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Said Elmarhoum, Sophie Mathieu, Komla Ako, William Helbert. Sulfate groups position determines the ionic selectivity and syneresis properties of carrageenan systems. Carbohydrate Polymers, 2023, 10.1016/j.carbpol.2022.120166 . hal-03812613

**HAL Id: hal-03812613**

**<https://hal.science/hal-03812613>**

Submitted on 12 Oct 2022

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# Sulfate groups position determines the ionic selectivity and syneresis properties of carrageenan systems

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## Abstract

The salt sensitivity and selectivity feature of  $\alpha$ -carrageenan ( $\alpha$ -Car) were investigated and compared with  $\kappa$ -carrageenan ( $\kappa$ -Car) and iota-carrageenan ( $\iota$ -Car). These carrageenans are identified by one sulfate group on the 3,6-anhydro-D-galactose (DA) for  $\alpha$ -Car, D-galactose (G) for  $\kappa$ -Car and on both carrabiose moieties (G and DA) for  $\iota$ -Car. The viscosity and temperature, where order-disorder transition have been observed, were greater in presence of  $\text{CaCl}_2$  for  $\alpha$ -Car and  $\iota$ -Car compared with KCl and NaCl. Conversely, the reactivity of  $\kappa$ -Car systems were greater in presence of KCl than  $\text{CaCl}_2$ . Unlike  $\kappa$ -Car systems, the gelation of  $\alpha$ -Car in presence of KCl was observed without syneresis. Thus, the position of sulfate group on the carrabiose determines the importance of counterion valency too. The  $\alpha$ -Car could be a good alternative to  $\kappa$ -Car to reduce the syneresis effects.

**Keywords:** Alpha-carrageenan; Sulfate group position; Anhydro-ring; Viscosity; Salt sensitivity; Syneresis

## 1 Introduction

Numerous applications of natural polymers are limited by the ability of the polymers to give a gel with exudation (syneresis) (Chen, Xu & Wang, 2007; Gonzalez, Loubes, Bertotto, Baeza & Tolaba, 2021; Laplante, Turgeon & Paquin, 2006). Some of the applications are relevant for human health management like cell cultures in hydrogels for disease diagnostics and tissue engineering (Divoux, Mao & Snabre, 2015; Hoffman, 2012; Jafari, Farahani, Sedighi, Rabiee & Savoji, 2022; Peppas, Bures, Leobandung & Ichikawa, 2000; Zhang et al., 2020). To understand the physicochemical causes of syneresis of natural polymers hydrogels, kappa-carrageenan polysaccharide is used as the material of this investigation, because this material exhibits severe syneresis in relatively short experimental time with a dependence to many physicochemical factors (Ako, 2015; Elmarhoum & Ako, 2021). In this study, the target is the sulfate,  $-\text{SO}_3^-$ , ionic group position in the monomer of this polysaccharide.

Carrageenan (Car) defines a family of sulfated galactans extracted from marine algae (Prechoux, Genicot, Rogniaux & Helbert, 2013; Souza, Hilliou, Bastos & Gonçalves, 2011; Usov, 2011). The disaccharide repetition moieties (i.e. carrabiose) of the polysaccharide is made with two galactose units identified as G and DA for respectively D-galactose and 3,6-anhydro-D-galactose, each of which can carry different number of sulfate functional group (Ueda, Saiki & Brady, 2001; Usov, 2011) (Fig.1). The number and position of the sulfate group on the carrabiose identifies the type of carrageenan (Campo, Kawano, da Silva Jr & Carvalho, 2009; Millane, Chandrasekaran & Arnott, 1988; Perez & Claudio, 2020; Souza, Hilliou, Bastos & Gonçalves, 2011; Viebke, Borgström & Piculell, 1995). Kappa-carrageenan ( $\kappa$ -Car) has one sulfate group at the position 4 of the G units and is coded as G4S-DA. Alpha-Carrageenan ( $\alpha$ -Car) has one sulfate group at the position 2 of the DA units and is coded as G-DA2S accordingly (Bourgoin, Zablackis & Poli, 2008). Iota-carrageenan ( $\iota$ -Car) has two

sulfates on the carrabiose units on the same carbon position as for  $\kappa$ - and  $\alpha$ -Car, and thus, is coded as G4S-DA2S. The number, the position and the distribution of ester sulfate along the carrageenans chain define the physicochemical properties of the polysaccharides (Millane, Chandrasekaran & Arnott, 1988; Souza, Hilliou, Bastos & Gonçalves, 2011). As an example, with the same concentration of positive charge counterions, the divalent counterions like calcium ( $\text{Ca}^{2+}$ ) influence strongly the flow behavior of  $\iota$ -Car solution than the monovalent counterions like potassium ( $\text{K}^+$ ) (Bui, Nguyen, Renou & Nicolai, 2019; Elfaruk, Wen, Chi & Li, 2021). Conversely,  $\text{K}^+$  ions change drastically the flow behavior of  $\kappa$ -Car solution compared with  $\iota$ -Car (Lai, Wong & Lii, 2000; Norton & Goodall, 1983b). The selectivity of  $\iota$ -Car and  $\kappa$ -Car to respectively divalent and monovalent counterions and the fact that trivalent counterion induced gelation in  $\lambda$ -Car, which carries three sulfate groups on the carrabiose, lead to claim that the number of sulfate on the carrabiose is what determines the importance of counterion valency (Running, Falshaw & Janaswamy, 2012).

The exact role of the sulfate position on the counterions binding and importance of the counterion valency in carrageenan systems are unclear (Klevens, 1950; Lindman, Karlstrom & Stigsson, 2010; Running, Falshaw & Janaswamy, 2012). The  $\kappa$ -Car hydrogels, for example, exhibit severe syneresis behavior in presence of  $\text{K}^+$  than  $\iota$ -Car and other types of salts which are commonly used in food and biological domains (Thrimawithana, Young, Dunstan & Alany, 2010). Therefore, in order to understand the influence of the sulfate position on the rheological behavior of the carrageenans, we took advantage of the recent discovery of the endo-4O- $\iota$ -carrageenan sulfatase secreted by the marine bacteria *Pseudoalteromonas atlantica* T6c. This enzyme removes specifically the sulfate ester group located at the position 4 of the  $\iota$ -carrabiose moiety and allows completely converting the  $\iota$ -Car into  $\alpha$ -Car (Prechoux, Genicot, Rogniaux & Helbert, 2013). We have investigated the rheological behavior of  $\kappa$ -Car

and  $\alpha$ -Car solution in presence of different types of salts to emphasize the role that the positions of  $-\text{SO}_3^-$  may have in the flow properties of the polysaccharide.

## 2 Material and methods

### 2.1 Samples characteristics

**Molecular weight.** The samples were solubilized in 0.1 M  $\text{NaNO}_3$  and filtered on 0.2  $\mu\text{m}$  membrane. The samples were injected on two OHPak SB 806 M HQ Shodex columns mounted in series and eluted at 30°C in 0.1 M  $\text{NaNO}_3$ . Detection was monitored by a Optilab rEX Wyatt refractometer and a mini DAWN TREOS Wyatt light scattering working at three angles. The  $\text{dn}/\text{dC}$  was 0.142 mL/g. Polydispersity indices was calculated  $\text{Ip} = \text{Mw}/\text{Mn}$ . All measurements were done in triplicate.

**Enzymatic preparation of alpha-carrageenan ( $\alpha$ -Car).** The  $\alpha$ -carrageenan (Fig1) was prepared according to the method reported by (Prechoux, Genicot, Rogniaux & Helbert, 2013) using recombinant 4O-iota-carrageenan sulfatase. Briefly, the genePatl\_0889 (GenBank accession number ABG39415) encoding the sulfatase with a N-terminal poly-histidine tag was over expressed in BL21-DE3 *Escherichia coli* strain and purified on a Nickel affinity column. Iota-carrageenan provided by Danisco (Brabrand, Denmark) was incubated with the purified sulfatase in 50 mM Tris-HCl pH 7.5. Incubation with additional fresh enzymes was repeated until the complete conversion to  $\alpha$ -carrageenan was achieved. The yield of desulfation was measured by  $^1\text{H}$  NMR (Prechoux, Genicot, Rogniaux & Helbert, 2013). The polysaccharide was ultra-filtered on 10 Da membrane in water and lyophilized. The average molecular weight of the polysaccharide was  $\text{M}_w = 3.8 \times 10^5$  g/mol, with an average polydispersity index of  $\text{M}_w/\text{M}_n = 1.78$ .

**Kappa-carrageenan (*k*-Car).** The  $\kappa$ -Car polysaccharide (Fig1) is a gift from Rhodia Food Switzerland, product name and reference: MEYPRO-GEL 01/2001 WG95-37 K-Car. The dialyzed product may contain a weak amount of glucose and was found in the pure potassium  $\kappa$ -Car form of total potassium at no more than 4.2 % (w/w); the other salts were considered to be only trace amounts. The measurement of its molecular weight was done in triplicate. The average molecular weight of the polysaccharide was  $M_w = 3.3 \times 10^5$  g/mol, with an average polydispersity index of  $M_w/M_n = 2.02$ .

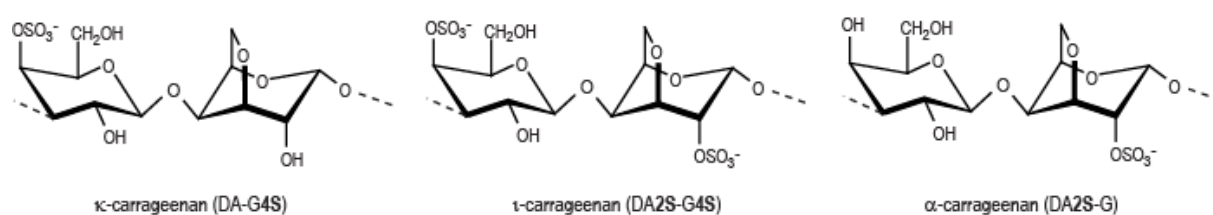


Figure 1: Structure of the carrabiose moieties composing kappa-, alpha- and iota-carrageenan.

## 2.2 Samples preparation

A 20 g/L solution was prepared by dissolving the  $\kappa$ -Car polysaccharides powder in demineralized water with 200 ppm sodium azide as the bacteriostatic agent under heat and stirring conditions. The resulting solution was dialyzed and filtered through 0.45  $\mu$ m pore size. A 9 g/L of solution was prepared by dilution of the 20 g/L solution and was stocked in fridge at 5  $^{\circ}$ C, with which, all the samples were prepared by dilution with demineralized water containing 200 ppm sodium azide with salt at the appropriate concentration. The dilution gives the desired final concentration of polysaccharide and salt concentration at the appropriate temperature. The  $\alpha$ -Car polysaccharide in the lyophilized form were dissolved in demineralized water with 200 ppm sodium azide under heat and stirring conditions to 9 g/l stock solution. The samples were prepared in the same way like the  $\kappa$ -Car using the appropriate salt.

## 2.3 samples characterization

**Viscosity measurements.** The viscosity measurements were performed in Couette geometry using a DHR3 Rheometer (TA Instrument). The Couette geometry consisted of a concentric cylinder geometry of an inner rotor cylinder (bob) and outer stator cylinder (cup) of respectively a radius  $R_1$  of 14 mm and  $R_2$  of 15 mm defining a horizontal gap ( $R_2 - R_1$ ) of 1 mm and an average radius  $R$  of 14.5 mm as  $(R_1 + R_2)/2$ . The height of the bob was 42 mm and the vertical gap between the bob and cup was set at 2 mm. The minimum and maximum torque of the instrument were  $5 \mu\text{Nm}$  and  $5 \times 10^3 \text{ Nm}$  respectively. Both the torque ( $M$ ) and the angular velocity ( $\Omega$ ) of the bob, which were proportional with respectively the stress ( $\sigma$ ) and shear rate ( $\dot{\gamma}$ ), were measured by the rheometer, from which the viscosity ( $\eta$ ) was displayed as  $\sigma / \dot{\gamma}$ . The viscosity was determined as a function of the shear rate from  $0.3 \text{ s}^{-1}$  to  $30 \text{ s}^{-1}$  to define the Newtonian flow domain at a constant temperature in the range between  $5 \text{ }^\circ\text{C}$  and  $60 \text{ }^\circ\text{C}$ . The viscosity was also measured at a constant shear rate of  $15 \text{ s}^{-1}$  while cooling or heating at  $2 \text{ }^\circ\text{C min}^{-1}$  or a lower rate  $0.5 \text{ }^\circ\text{C.min}^{-1}$ .

**Viscoelastic modulus measurements.** The rheological measurements were performed using a DHR3 Rheometer (TA Instrument) with etching conic geometry ( $\text{Ø}50 \text{ mm}$ ,  $2.0^\circ$ , TADC2°InR500) and etching plate. The conic geometry has a truncation gap of  $110.0 \mu\text{m}$ . A portion of the prepared hot sample solution was loaded onto the Peltier of the rheometer and set at a temperature above the gelation temperature of the sample. After loading the sample, the surface was covered with a thin layer of mineral oil to avoid evaporation. Then, the temperature and the measurement program were run. The condition of each measurement will be specified in the text.

### 3 Results and discussion

#### 3.1 Flow behavior of $\alpha$ -carrageenan solution

The viscosity of  $\alpha$ -Car in presence of 80 mM KCl is displayed in Fig. 2a for cooling and heating ramps. The viscosity increases when the temperature is decreased, and sharply from  $\approx 11.5$  °C. The sharp increase of the viscosity was shown by Rochas and Rinaudo (1984) to be concomitant with the coil-helix transition. If the temperature is increased immediately from 5 °C, the viscosity decreases sharply and the helix-coil transition was found at  $\approx 14.3$  °C above the coil-helix transition (Rochas & Rinaudo, 1984). The thermal hysteresis was reported as a result of inter-helical aggregation (Morris, Rees & Robinson, 1980; Piculell, Nilsson & Muhrbeck, 1992).

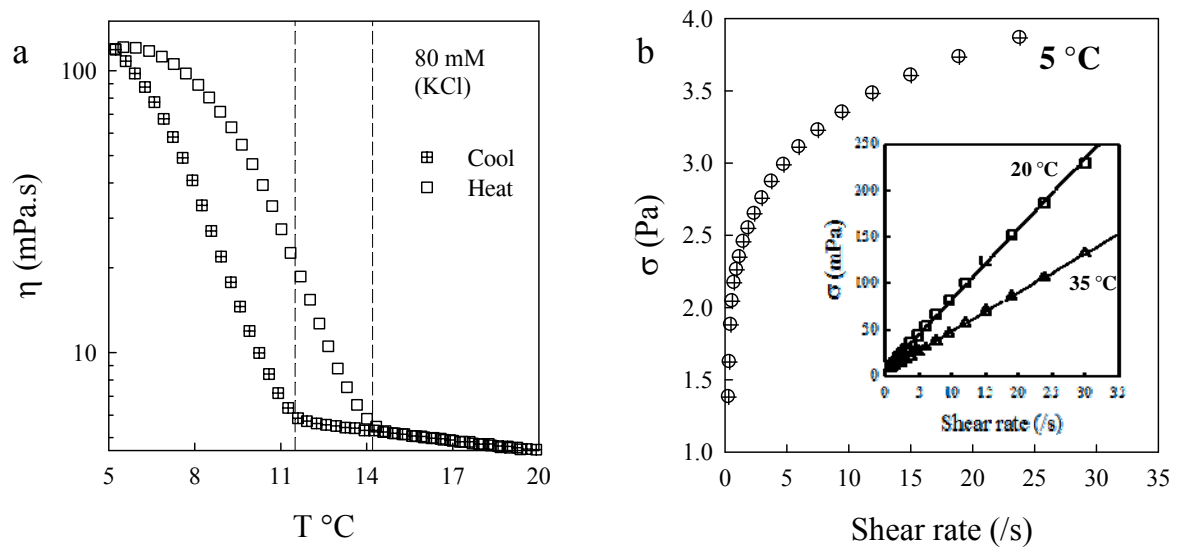


Figure 2: a) temperature dependence of  $\alpha$ -Car solution viscosity for 3 g/L, 80 mM KCl, b) shear rate and stress relation displayed for the solution to show the viscosity behavior, at 5 °C in the helices domain the viscosity changes with the shear rate, in the coil domain the viscosity is constant (insert).

The thermal hysteresis defines three temperature domains which are identified in the Fig.2a as: (1) domain below the coil-helix transition temperature,  $< 11.5\text{ }^{\circ}\text{C}$ , (2) domain between the coil-helix and helix-coil transition temperature,  $[11.5\text{ }^{\circ}\text{C}, 14\text{ }^{\circ}\text{C}]$  and (3) domain above the helix-coil transition temperature,  $> 14\text{ }^{\circ}\text{C}$ . The conformational state of the carrageenan in temperature domain (2) depends on the temperature ramp (cooling or heating) but in the domain (1) or (3) the conformations of the polysaccharide chains were respectively either helices or coils. The shear rate dependence of the applied stress plots is shown in the Fig.2b. A non-linear relationship is observed, at  $5\text{ }^{\circ}\text{C}$ , in the domain below the coil-helix transition temperature and a linear relationship is observed at temperatures in the domain above the helix-coil transition temperature (insert of Fig.2b). We have observed that the flow behavior of  $\kappa$ -Car turns from Newtonian to non-Newtonian flow when the conformations of the polysaccharides turn from coils to helices (Elmarhoum & Ako, 2021). We think that similar conformational change could explain the temperature dependence of the flow behavior of  $\alpha$ -Car. The impact of salt on the flow behavior of carrageenan solutions depends mainly on the kinetics of the helices aggregation and the kinetics increases with decreasing temperature below the coil-helix transition temperature (Meunier, Nicolai, Durand & Parker, 1999). We didn't observe the gelation for  $\alpha$ -Car solutions in the interval of temperature explored in Fig.2a, when in the same KCl and polysaccharide concentration the  $\kappa$ -Car solutions gelled at temperature below  $50\text{ }^{\circ}\text{C}$  with syneresis (Ako, 2015, 2017). A lower helices aggregation kinetics is expected in relation with the weak coil-helix transition temperature in comparison with  $\kappa$ -Car. We didn't exclude the fact that the  $\alpha$ -Car solutions at temperature below  $12\text{ }^{\circ}\text{C}$  behave like a suspension of aggregates (Garrec, Guthrie & Norton, 2013).

### *3.2 Coil-helix and helix-coil transition temperature: effect of salt*

The transition from random coils to helices conformation, which is measured using optical rotation technique, have been observed to be concomitance with the steep rising of the

viscosity (Rochas & Rinaudo, 1984). Therefore, the onset of helix formation during cooling ramp is characterized by the temperature at which the viscosity starts to increase and is denoted as the coil-helix temperature ( $T_{ch}$ ). Salt concentration dependence of the  $T_{ch}$  is shown in the Fig.3. The results for  $CaCl_2$  salt are displayed for  $\alpha$ -Car and the result for KCl salt is displayed in the insert of the Fig.3 for  $\alpha$ -Car and  $\kappa$ -Car. The polysaccharides sensitivity to salt as a polyelectrolyte is expected (Ako, 2015; Ciancia, Milas & Rinaudo, 1997), but the fact that the coil-helix transition of  $\alpha$ -Car is more sensitive to  $CaCl_2$  than KCl was not obvious regarding the literature on  $\kappa$ -Car (Elmarhoum & Ako, 2021). Moreover, the sensitivity of  $\kappa$ -Car to KCl result in a greater coil-helix transition temperature than the sensitivity of  $\alpha$ -Car to both KCl and  $CaCl_2$ . This finding emphasis the importance of the  $-SO_3^-$  position in the monomer as a physicochemical factor, which determines the polysaccharide reaction to salt.

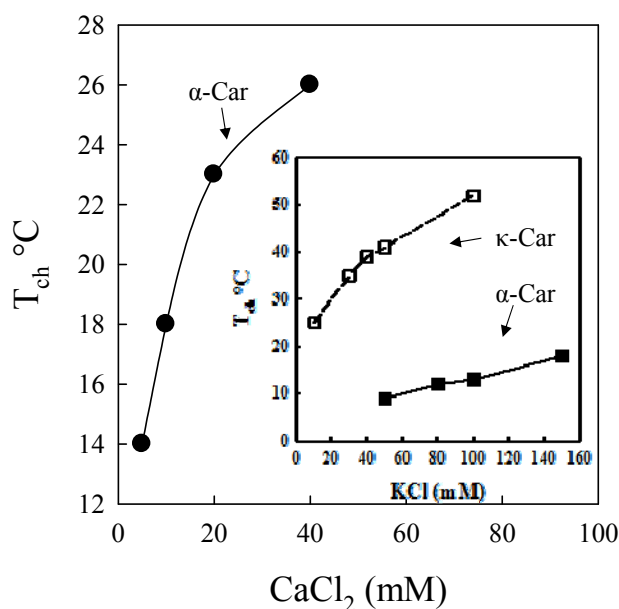


Figure 3:  $CaCl_2$  dependence of the coil-helix transition temperature of  $\alpha$ -Car and in the insert the KCl concentration dependence of the coil-helix transition of  $\alpha$ -Car and  $\kappa$ -Car.

For  $\alpha$ -Car, if  $\text{CaCl}_2$  concentration is increased from 5 mM to 40 mM, the  $T_{\text{ch}}$  increases from  $\approx 14^\circ\text{C}$  to  $\approx 26^\circ\text{C}$ . The  $T_{\text{ch}}$  increases from  $\approx 10^\circ\text{C}$  to  $20^\circ\text{C}$ , if the KCl concentration is increased from 45 mM to  $\approx 150$  mM and the coil-helix transition was not observed for 20 mM KCl concentration, which is two time greater than 5 mM  $\text{CaCl}_2$  in term of the concentration of positive charge counterions. Therefore,  $\alpha$ -Car is more sensitive to divalent than the monovalent counterions. Based on this result, the behavior of  $\alpha$ -Car with one sulfate anionic functional group on its monomer is rather similar to that of  $\iota$ -Car with two sulfates anionic functional groups than  $\kappa$ -Car (Morris, Rees & Robinson, 1980). The  $\kappa$ -Car systems with one sulfate anionic functional group on its monomer react strongly in presence of KCl salt than the  $\alpha$ -Car systems. The  $T_{\text{ch}}$  increases from  $\approx 25^\circ\text{C}$  to  $\approx 50^\circ\text{C}$ , if the KCl concentration is increased from 10 mM to 100 mM, with the hysteresis loop effect.

### 3.3 Monovalent salt sensitivity of $\alpha$ -Car

The fact that the conformational change of  $\alpha$ -Car happens at a greater temperature in presence of calcium than potassium counterions, characterizes its greater sensitivity for the divalent salt than the monovalent salt (Morris, Rees & Robinson, 1980). At any rate, in the conditions where the coils conformations prevail, the viscosity of the solution doesn't depend on the cooling or heating ramps and the solution flow is Newtonian. In this section we have tried to bring some more results on the sensitivity of the polysaccharide for monovalent salts like; NaCl, KCl and KI.

In the Fig.4a, are shown the temperature dependence of the viscosity for the solution of 3 g/L as the reference (ref.) to show the effect of 10 mM of the selected monovalent salt. The results show a decrease of the viscosity in presence of the salts with no particular effect due to the type of salt for the concentration of 10 mM. Moreover, the KCl salt concentration is not enough to induce the coil-helix transition in the temperature range of the measurements as it

was observed for  $\kappa$ -Car. Based on the fact that, the temperature dependence of  $\kappa$ -Car solutions viscosity for 10 mM of NaCl, KI or KCl are different (Ciancia, Milas & Rinaudo, 1997), we didn't expect that the temperature dependence of  $\alpha$ -Car solutions viscosity being similar. We cannot explain the difference between the behavior of  $\alpha$ -Car and  $\kappa$ -Car, without considering the sulfate group position. If the NaCl concentration is increased to 100 mM, the solutions exhibited a coil-helix transition around 15 °C and a helix-coil transition around 17 °C. This may appear to be a small hysteresis but when scan rate dependence is accounted for these would be superimposable (Fig.4b). In presence of 100 mM of KCl, the coil-helix transition is observed around 12 °C and two transitional temperatures were observed for the heating ramp; the first one around 17 °C and the second one correspond to the helix-coil transition around 42 °C.

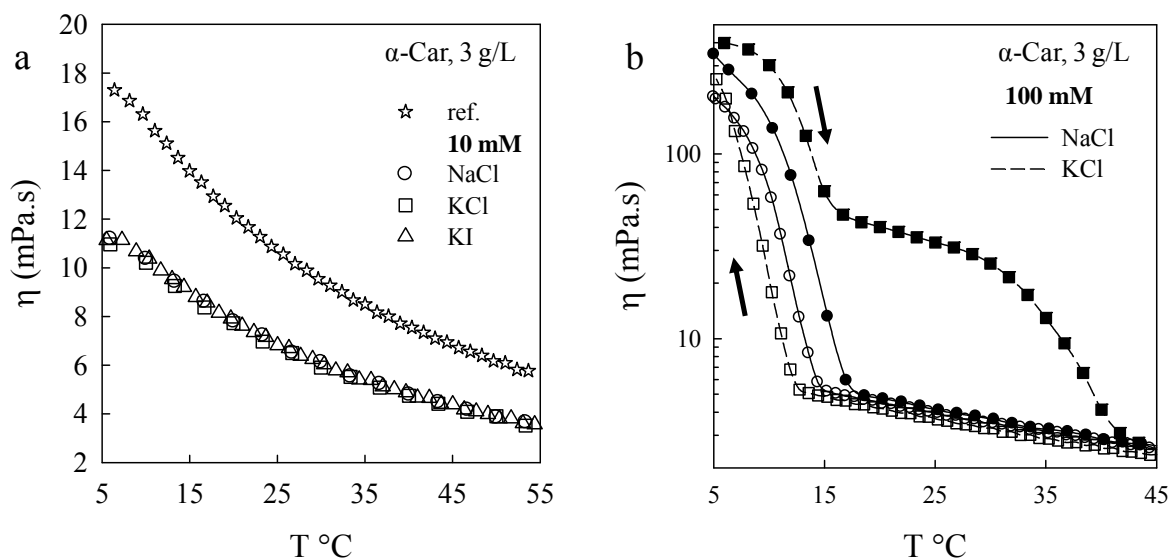


Figure 4: temperature dependence of the viscosity of  $\alpha$ -Car solution, 3 g/L in presence of a) 0 mM (ref.) and 10 mM of NaCl, KCl or KI, the cooling and heating ramps give similar results and b) 100 mM of NaCl or KCl, the cooling (open symbols) and heating (full symbols) results are displayed.

The thermal hysteresis of the solution of  $\alpha$ -Car with 100 mM KCl indicates an extensive aggregation of the helices which led the conserved solution to gel at 5 °C. The solution with 100 mM NaCl didn't gel after several days at 5 °C. The shift of the coil-helix transition temperature  $T_{ch}$  from 12 °C to 15 °C, when KCl is replaced by NaCl, leads to say that the conformational change of  $\alpha$ -Car would be rather sensitive to NaCl than KCl. The role of monovalent salt to promote helix formation and then gelation for  $\kappa$ -Car systems was reported to follow the sequence  $RbCl > CsCl, KCl > NaCl > LiCl, HCl$  (Ciancia, Milas & Rinaudo, 1997; Rochas & Rinaudo, 1984). The superiority of the potassium on sodium to induce coil-helix conformational change of  $\kappa$ -Car is inversed in the case of  $\alpha$ -Car. This inversion may indicate that the anhydro-ring promotes the accessibility to the sulfate group on the 3,6-anhydro-D-Galactose unit by small size counterions. However, extensive aggregation of the helices and gelation in presence of 100 mM KCl, indicate that the ion-pair formation of  $K^+$  with the  $-SO_3^-$  is a time dependent process (Grasdalen & Smidsrod, 1981; Norton & Goodall, 1983a). The reduction of the sulfate charge by counterions would be fast for  $\kappa$ -Car, and thus, promotes immediate coil-helix transition and aggregation of the helices to gelation.

### *3.4 Influence of the degree of carrageenan sulfation*

The gelation of different types of mono-composite (MC) of carrageenan polysaccharides made of  $\alpha$ -carrabiose and  $\iota$ -carrabiose on the same backbone are proposed in this section to show how the degree of conversion of  $\iota$ -Car to  $\alpha$ -Car can influence the coil-helix transition temperature. The gelation of a bi-composite (BC) solution, which is a blending of  $\alpha$ -Car and  $\iota$ -Car solution to the proportion of 67 wt.% and 33 wt.% respectively, is also shown. Given that the  $\alpha$ -Car is obtained by conversion of  $\iota$ -Car, the result of the bi-composite will indicate the influence that could have a few content of  $\iota$ -Car in the  $\alpha$ -Car solution on the coil-helix transition temperature (Piculell, Nilsson & Muhrbeck, 1992). The concentration of all the

solutions is 6 g/L and were prepared with 20 mM of  $\text{CaCl}_2$  then cooled to give the results in Fig.5. The temperature dependence of the  $G'$  of the solutions were measured during the cooling ramp. The result of the BC solution is marked with a star in the legend of Fig5.b. The gelation of carrageenan systems is a time dependent process which is connected to the helices aggregation kinetic, thus the gelling point as the point where  $G' = G''$  depends on the scan rate. The onset of elastic behavior of aqueous iota-carrageenan has been observed by Piculell et al., (1992) to closely follow the onset of helix formation, and the modulus of elasticity increases monotonically with an increasing helical content (Piculell, Nilsson & Muhrbeck, 1992). Therefore, if  $G' > G''$  is observed, the temperature at which the helices are formed, is considered here as the gelation temperature. In this section, we have worked in the conditions where for all the samples,  $G' > G''$  was observed during the cooling ramps.

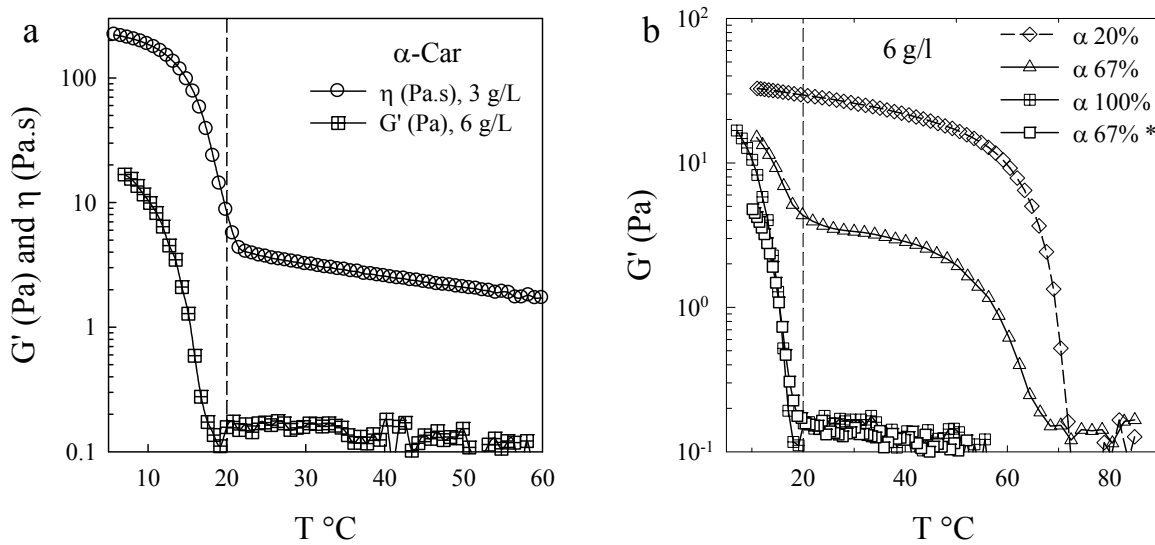


Figure 5: Temperature dependence of the  $G'$  and the viscosity of a)  $\alpha$ -Car in presence of 20 mM  $\text{CaCl}_2$  and b) different composition of  $\alpha$ -Car and  $\iota$ -Car carrabiose in the polysaccharide backbone. The composition  $\alpha$  67 %\* is a solution made with 67 wt% of  $\alpha$ -Car and 33 wt% of  $\iota$ -Car. The solutions were prepared with 20 mM  $\text{CaCl}_2$ .

275 In Fig.5a, the gelation temperature is observed to be almost equal to the temperature at which  
276 the G' of the system increases sharply while cooling the solution. We can see that the domain  
277 of temperature where the viscosity increases coincides with that of G', which we said is  
278 concomitant with the formation of helices (Belton, Chilvers, Morris & Tanner, 1984; Ciancea,  
279 Milas & Rinaudo, 1997; Elmarhoum & Ako, 2021; Meunier, Nicolai, Durand & Parker, 1999;  
280 Norton & Goodall, 1983b; Rochas & Rinaudo, 1984). The  $\alpha$ -Car solution and the solution of  
281 67 wt.%  $\alpha$ -Car with 33 wt.%  $\iota$ -Car, indicated in the Fig.5b respectively as  $\alpha$  100 % and  $\alpha$  67  
282 %\*, show the same gelation temperature. Therefore, presumably a few content of  $\iota$ -Car in the  
283  $\alpha$ -Car solution would not affect the coil-helix transition temperature of  $\alpha$ -Car and its  
284 sensitivity to salt. The thermal behavior of  $\alpha$ -Car solution seems to be very stable regarding a  
285 small variation of  $\iota$ -Car concentration in wt.%. Considering the molecular weight of each  
286 composite of the blending solution, the 67 wt.% of  $\alpha$ -Car may correspond to 90 mol%, which  
287 means that one molecules of  $\iota$ -Car is surrounded by nine molecules of  $\alpha$ -Car and this could  
288 explain the similarity between the thermal properties of the 100 mol% and 90 mol%  $\alpha$ -Car  
289 solution. In the MC polysaccharide, the proportion of  $\alpha$ -carrabiose and  $\iota$ -carrabiose are in  
290 mol% and 67 % of  $\alpha$ -Car in the MC shows the gelation temperature of the part related to both  
291  $\alpha$ -Car and  $\iota$ -Car. This result, may indicate that the coil-helix transition would be rather an  
292 intramolecular mechanism. In the condition where the  $\alpha$ -carrabioses are randomly distributed  
293 along the hybrid carrageenan chain (Prechoux, Genicot, Rogniaux & Helbert, 2013), we  
294 should expect one gelation temperature. However, the probability to have a bloc of  $\alpha$ -  
295 carrabioses along the hybrid chain which behave like a free chain of  $\alpha$ -Car is increased by  
296 increasing the degree of  $\iota$ -Car conversion to  $\alpha$ -Car and this fact seems to correspond to the  
297 results in Fig.5b. Below 50 % of  $\alpha$ -Car in the MC, the gelation temperature of  $\alpha$ -Car was not  
298 clearly detectable. Supposed that the 20 mol% / 80 mol% of  $\alpha$ -/ $\iota$ -Car were distributed like a  
299 bloc copolymer, then the temperature dependence of G' would have shown distinctly the

gelation temperature of  $\alpha$ -Car and  $\iota$ -Car. Bui et al., (2019) have mixed 50 wt.% of  $\kappa$ -Car and 50 wt.% of  $\iota$ -Car in 50 mM  $\text{CaCl}_2$  aqueous solution and they have observed two distinct gelation temperatures of the BC solution indicating independent coil-helix transition (Bui, Nguyen, Renou & Nicolai, 2019). However the authors observe that  $\kappa$ -Car network is more homogeneous in the mixed gels than in the corresponding individual gels and the mixed gels were stiffer than the individual gels. The independent coil-helix transition of the polysaccharide which occurs in both BC and MC systems may indicate that the reduction of the polymers net charge is preferentially intramolecular by which the helices and aggregates are formed.

The claim of intramolecular mechanism to explain the reduction of the polysaccharide charge or ion-pair formation could also refer to the finding of Rochas and Rinaudo (1984). The authors found that the ionic selectivity for potassium counterions work also even if the  $\kappa$ -Car is in the coil conformation. If so, then the coil-helix conformational change may take place in concentration well below the critical concentration, where the polysaccharide are supposed to be individual. Hence, the polysaccharide conformation would be single helix. However, the authors have rejected the single-helix formation presumably because they didn't observe a specific role of the potassium ion in the crosslinking mechanism of the polysaccharide. It is not clear the way the intramolecular charge neutralization mechanism conciliates the hypothesis of double helices and the aggregation of the helices in BC and MC model, except if the double helices are considered as aggregate of two single-helices.

We can note the absence of peak or maximum point for the  $G'$  plots in Fig.5, which several authors reported for  $\kappa$ -Car gel systems as characteristic of slipping effect due to sever syneresis (release of liquid phenomena by the gels). The gelation of  $\kappa$ -Car solutions with  $\text{K}^+$ ,  $\text{Cs}^+$ ,  $\text{Ca}^{2+}$  gives gels with syneresis was reported by several authors (Ako, 2015; Lai, Wong & Lii, 2000; Piculell, Borgström, Chronakis, Quist & Viebke, 1997; Richardson & Goycoolea,

1994). The salt concentrations at which the syneresis was observed are relatively very weak compared with ι-Car (Piculell, Nilsson & Muhrbeck, 1992; Thrimawithana, Young, Dunstan & Alany, 2010) and if the syneresis mechanism may exist for α-Car, then it should be caused by salt concentrations larger than those used for κ-Car and presumably over the experimental time conditions of this study. The gels coarsening and contraction cause syneresis in general, but in condition which is determined by the polysaccharide net charge reduction or neutralization, extensive helices aggregation induced syneresis reflects the salt ability to neutralize or reduce the intra and/or intermolecular repulsion forces. Therefore, sulfate group position on D-galactose unit is more favorable to neutralization by salt than the sulfate group position on 3,6-anhydro-D-galactose unit.

### 3.5 Discussion

Alpha-carrageenan and kappa-carrageenan are characterized respectively, by a mono sulfate group on the 3,6-anhydro-D-galactose unit and a mono sulfate group on the D-galactose unit. Zablackis et al., have studied the carrageenan extracted from *Catenella nipae Zanardini*, which according to the work of Falshaw et al., (1996) may correspond to hybrid α-/ι-Car of variable proportion (Bourgoin, Zablackis & Poli, 2008; Falshaw, Furneaux, Wong, Liao, Bacic & Chandkrachang, 1996), and the content of α-carrabiose in the native hybrid polysaccharide would not exceed 40 mol% (Prechoux, Genicot, Rogniaux & Helbert, 2013). The sodium form of this polysaccharide (in 100 mM NaCl) did not gel but exhibit a greater viscosity than κ-Car (Zablackis & Santos, 1986), while the sodium form of ι-Car (in 100 mM NaCl) has been observed to form a gel (Piculell, Nilsson & Muhrbeck, 1992). In presence of salt the viscosity of the solutions of α-Car and κ-Car depends on the degree of their helices formation and aggregation. However, the decrease of the gelling capacity when the proportion of α-carrabiose increases in the hybrid polysaccharide indicates that the sulfate on the anhydro-D-galactose unit is less favorable for the inter-helical aggregation compared with the

sulfate on the D-galactose unit. Therefore, lower inter-helical aggregation activity and higher inter-helical aggregation activity, respectively, characterize the function of the sulfate on the anhydro-D-galactose unit and the function of the sulfate on the D-galactose unit, which explain, respectively, the weak gelling capacity of  $\alpha$ -Car and the strong gelling capacity of  $\kappa$ -Car.

The viscosity of the solution of 3 g/L of  $\alpha$ -Car and  $\kappa$ -Car at 20 °C were respectively 12.2 mPa.s and 7.8 mPa.s. The viscosity of the free salt solution of  $\alpha$ -Car is observed to be greater than the viscosity of the free salt solution of  $\kappa$ -Car at a similar molecular weight, polysaccharide concentration and temperature conditions. Intrachain repulsive forces and water molecules binding capacity would be greater for  $\alpha$ -Car than  $\kappa$ -Car (Ako, Elmarhoum & Munialo, 2022), and accordingly, the sulfate on the anhydro-D-galactose unit promotes more expansion of the polysaccharide than the sulfate on the D-galactose unit.

It is appropriate to emphasize that the  $\alpha$ -Car system ionic selectivity for  $\text{CaCl}_2$  rather than  $\text{KCl}$  was not expected, because the  $\kappa$ -Car system, which could derive from  $\iota$ -Car too, is known to be more sensitive to  $\text{KCl}$  than  $\text{CaCl}_2$ . Therefore, the sensitivity of carrageenan systems to counterions is not only a matter of number of sulfate groups on the carrabiose (Running, Falshaw & Janaswamy, 2012) but a result of many physicochemical factors. The size of hydrated counterions is quoted to play a role in the distance between the cation and the sulfate group (Piculell, Nilsson & Muhrbeck, 1992). The cation mobility can also affect the ion-pair formation stability or equilibrium constant, i.e., the small size cations could have a greater mobility, and thus, lead to a less stable or strong ion-pair formation (Ciancia, Milas & Rinaudo, 1997; Grasdalen & Smidsrod, 1981). Although  $\alpha$ -Car and  $\iota$ -Car system are rather sensitive to  $\text{CaCl}_2$ , the conformational transition temperature of  $\alpha$ -Car systems is found well below that of  $\iota$ -Car systems, which reflect the intramolecular characteristic of the charge reduction mechanism in carrageenan systems. The ability of salt to reduce the charge carried

by carrageenan polysaccharides could be pictured as an in-to-out process. The in-to-out neutralization process means that the system tries to neutralize first the charge carried by the carrabiose (intramolecular process), and if this fails like in the case of  $\alpha$ -Car with calcium, the system tries to neutralize the charge carried by the carrageenan chain (intrachain process). In the intrachain process or interaction between carrabiose of same chain, if a block of carrabiose behave the same, like a block of  $\alpha$ -Car in  $\alpha$ -/ $\iota$ -Car hybrid polysaccharide, then the conformation transition characterizing the block could be detectable. The neutralization process could continue or stop by interchain/intermolecular process (aggregation), in this process the two sulfates or carrabiose which bind the multi valence counterions do not belong to the same chain.

#### 4. Conclusion

The importance of counterions in the helical formation of carrageenan depends on many physicochemical factors. We show in this treatise that the influence of the counterion valency depends on the position and number of the sulfate group carried by the carrabiose. The sulfate on the 3,6-anhydro-D-galactose unit, which characterizes  $\alpha$ -carrageenan (G-DA2S), is rather sensitive to divalent counterions and the sulfate group on the D-galactose unit, which characterizes  $\kappa$ -carrageenan (G4S-DA), is rather sensitive to monovalent counterions. The two types of sulfate, which characterize  $\iota$ -carrageenan (G4S-DA2S), is more sensitive to divalent counterions than the one type of sulfate carried by  $\alpha$ -carrageenan (G-DA2S). The temperature dependence of the  $G'$  of  $\alpha$ -/ $\iota$ -carrageenan, as a carrageenan block copolymer system in  $\text{CaCl}_2$  solution, has shown distinctly the helical formation temperature of  $\alpha$ -carrageenan and  $\iota$ -carrageenan. Based on the results of this study, the counterion influence, which we qualify as in-to-out process, would be preferentially and gradually intramolecular, then intrachain and finally interchain process.

## 5 Acknowledgements

We thank Vincent VERDOOT for his technical assistance with the rheometer instruments. We thank Laurine Buon and Eric Bayma from Cermav for their kind assistance with the products composition. This work was financially supported by I-MEP2 PhD graduate school and the PolyNatCarnotInstitut. The LaboratoireRhéologie et Procédés (LRP) is part of the LabEx Tec 21 (Investissementsd’Avenir - grant agreement n°ANR-11-LABX-0030) and of the PolyNat Carnot InstitutInvestissementsd’Avenir - grant agreement n°ANR-11-CARN-030-01).

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