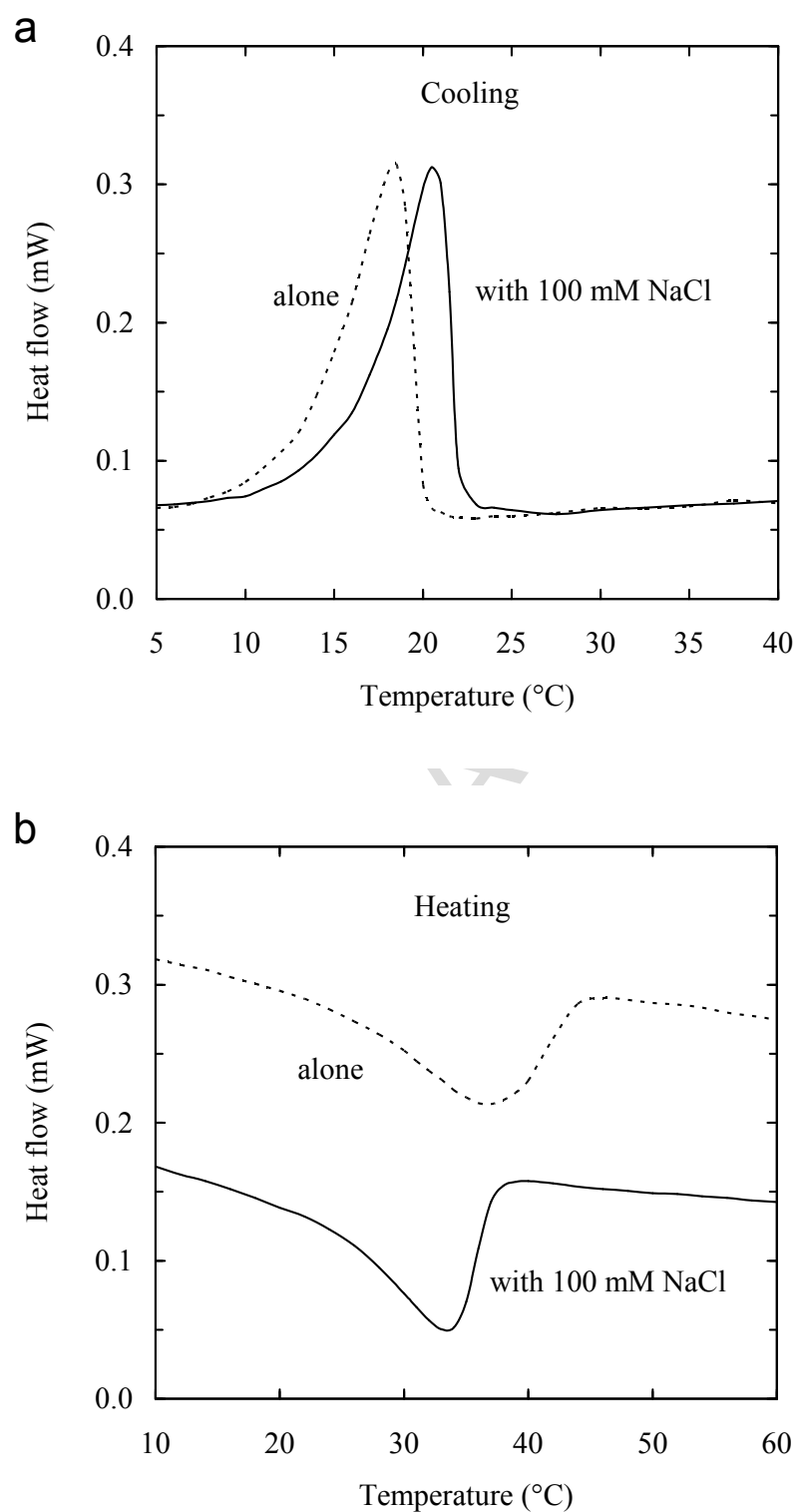
**Fig. 4.** Effect of ionic strength on the extent of thermal hysteresis between DSC cooling and heating scans (Fig. 3) for 1.0 wt %  $\text{Na}^{+}$  kappa carrageenan in the presence of increasing concentrations of NaCl ( $\blacktriangle$ ), KCl ( $\blacksquare$ ) or  $\text{CaCl}_2$  ( $\bullet$ ).



**Fig. 5.** Variation of peak-maximum temperature with ionic strength on addition of increasing concentrations of KCl to 1.0 wt %  $\text{Na}^{+}$  kappa carrageenan in mixtures with 5 mM (●) or 12.5 mM (▲)  $\text{CaCl}_2$  on (a) cooling and (b) heating. The corresponding values from addition of KCl (□) or  $\text{CaCl}_2$  (○) alone (Fig. 3) are shown for comparison.



**Fig. 6.** Variation of peak-maximum temperature with ionic strength on addition of increasing concentrations of NaCl to 1.0 wt % Na<sup>+</sup> kappa carrageenan in mixtures with 5 mM (●) or 12.5 mM (▲) CaCl<sub>2</sub> on (a) cooling and (b) heating. The corresponding values from addition of NaCl (Δ) or CaCl<sub>2</sub> (○) alone (Fig. 3) are shown for comparison.



**Fig. 7.** DSC traces recorded on (a) cooling and (b) heating for 1.0 wt % Na<sup>+</sup> kappa carrageenan with 12.5 mM CaCl<sub>2</sub> in the presence (solid line) or absence (dashed line) of 100 mM NaCl.

**Highlights**

- Site-binding of calcium cations between kappa carrageenan double helices
- Consequent large hysteresis between ordering and disordering transitions in DSC
- Bound  $\text{Ca}^{2+}$  displaced progressively by increasing concentrations of NaCl or KCl
- KCl moves order–disorder transition towards higher temperatures seen with  $\text{K}^+$  alone
- High concentrations of NaCl *lower* transition temperature rather than raising it