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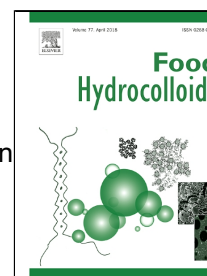
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# Accepted Manuscript

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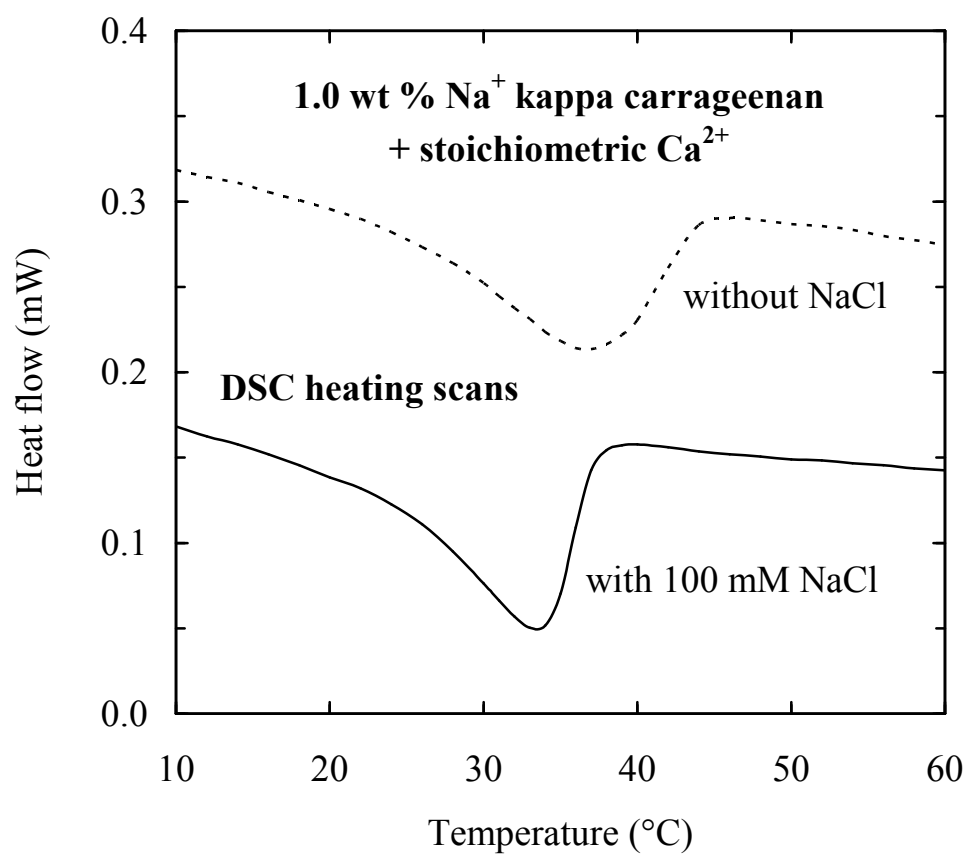


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## Graphical abstract



ACCEPTED

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

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## 1 Abstract

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

## 20 Keywords

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

## 23 1. Introduction

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

27 It is one of a family of sulfated galactans (Painter, 1983) with primary structures based  
28 on a linear disaccharide repeating sequence of alternating 1,3 and 1,4-linked residues. The  
29 other gelling carrageenan used commercially is iota. In both iota and kappa carrageenan the  
30 1,4-linked residues occur predominantly as the 3,6-anhydride (Fig. 1), and the 1,3-linked  
31 residues have a sulfate group at C(4). In nature, the anhydride bridge is absent in a significant  
32 proportion of the 1,4-linked residues, but in commercial production structural regularity is  
33 normally enhanced by treatment with alkali (Smidsrød, Larsen, Penas & Haug, 1967;  
34 Stanley, 1963), giving structures close to the idealised repeating sequence in Fig. 1. Unlike  
35 kappa carrageenan, iota has an additional sulfate substituent at C(2) of the 1,4-linked residue  
36 (Anderson, Dolan & Rees, 1973). Thus iota carrageenan has two negative charges per  
37 disaccharide, whereas kappa has only one.

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

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

54 It has been demonstrated by NMR (Belton, Morris & Tanner, 1986; Grasdalen & Smidsrød,  
55 1981; Piculell, Nilsson & Ström, 1989) that, unlike  $\text{Li}^+$  and  $\text{Na}^+$ ,  $\text{K}^+$  (and larger Group I  
56 cations) bind to kappa carrageenan double helices. This reduces the net negative charge  
57 on the helices, and promotes helix–helix aggregation by suppressing electrostatic repulsion.  
58 Aggregation increases gel strength by giving an additional mechanism of crosslinking  
59 (Viebeck, Piculell & Nilsson, 1994), and commercial kappa carrageenan is therefore normally  
60 produced and supplied with  $\text{K}^+$  as the predominant cation.

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

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

## 75 **2. Materials and methods**

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

80 Differential scanning calorimetry (DSC) measurements were made at a fixed carrageenan  
81 concentration of 1.0 wt % (~25 mM w.r.t. sulfate groups) using a Setaram DSC III scanning  
82 microcalorimeter at heating and cooling rates of  $0.5^\circ\text{C}/\text{min}$ . Water was used as thermal  
83 reference, and sample and reference pans were balanced to within 0.05 mg (typical loading  
84 ~850 mg).

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

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

### 92 3. Results

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

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

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

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



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

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

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

#### 132 **4. Discussion and conclusions**

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

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

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

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

#### 160 **Acknowledgements**

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232 **Legends to figures**

233

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

236

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

239

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

243

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

247

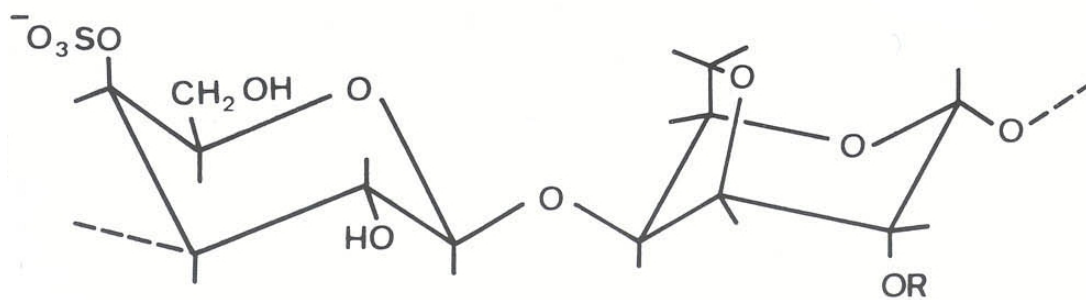
248 **Fig. 5.** Variation of peak-maximum temperature with ionic strength on addition of increasing  
249 concentrations of KCl to 1.0 wt % Na<sup>+</sup> kappa carrageenan in mixtures with 5 mM (●) or  
250 12.5 mM (▲) CaCl<sub>2</sub> on (a) cooling and (b) heating The corresponding values from addition  
251 of KCl (□) or CaCl<sub>2</sub> (○) alone (Fig. 3) are shown for comparison.

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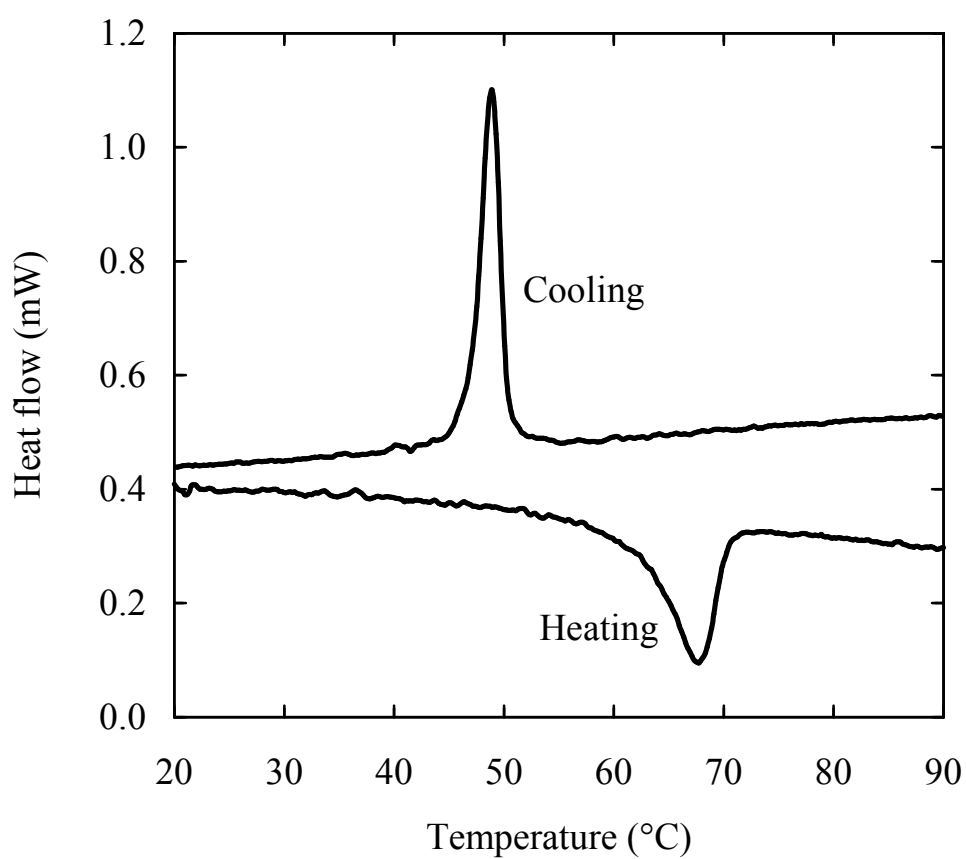
253 **Fig. 6.** Variation of peak-maximum temperature with ionic strength on addition of increasing  
254 concentrations of NaCl to 1.0 wt % Na<sup>+</sup> kappa carrageenan in mixtures with 5 mM (●) or  
255 12.5 mM (▲) CaCl<sub>2</sub> on (a) cooling and (b) heating The corresponding values from addition of  
256 NaCl (Δ) or CaCl<sub>2</sub> (○) alone (Fig. 3) are shown for comparison.

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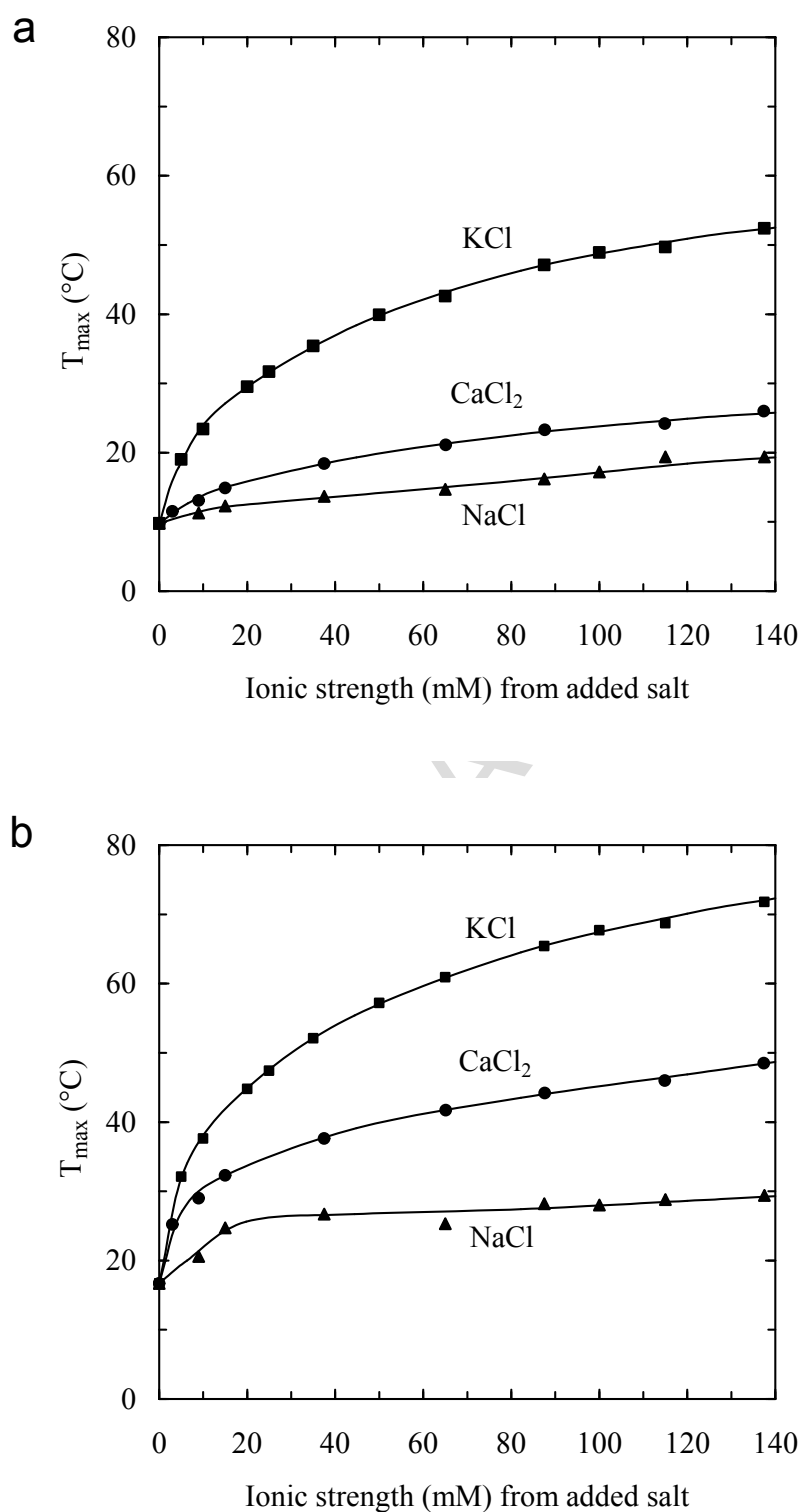
258 **Fig. 7.** DSC traces recorded on (a) cooling and (b) heating for 1.0 wt % Na<sup>+</sup> kappa  
259 carrageenan with 12.5 mM CaCl<sub>2</sub> in the presence (solid line) or absence (dashed line)  
260 of 100 mM NaCl.



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

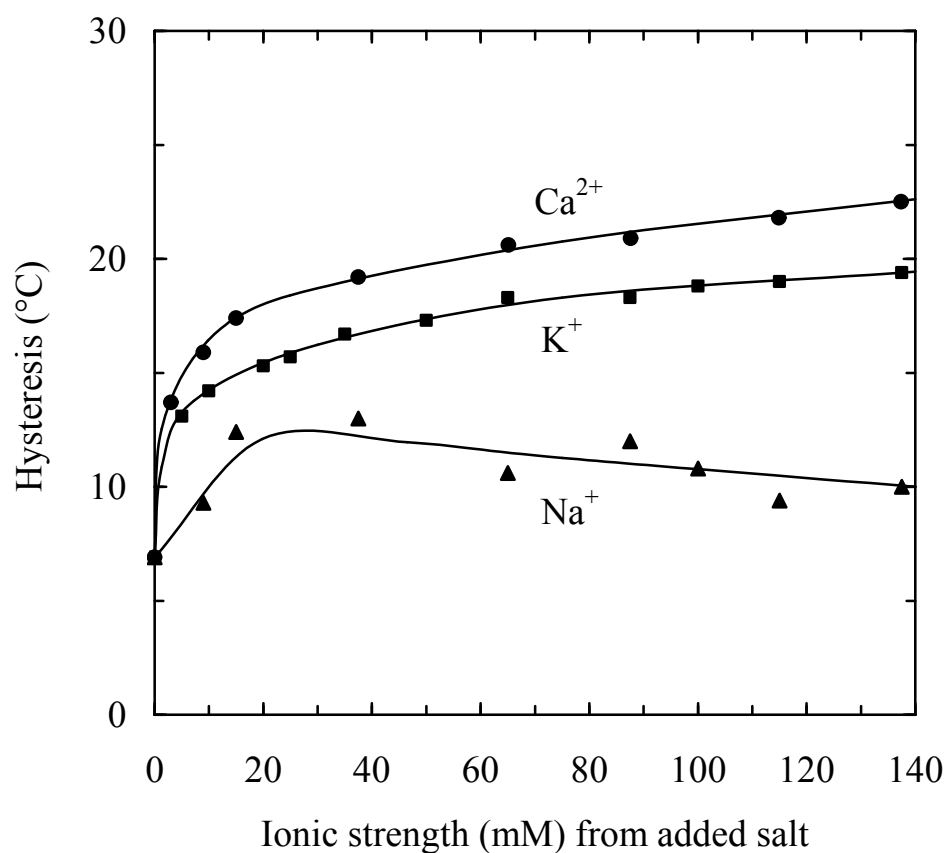


**Fig. 2.** DSC traces recorded on cooling and heating at  $0.5^\circ\text{C}/\text{min}$  for 1.0 wt %  $\text{Na}^+$  kappa carrageenan with 100 mM added KCl.

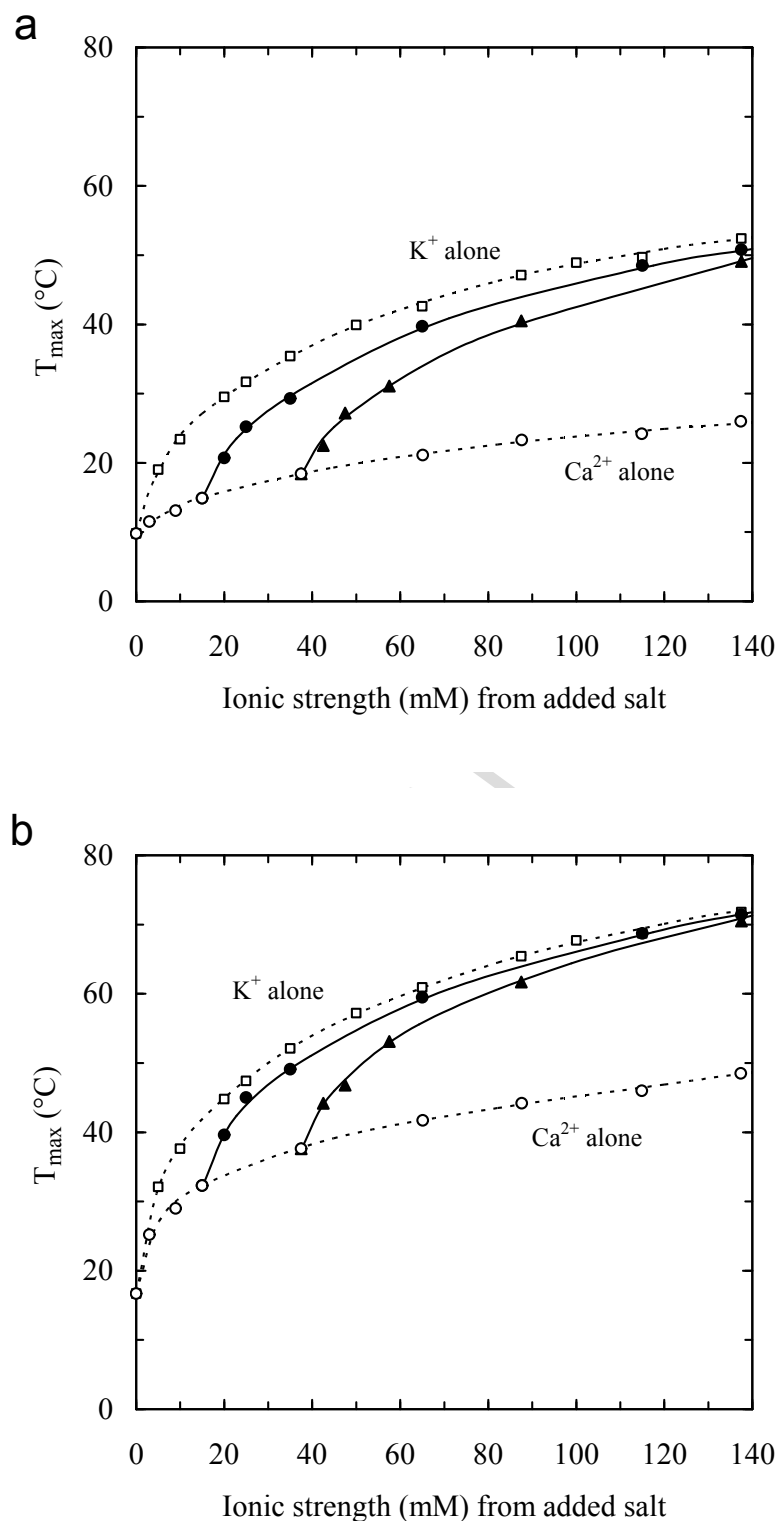


**Fig. 3.** Effect of ionic strength on peak-maximum temperature ( $T_{\max}$ ) for 1.0 wt % Na<sup>+</sup> kappa carrageenan on (a) cooling and (b) heating in the presence of increasing concentrations of NaCl (▲), KCl (■) or CaCl<sub>2</sub> (●).

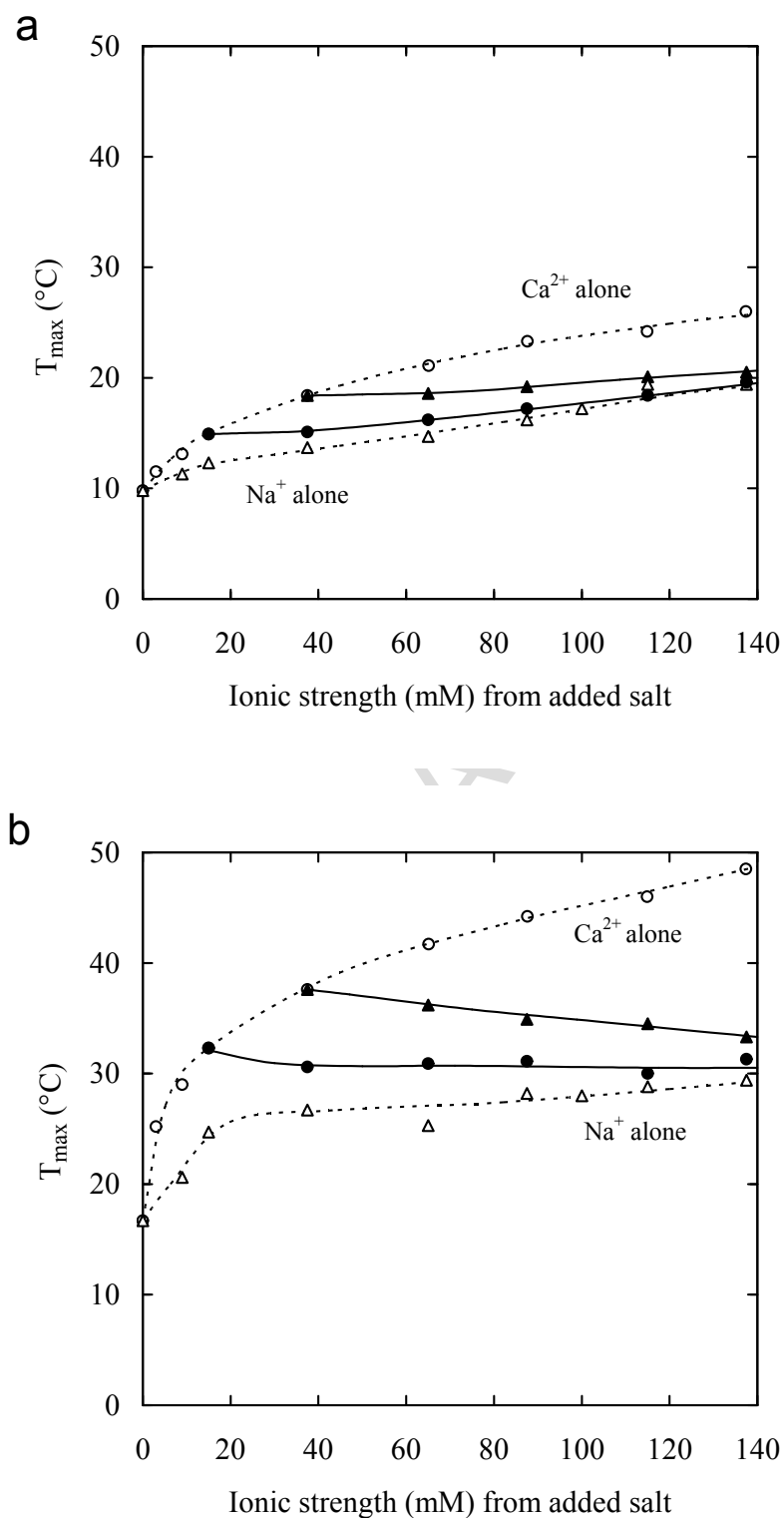




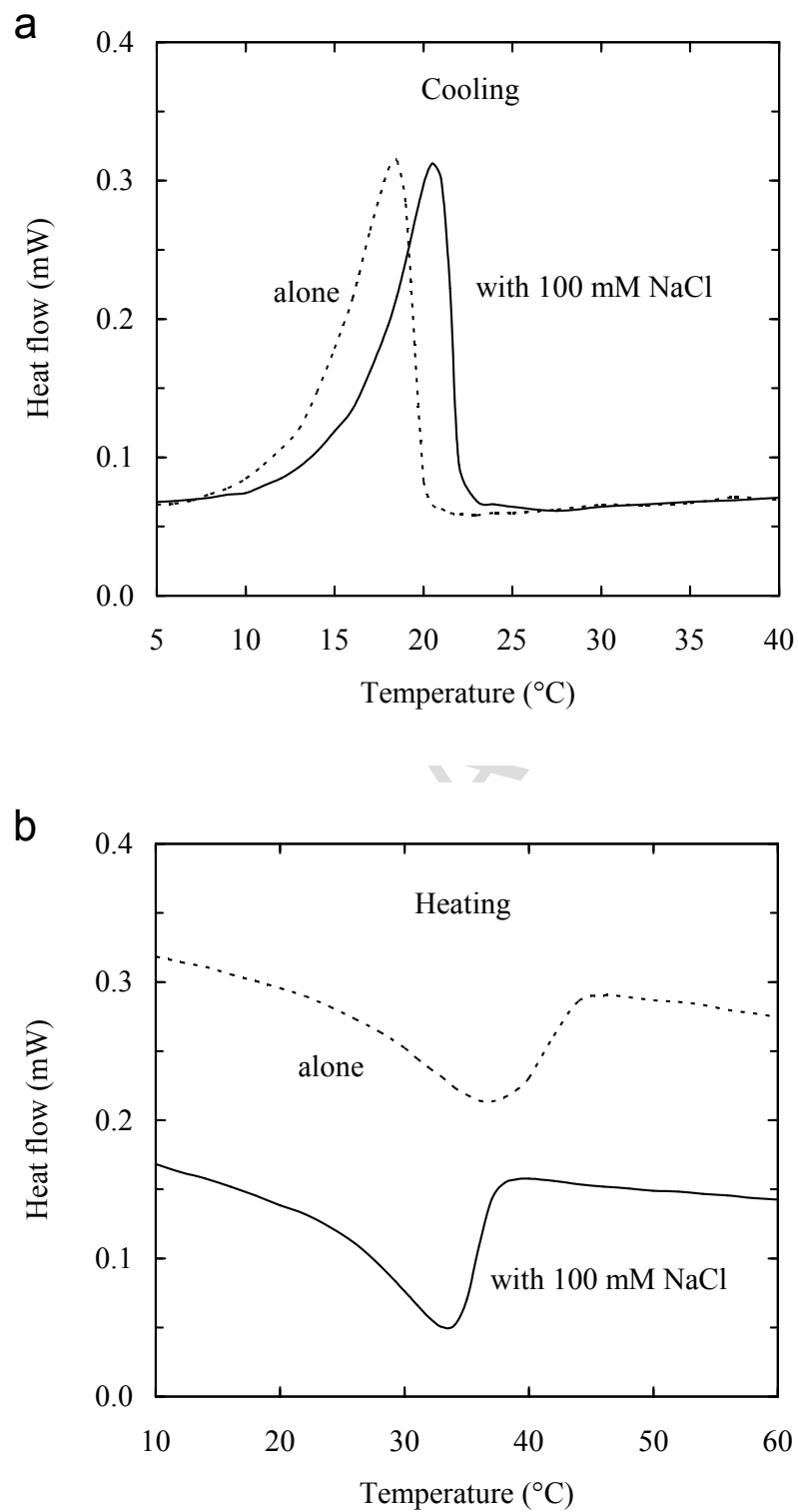
**Fig. 4.** Effect of ionic strength on the extent of thermal hysteresis between DSC cooling and heating scans (Fig. 3) for 1.0 wt % Na<sup>+</sup> kappa carrageenan in the presence of increasing concentrations of NaCl (▲), KCl (■) or CaCl<sub>2</sub> (●).



**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 %  $\text{Na}^+$  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 NaCl (Δ) or  $\text{CaCl}_2$  (○) 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