Understanding nanostructural differences in hydrogels from commercial carrageenans: Combined small angle X-ray scattering and rheological studies

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ABSTRACT

Hydrogels from commercial carrageenans (κ-C, ι-C, λ↑-C (high viscosity) and λ↓-C (low viscosity)) were prepared with and without the addition of salts (KCl and CaCl$_2$). FT-IR and ^{1}H NMR characterization evidenced that while the κ-C and ι-C grades were relatively pure carrageenans, the two λ-C grades were λ-, κ-, θ- and μ-carrageenan hybrids. The effect of carrageenan and salt concentration on the hydrogel strength were evaluated through a response surface design and a detailed structural characterization was carried out by small angle X-ray scattering (SAXS) and rheology. The low amount of sulphate substitution in κ-C enabled intramolecular association, giving rise to strong hydrogels, even in the absence of salts. On the other hand, ι-C, λ↑-C and λ↓-C produced much weaker hydrogels and required the addition of salts to induce intramolecular association by ionic cross-linking. SAXS results suggested the formation of similar structures of double helices in κ-C and ι-C with the addition of salts; however, distinct network structures were attained. In the case of κ-C, a Gauss-Lorentz gel model was suitable to describe the hydrogel structure and the addition of K$^+$ promoted the formation of more ordered and densely packed structures. On the other hand, larger but weaker aggregates, with marked periodicity, were observed in ι-C, with Ca^{2+} inducing the formation of more densely packed networks. The complex composition of the λ-C grades gave rise to more heterogeneous branched network structures, properly described by a correlation length model, where the gelation mechanism was mostly governed by the κ-carrageenan component.

1. Introduction

Carrageenans are sulphated polysaccharides present in the cell walls and in the intercellular matrix of red seaweeds (*Rhodophyta*). These polysaccharides contain a backbone of alternating disaccharide repeating units of β-D-galactose (G-units) linked at position 3 and α-D-galactose (D-units) or 3,6-anhydro-α-D-galactose (DA-units) linked at position 4. Depending on the content and position of sulphate groups (SO$_3{}^-$), they can be classified into different forms, being the kappa (κ)-, iota (ι)- and lambda (λ)-carrageenans, which contain one, two and three sulphate groups per disaccharide unit, respectively, the three main types commercially available. These carrageenans are obtained from different seaweed species and using different extraction procedures. κ-Carrageenan is typically extracted from the *Eucheuma cottonii* seaweed species, while ι-carrageenan is mainly obtained from *Eucheuma spinosum* [1]. Their extraction process involves the application of

physical separation methods, followed by alkali treatments at high temperatures to transform the naturally occurring μ- and ν-carrageenans (whose backbones are composed of G- and D-units) into κ- and ι-carrageenans. The alkali treatments produce the removal of one sulphate group from the D-units, originating an anhydride bridge and giving rise to the formation of DA-units. It should be noted that most of the extraction protocols described in the literature typically yield κ-/ι-hybrids rather than pure carrageenans [2–4]. On the other hand, λ-carrageenan is obtained from different species of the *Gigartina* and *Chondrus* genera, although the materials obtained from these seaweeds are typically hybrids of several types of carrageenan [1,5–7].

Carrageenans are widely used in the food industry due to their texturizing properties, highlighting their thickening and gelling capacity and their ability to stabilize proteins [8,9]; additionally, they also find applications in the pharmaceutical [10], cosmetic [11], printing and textile industries [12]. Of special interest is their use for the

production of hydrogels. Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of holding large amounts of water. In the food industry, hydrogels are used as control release systems and as additives for satiety control and texture modification in food formulations [13–16]. The formation of carrageenan hydrogels is a complex process that depends on the proportion and location of sulphate groups, the carrageenan concentration and the presence of counter ions, amongst others. These factors will determine the viscosity of solutions and the strength of the hydrogels formed [17,18]. The gelation of carrageenans is generally explained by the 'domain model' [19], which describes gelation as a two-step process: (i) the transition from random coils in the solution state to double helices, followed by (ii) the aggregation of double helices to form cross-linking domains which originate the hydrogel network structure [20]. This general mechanism seems to be valid for the most studied gelling carrageenans (e.g. κ- and ι-); however, these two carrageenan types form hydrogels with very distinct properties: whereas κ-carrageenan forms hydrogels that are hard, strong and brittle, ι-carrageenan forms soft and weak hydrogels [21]. This must be attributed to structural differences in the hydrogel network, which in turn, should be the result of different processes taking place upon gelation. The presence of the anhydro-galactose bridge in the 4-linked galactose residue (as in κ- and ι-carrageenans) has been proposed to be crucial for the formation of the helical structures, initiating the gelation process [1,7]. In the case of λ-carrageenan, lacking the 3,6-anhydro bridge, the C2-sulphate group from the 4-linked residue is oriented towards the internal part, thus hindering the formation of double helices [1]. As a result, λ-carrageenan is thought to adopt a coil conformation whatever the temperature conditions and is unable to form hydrogels [7], although it can be used as thickening agent due to its high viscosity. Furthermore, due to the existence of electrical charges in the carrageenan chains, provided by the sulphate groups, the presence of cations plays a very important role in the gelation process [22]. While monovalent cations have been seen to promote more efficiently hydrogel formation than divalent cations in κ-carrageenan [22–25], divalent cations have been proposed as more suitable for the formation of stronger ι-carrageenan hydrogels [21,26]. The exact mechanism through which specific cations interplay in the gelation mechanism of κ- and ι-carrageenan is still not fully understood and deserves further investigation. Moreover, λ-carrageenan has also been claimed to show gel-like behaviour in presence of trivalent cations [27]; the authors proposed that the neutralization of the sulphate groups by the trivalent cations induced the formation of the hydrogel network structure, which goes against the hypothesis of double helices being essential for the gelation process.

Given the relevance of the hydrogel properties for practical applications, it is of importance to evaluate how the network structure is modified depending on the type of carrageenan and type of cation used. While there are several studies which have focused on these issues for a specific carrageenan type, models for the mechanisms driving the gelation processes of commercial carrageenans, which are typically hybrid non-purified carrageenans, are still lacking. In this context, the aim of this work was to implement a proper characterization protocol, based on the combination of different techniques, to determine the composition and structural characteristics of different commercial carrageenan grades and optimize the formulation of hydrogels in the presence of mono- and divalent cations (K^+ and Ca^{2+}). For this purpose,

the composition of the commercial grades was firstly studied by means of spectroscopy (FT-IR and ^{1}H NMR) and the influence of the concentration of carrageenan and salts in the hydrogels formed were evaluated through a response surface design, obtaining the optimal formulations for the strongest hydrogels. Moreover, a detailed structural characterization of selected carrageenan hydrogels was carried out using small angle X-ray scattering (SAXS) and rheology. Based on these results and on the models proposed in the literature for the gelation of pure carrageenans, the gelation mechanism for each commercial grade was proposed, which will be particularly useful for the design of hydrogels with target properties for their use in food-related applications.

2. Materials and methods

2.1. Materials

Different commercial carrageenan grades, namely, κ-carrageenan (Ceamgel 90-093) (extracted from *Euchema cottonii*), ι-carrageenan (Ceamvis 3383) (extracted from *Euchema spinosum*) and λ-carrageenan (low viscosity grade Ceamlacta 2270 and low viscosity grade Ceamlacta 2170) (extracted from *Gigartina pistillata* (from Chilean origin for the low viscosity grade and Moroccan origin for the high viscosity grade)), in the form of powders, were kindly donated by CEAMSA (Pontevedra, Spain). The cationic composition of the commercial carrageenans was determined by inductively coupled plasma mass spectrometry (ICP-MS) for the measurement of Ca^{2+}, Na^+, K^+ and Mg^{2+}. The samples were subjected to an acid digestion in an oven at 200 °C and analysed using an Agilent 7900 instrument.

$CaCl_2 \cdot 2H_2O$ and KCl were obtained from Panreac and Sigma-Aldrich (Spain), respectively.

2.2. Preparation of carrageenan hydrogels

Hydrogels from the different commercial carrageenan grades (κ-carrageenan (κ-C), ι-carrageenan- (ι-C) and λ-carrageenan with high (λ↑-C) and low viscosity (λ↓-C)) were prepared with and without the addition of two different salts (KCl and $CaCl_2$). The range of carrageenan and salt concentrations tested (cf. Table 1) were established by preliminary experiments and a central composite design was carried out for each combination of carrageenan type and salt. The required amount of carrageenan powder was added to distilled water and heated up to 90 °C for 30 min. After that, the required amount of salt was added and the solutions were stirred until the salt was completely dissolved. Aliquots of the hot solutions were directly transferred to quartz capillaries for SAXS analyses or to the rheometer plate. For the penetration tests, the hot solutions were transferred to cylindrical containers (30 mm diameter, 40 mm height) and were cooled down to room temperature and subsequently stored at 4 °C for 24 h prior to the analyses.

2.3. Hydrogel strength

The hydrogel strength was evaluated at room temperature (20–25 °C) through penetration tests in a texture analyser (Stable Micro Systems model TA-XT2, Surrey, UK) equipped with a cylindrical Teflon

Table 1
Minimum and maximum level considered in the study for carrageenan and salt concentrations (% wt./v).

	κ-C			ι-C			λ↑-C			λ↓-C			
	κ	KCl	CaCl₂	ι	KCl	CaCl₂	λ↑	KCl	CaCl₂	λ↓	KCl	λ↓	CaCl₂
Min	1.0	0.0	0.1	1.0	0.5	0.1	1.0	0.25	0.25	1.0	0.5	2.0	0.5
Max	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	3.0	2.5	3.0	2.5

plunger (1 cm diameter) and operating at a penetration rate of 1 mm/s to a depth of 5 mm. All measurements were performed, at least, in triplicate.

2.4. Statistics

A statistical central composite design was carried out to evaluate the influence of the carrageenan concentration (C) and the concentration of two different added salts (KCl and CaCl$_2$) (S$_{KCl}$ and S$_{CaCl2}$) in the hydrogel strength (HS). The considered ranges for each variable in this model, which were determined empirically through preliminary tests, are shown in Table 1. According to the central composite design, 13 runs for each combination of carrageenan type and type of salt were carried out. The matrix designs and the graphical analysis were performed using IBM SPSS Statistic software (v.24) (IBM corp., USA) and Matlab (Mathworks, Inc., USA), respectively.

The response values were predicted by the quadratic polynomial equation as follows:

$$\gamma = \beta 0 + \sum_{i=1}^{k} \beta_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i \neq j}^{k} \beta_{ij} X_i X_j \tag{1}$$

where γ corresponds to the predicted response: β_0, β_i, β_{ii} and β_{ij} were the constant regression coefficients of the model; X_i and X_j represent the independent variables. The results obtained were analysed by ANOVA to investigate the significance and fitness of the model, as well as the effect of significant individual terms and the interactions on the responses. The optimization of the response was obtained using a response surface methodology.

2.5. Oscillatory rheological measurements

The rheological measurements were performed on a DHR-3 rheometer from TA instruments (USA) using a cone-plate geometry (4 cm diameter, 1° angle and 26 μm of gap). Temperature was controlled using a Peltier plate. The cone was equipped with a solvent trap and an evaporation blocker from TA Instruments. In addition, the samples were covered with a layer of paraffin oil. Freshly prepared hot carrageenan solutions were loaded onto the rheometer, which plate was pre-heated to 90 °C. After an equilibration time of 5 min, a cooling step from 90 to 20 °C was performed at a constant rate of 1 °C/min. The storage (G′) and loss (G″) moduli were recorded at a strain of 1% and frequency of 1 Hz. Additionally, the κ-C hydrogel with 1.5% KCl added was also subjected to the same cooling step but with lower strains of 0.05% and 0.1% and frequency of 1 Hz. A rough plate-plate geometry (4 cm diameter and 300 μm of gap) was also used to run the sample at a strain of 0.5% and frequency of 1 Hz.

2.6. High performance size exclusion chromatography (HPLC-SEC)

The molecular weight of the different carrageenan grades was estimated by HPLC-SEC analyses, following a method adapted from [28]. The HPLC system was equipped with a Waters 2695 separations module and a Waters 2414 refractive index detector (Waters, USA). The samples (1 mg/mL) were dissolved in the mobile phase (0.01 M LiCl) at 45 °C for 1 h, filtered through 0.8 μm pore syringe filters for aqueous media (Sartorius, Germany) and injected into an OHpak SB-806 HQ (8 mm × 300 mm) SEC column (Shodex, Japan) equilibrated at 45 °C. The injection volume was 20 μL and the flow rate was 0.5 mL/min. Calibration was performed using P-82 pullulan standards (Shodex, Japan), and peak molecular weights (M$_p$) are reported.

2.7. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra from the commercial carrageenan powders were recorded in attenuated total reflectance (ATR) mode in a controlled

chamber at 21 °C and dry air to avoid humidity and CO$_2$ using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm^{-1} resolution in a wavelength range between 650 and 4000 cm^{-1} and averaging a minimum of 32 scans.

2.8. ^{1}H NMR analysis

The ^{1}H NMR experiments were performed at a ^{1}H frequency of 500.13 MHz using a Neo-500 Bruker spectrometer, equipped with a 5 mm BBO-F-plus probehead. The samples were dissolved in D$_2$O at 70 °C, at a concentration of 1% (wt./v), and transferred to 5 mm NMR tubes. The spectra were acquired at 70 °C, with a 30° pulse sequence (pulse 90° 10 μs), with 2 s of relaxation delay and 256 scans. All chemical shifts were referenced to residual non-deuterated solvent (4.82 ppm).

2.9. Small angle X-ray scattering (SAXS)

SAXS experiments were carried out in the Non Crystalline Diffraction beamline, BL-11, at ALBA synchrotron light source (www.albasynchrotron.es). Aliquots of carrageenan solutions (containing 2% (wt./v) of carrageenan and 1.5% (wt./v) of salt) were placed in sealed 2 mm quartz capillaries (Hilgenberg Gmbh, Germany) and were left to cool down at 25 °C for 24 h to form gels prior to the experiments. The energy of the incident photons was 12.4 keV or equivalently a wavelength, λ, of 1 Å. The SAXS diffraction patterns were collected by means of a photon counting detector, Pilatus 1 M, with an active area of 168.7 × 179.4 mm^2, an effective pixel size of 172 × 172 μm^2 and a dynamic range of 20 bits. The sample-to-detector distance was set to 6425 mm, resulting in a q range with a maximum value of q = 0.23 Å^{-1}. An exposure time of 0.5 s was selected based on preliminary trials. The data reduction was treated by pyFAI python code (ESRF) [59], modified by ALBA beamline staff, to do on-line azimuthal integrations from a previously calibrated file. The calibration files were created from a silver behenate (AgBh) standard. The intensity profiles were then represented as a function of q using the IRENA macro suite [60] within the Igor software package (Wavemetrics, Lake Oswego, Oregon). A scattering background (corresponding to a quartz capillary filled with distilled water) was subtracted from all the samples.

Different models were used to fit the experimental data, depending on the type of carrageenan used to prepare the hydrogels. In the case of the κ-C hydrogels, a Gauss-Lorentz gel model was used. This model, described by the scattering function (Eq. (2)), calculates the scattering from a gel structure (typically physical networks) [29–31] as the sum of a low-q exponential decay plus a Lorentzian at higher q-values:

$$I(q) = I_G(0) \cdot \exp\left[-\frac{q^2 \Xi^2}{2}\right] + I_L(0)/(1 + q^2 \xi^2) + bkg \tag{2}$$

where $I_L(0)$ and $I_G(0)$ are, respectively, the linear coefficients of the Lorentzian and Gaussian terms, Ξ is the characteristic mean size of the static heterogeneities in the system under study, ξ is the correlation length of polymer–polymer interactions between the fluctuating chains of polymer and bkg is the background.

The Gauss-Lorentz gel model was not able to provide satisfactory fits for the scattering curves from the ι-C hydrogels and a two-level unified model was used instead (Eq. (3)). This model considers that, for each individual level, the scattering intensity is the sum of a Guinier term and a power-law function [32,33]:

$$I(q) = \sum_{i=1}^{N} G_i \exp\left(-q^2 \cdot \frac{R_{g,i}^2}{3}\right) + \frac{B_i [\text{erf}(qR_{g,i}/\sqrt{6})]^{3P_i}}{q^{P_i}} + bkg \tag{3}$$

where $G_i = c_i V_i \Delta SLD_i^2$ is the exponential prefactor (where V_i is the volume of the particle and ΔSLD_i is the scattering length density (SLD) contrast existing between the ith structural feature and the surrounding solvent), $R_{g,i}$ is the radius of gyration describing the average size of the

ith level structural feature, B_i is a q-independent prefactor specific to the type of power-law scattering with power-law exponent, P_i, and *bkg* is the background. An additional term consisting in a Gaussian peak was added to fit the data from the ι-C hydrogel with KCl, resulting in the following function:

$$
\begin{aligned}
I(q) &= \sum_{i=1}^{N} G_i \exp\left(-q^2 \cdot \frac{R_{g,i}^2}{3}\right) + \frac{B_i \left[\mathrm{erf}(qR_{g,i}/\sqrt{6})\right]^{3P_i}}{q^{P_i}} + I_0 \\
&\quad \cdot \exp\left[-\frac{1}{2} \cdot \left(\frac{q-q_0}{B}\right)^2\right] + bkg
\end{aligned}
\tag{4}
$$

where q_0 is the peak position, I_0 is the intensity of the peak and B is the standard deviation of the peak position.

In the case of the λ-C hydrogels, a much simpler correlation length model, described by Eq. (5), could be used to fit the scattering data. This model contains a first term, described by a power-law function, which accounts for the scattering from large clusters in the low q region and a second term, consisting of a Lorentzian function, which describes scattering from polymer chains in the high q region:

$$
I(q) = \frac{A}{q^n} + \frac{C}{1 + (q\xi_L)^m} + bkg
\tag{5}
$$

where n is the power-law exponent, A is the power-law coefficient, m is the Lorentzian exponent, C is the Lorentzian coefficient and ξ_L is the correlation length for the polymer chains (which gives an indication of the gel's mesh size).

3. Results and discussion

3.1. Molecular structure of the different carrageenan grades

The mechanical properties of carrageenan hydrogels are known to be strongly dependent on the content and position of sulphate groups [34]. Furthermore, some studies pointed out towards a possible effect of the molecular weight in some of the parameters describing the hydrogel mechanical performance [35,36]. Accordingly, different commercial carrageenan grades, potentially presenting different composition and molecular weights, were selected in this work to evaluate the effect of their structural features in the strength of hydrogels formed with and without the addition of salts.

FTIR analyses were firstly carried out to assess the differences between the various carrageenan grades in terms of sulphate group content and distribution. The obtained spectra in the wavelength region where the sulphate-characteristic bands are typically detected (1400–600 cm^{-1}) are shown in Fig. 1A. The bands located at 1065 cm^{-1} and 920 cm^{-1}, which are attributed to C-O-C vibrations of the 3,6 anhydrogalactose ring [37], were observed in all the samples. Although the position of these bands was slightly shifted in the two λ-C grades, their appearance indicates that these materials were not of high purity, since no 3,6-anhydride bridges are present in pure λ-carrageenans [38]. The appearance of these bands has been reported for θ-carrageenans [38], which can be produced by alkali treatment of λ-carrageenan or found in native seaweed species such as *Sarcothalia crisptata*, *Gigartina skottsbergii* [39] and *Callophyllis hombroniana* [40]. In addition, the band at 890 cm^{-1}, related to the rocking mode of the C6 group of the β-D-galactose unit [41], was also detected in all carrageenan grades, but it appeared more intense for κ-C and ι-C. As observed, several sulphate-characteristic bands could be detected for all the carrageenan grades, although not all of them were visible for all the samples and their relative intensity was not the same. The absorption band at 1220 cm^{-1}, characteristic of ester sulphate groups from galactans [18], was apparent for all the carrageenans; however, its relative intensity was the lowest for κ-C. This is related to the lower amount of sulphate group substitutions in κ-carrageenan (i.e. one

sulphate group per disaccharide repeating unit, versus two sulphate groups in ι-C and three sulphate groups in λ-C), which was expected to be the major component in this carrageenan grade. The band located at 840 cm^{-1}, indicative of an axial sulphate ester at the O-4 of a 3–linked galactose [42], was visible in κ-C and ι-C, while a broader shoulder was apparent for the two λ-C grades. This shoulder has been assigned to the existence of C–O–SO$_3$ on the C2 of galactose [37], which is present in λ-, ξ- and θ-carrageenans. The band at 796 cm^{-1}, which is assigned to the sulphate ester substitution at the O-2 of a 4-linked, 3–6 anhydrogalactose [42,43], was clearly visible in ι-C, although it also appeared as a weak shoulder in the two λ-C grades, supporting the presence of a certain fraction of θ-carrageenan. For λ-carrageenan, a broad band corresponding to sulphate ester substitution at the O-6 of a 4-linked galactose has been reported to appear at 867 cm^{-1} [37], but the λ-C samples analysed in this work did not show any sharp peak at this position and instead, they presented a broad band between 780 and 865 cm^{-1}. This evidences the heterogeneity of the λ-C grades, which seemed to be hybrids of different types of carrageenan. In fact, several studies have reported that the carrageenans extracted from the seaweed species *Gigartina pistillata* consist of λ-, κ- and ξ-carrageenans hybrids [6,37].

To gain further insights on the composition of the commercial carrageenan grades, ^{1}H NMR analyses were also performed and the obtained results are shown in Fig. 1B. Based on the existing literature [6,44,45], the bands appearing in the region of anomeric protons could be assigned to different carrageenan types. The κ-C grade was characterized by the appearance of a peak at 5.09 ppm, corresponding to the anomeric proton of κ-carrageenan, and a very weak signal at 5.24 ppm, assigned to ι-carrageenan. The relative amount of each carrageenan type was estimated by integrating of the areas under the peaks, suggesting that the material was composed of 92% κ-carrageenan and 8% ι-carrageenan. The ι-C grade showed the ι-carrageenan characteristic band (accounting for 82%) and a smaller peak at 5.35 ppm, which indicates the presence of floridean starch (corresponding to 18%) [6]. The two λ-C grades showed a very heterogeneous composition, as reflected by the appearance of multiple peaks in their spectra. In both materials, the λ-carrageenan band was detected at 5.55 ppm; however, additional peaks at 5.09 ppm (κ-carrageenan), 5.14 ppm (non-identified), 5.24 ppm (μ-carrageenan), 5.30 ppm (θ-carrageenan) and 5.35 ppm (floridean starch) were also visible. Accordingly, the composition was determined as 26% λ-carrageenan, 28% κ-carrageenan, 13% μ-carrageenan, 18% θ-carrageenan and 11% starch for λ↓-C and 29% λ-carrageenan, 27% κ-carrageenan, 2% μ-carrageenan, 20% θ-carrageenan and 19% starch for λ↑-C. Thus, the NMR results confirm that while the κ-C and ι-C grades were relatively pure carrageenans, containing a certain amount of starch, the two λ-C grades were actually hybrids.

The cationic composition of all the carrageenan grades was also determined by means of ICP-MS to quantify the amounts of K^+, Na^+, Ca^{2+} and Mg^{2+} in the commercial products. The results, compiled in Table S1, evidence the presence of cations in the four commercial grades, being the total cationic content slightly higher in the ι-C grade. K^+ was the most abundant cation in the κ-C and ι-C grades, while the two λ-C grades contained more Na^+.

The molecular weight of the different carrageenan grades was analysed by means of HPLC-SEC. The comparison of the molecular weight of different carrageenans through this technique is complex due to their different aggregation behaviour in the presence of salts, while a certain ionic strength for the eluent is usually required for hydrophilic samples, especially polyelectrolytes [46]. The transition of carrageenans from a disordered to an ordered conformation, and subsequent aggregation or gelation, is generally induced by increasing the ionic strength or lowering the temperature, so low ionic strengths and high temperatures were most desirable for the analysis. Accordingly, 0.01 M LiCl was selected as eluent, since ι- and κ-carrageenans have been previously described to exhibit a disordered structure in this medium [47] (less information is available for λ-carrageenans), and the
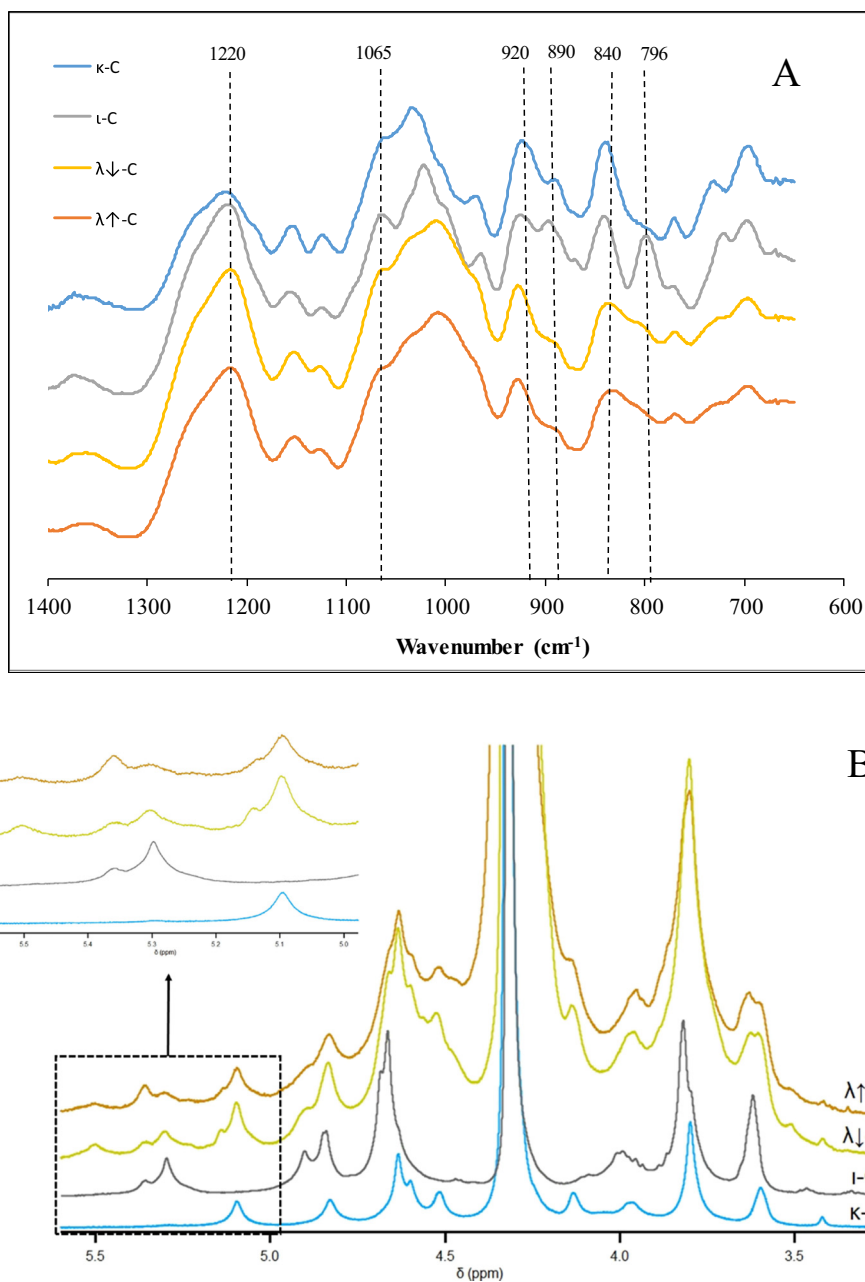
Fig. 1. (A) FT-IR spectra and (B) ^{1}H NMR spectra of the various commercial carrageenan grades used in this work. Spectra have been offset for clarity. Characteristic bands are marked with discontinuous lines.
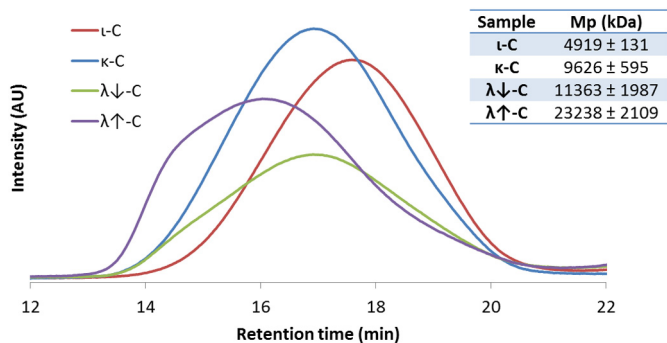

Fig. 2. HPLC-SEC chromatograms of the commercial carrageenan grades and the estimated peak molecular weights (Mp). Data shown as mean ± SD, n = 3.

Sample	Mp (kDa)
ι-C	4919 ± 131
κ-C	9626 ± 595
λ↓-C	11363 ± 1987
λ↑-C	23238 ± 2109

experiments were carried out at 45 °C, above the aggregation temperature. Fig. 2 shows the HPLC-SEC chromatograms obtained for the different carrageenan grades used in this work. As observed, all the samples presented significantly lower retention times than the standard pullulan samples used for calibration, suggesting that their molecular weights were considerably higher. However, it is worth mentioning that, although pullulan standards are commonly used as references for SEC of polysaccharides, the conformation of these neutral carbohydrates in solution is notably different from that of more complex polysaccharides such as carrageenans, precluding the accurate determination of absolute molecular weight values. Nevertheless, relative molecular weight data could be estimated for comparative analysis, and peak molecular weights for each carrageenan grade were calculated by extrapolation from the calibration curve built using the pullulan standards (R^2 = 0.992). The results, shown in Fig. 2, evidence strong differences between the carrageenan grades. In particular, the ι-C

presented the lowest molecular weight, while the $\lambda\uparrow$-C, with the highest molecular weight, showed ca. 2-fold increased value with respect to $\lambda\downarrow$-C.

3.2. Optimization of hydrogel strength: Effect of carrageenan and salt concentration

The four different commercial carrageenan grades (κ-C, ι-C, $\lambda\downarrow$-C and $\lambda\uparrow$-C) were used to produce hydrogels, with and without the addition of salts (a monovalent salt such as KCl and a divalent salt such as $CaCl_2$) and the effect of the carrageenan concentration and the salt concentration on the strength of the obtained materials was evaluated. To do so, a central composite design was performed, with the measured hydrogel strength being set as the response variable. Tables S2, S3, S4 and S5 (in the Supplementary Material) present the measured hydrogel strength values for each of the carrageenan and salt concentration combinations determined by the design. For each carrageenan grade and each type of salt, the obtained results were fitted using a quadratic model. The high model correlation coefficients obtained for all the types of hydrogels ($R^2 > 0.99$) evidence the accuracy of the theoretical models in describing the experimental data, i.e. they provide a good agreement between the experimental and predicted strength values (see Table S6 for the associated statistical parameters). The response surface plots were also obtained to show visually the dependence of the hydrogel strength on the two studied parameters (i.e. carrageenan and salt concentration) and the results are shown in Fig. 3.

In general, it was observed that, as already anticipated due to the lower amount of sulphate substitution in κ-carrageenan, κ-C hydrogels showed the highest strength values from the four commercial grades tested. The results from the statistical analysis indicated that all the factors, except for the quadratic effect of the $CaCl_2$ concentration ($P = 0.38$), had a significant effect on the κ-C hydrogel strength ($P \leq 0.05$) (cf. Fig. 3A and B). According to the results, higher hydrogel strength values were obtained when increasing the κ-C and salt concentration, where KCl had a stronger effect than $CaCl_2$. This is in agreement with a previous work in which higher strength values were reported for κ-carrageenan hydrogels prepared using monovalent salts as compared to those obtained with the addition of divalent salts [21]. This behaviour was explained on the basis of the interference of these salts with the carrageenan double helix association mechanism. While K^+ has been hypothesised to facilitate the formation of strong κ-carrageenan double helix aggregates by neutralizing the repulsive electrostatic forces provided by sulphate groups, Ca^{2+} is thought to induce ionic cross-links between the κ-carrageenan chains, generating softer hydrogels [48]. It should be noted that the surface response graphs plotted as a function of ionic strength (cf. Fig. S1) showed the same behaviour, in line with KCl and $CaCl_2$ promoting different supramolecular structures. The models obtained for the strength of the κ-C hydrogels with the addition of KCl and $CaCl_2$ could be described by the following equations:

$$HS_{KCl} = -16.72 + 16.19 * C + 14.79 * S + -1.16 * C^2 - 4.13 \\ * S^2 + 1.14 * CS \tag{6}$$

$$HS_{CaCl_2} = -13.45 + 26.62 * C + 3.17 * S - 6.13 * C^2 - 3.36 * S^2 + 2.47 \\ * CS \tag{7}$$

Based on these equations, the maximum hydrogel strength values within the range of the carrageenan and salt concentrations studied were estimated, being ~29 N with the addition of KCl and ~20 N with $CaCl_2$ (cf. Table 2). Hydrogels with the corresponding κ-C and salt concentrations (2.0% κ-C + 2.0% KCl and 2.0% κ-C + 1.2% $CaCl_2$) were prepared and their strength values were measured. As deduced from the values listed in Table 2, the experimental and model-predicted strength values were very similar, highlighting the accuracy of the model.

Regarding the ι-C hydrogels, all the measured strength values were significantly lower than those from the κ-C hydrogels. This can be related to the greater sulphate substitution in ι-carrageenan (the main component in the ι-C grade), which in turn impairs the association of the carrageenan double helices due to electrostatic repulsive forces. The statistical analyses indicated that all the considered factors had a significant effect on the hydrogel strength. As deduced from the response surface plots (cf. Fig. 3B), the ι-C hydrogels with KCl presented greater strength values when increasing the carrageenan and salt concentrations. On the other hand, the ι-C hydrogels with $CaCl_2$ presented a different behaviour (cf. Fig. 3C), showing a maximum strength at intermediate salt concentrations, in agreement with previous work [17,21]. Moreover, as previously reported [21], lower concentrations were required for hydrogel formation in the presence of $CaCl_2$ as compared with KCl. This observation has been ascribed to the greater capacity of Ca^{2+} to form intramolecular bridges between the sulphate groups of adjacent anhydro-D-galactose and D-galactose residues of ι-carrageenan, as opposed to K^+, which is thought to form an ionic bond with the sulphate group of D-galactose residues [49]. The models obtained for the ι-C hydrogels with the addition of KCl and $CaCl_2$ were described by the following equations:

$$HS_{KCl} = -0.22 + 0.34 * C + 0.07 * S - 0.07 * C^2 - 0.036 * S^2 + 0.04 * CS \tag{8}$$

$$HS_{CaCl_2} = 0.29 - 0.18 * C - 0.051 * S + 0.10 * C^2 - 0.03 * S^2 + 0.06 * CS \tag{9}$$

The formulations providing the maximum strength values were predicted as 2.0% ι-C + 2.0% KCl and 2.0% ι-C + 1.0% $CaCl_2$. As shown in Table 2, the corresponding hydrogel strength values (~0.3 N and ~0.4 N for KCl and $CaCl_2$, respectively) were again very close to the theoretical values predicted by the model equations.

The two λ-C grades presented a slightly different behaviour. In particular, $\lambda\downarrow$-C required higher carrageenan and salt concentrations to form hydrogels than $\lambda\uparrow$-C, which is reasonable considering the lower molecular weight of the former. Although the strength values were in general greater for the $\lambda\uparrow$-C hydrogels, the differences were not remarkable as compared with the $\lambda\downarrow$-C hydrogels. The statistical analysis evidenced that all the factors except the linear term of KCl concentration presented a significant effect on the strength of $\lambda\downarrow$-C hydrogels. On the other hand, in the case of the $\lambda\uparrow$-C hydrogels, all the factors presented a significant effect in the response. Fig. 3F and G show that the highest values of strength for the $\lambda\downarrow$-C hydrogels were obtained increasing the carrageenan concentration and at an intermediate level of KCl concentration, whereas in the case of $CaCl_2$ the highest strength values were obtained by increasing the salt concentration. For the $\lambda\uparrow$-C, the strongest hydrogels could be obtained by increasing the concentration of carrageenan and salts (Fig. 3H and I). Interestingly, KCl yielded stronger hydrogels than $CaCl_2$ for both $\lambda\uparrow$-C and $\lambda\downarrow$-C. Gelation of λ-C using mono- or divalent salts has not been reported to date and only the addition of a trivalent salt ($FeCl_3$) has been described to produce the gelation of λ-C [27]. It was suggested that this type of salt was able to equilibrate the negative charges of sulphate groups in λ-C (three sulphate groups per disaccharide repeating unit), promoting the interaction between the chains and forming a strong gel. The fact that the λ-C grades used in this work were able to form hydrogels confirms that these were actually hybrids of the λ-carrageenan form with other types of carrageenan naturally occurring in the seaweed used for the extraction, such as κ-, μ- and θ-carrageenans, as previously suggested by the FT-IR and ^1H NMR results. In particular, the presence of gelling κ-carrageenan may have contributed to the capacity of the λ-C grades to form gels. The models that describe the relationship between the hydrogel strength and the factors for $\lambda\downarrow$-C (Eqs. 10 and 11) and $\lambda\uparrow$-C (Eqs. 12 and 13) were the following:
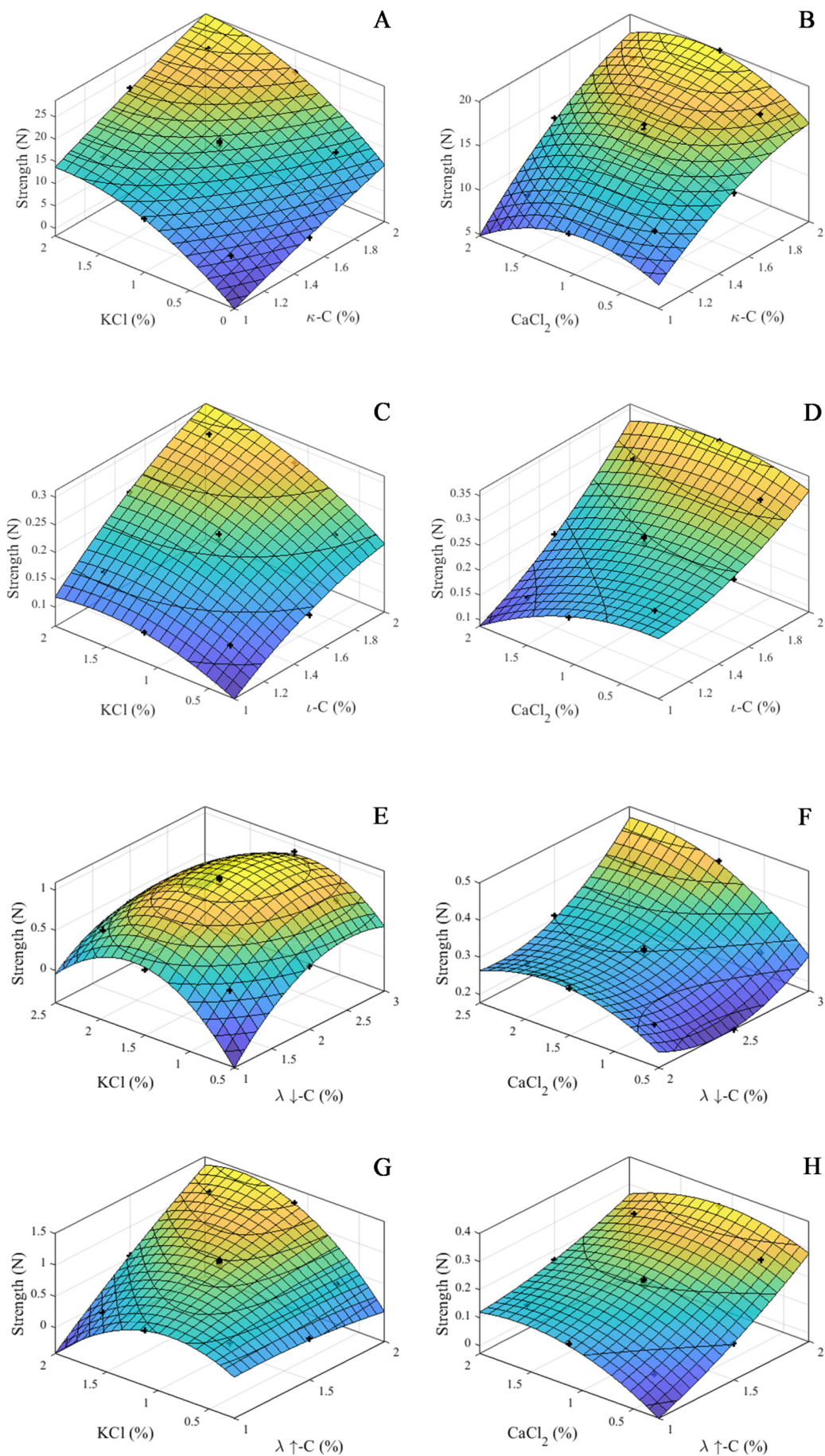
Fig. 3. Response surface plots of the hydrogel strength for the different combinations of commercial carrageenans and salts.

Table 2

Model-predicted and experimental strength values for the optimum hydrogel formulations, being C (%) the carrageenan concentration, S (%) the salt concentration and HS (N) the theoretical hydrogel strength. Data shown as mean ± SD, n = 3.

		C (%)	S (%)	HS (N)	Experimental strength (N)
κ-C	KCl	2.0	2.0	28.6	27.5 ± 1.3
	CaCl$_2$	2.0	1.2	20.1	19.9 ± 0.4
ι-C	KCl	2.0	2.0	0.31	0.34 ± 0.01
	CaCl$_2$	2.0	1.0	0.36	0.35 ± 0.01
λ↓-C	KCl	2.2	1.5	1.07	1.1 ± 0.04
	CaCl$_2$	3.0	2.3	0.47	0.46 ± 0.04
λ↑-C	KCl	2.0	1.6	1.45	1.4 ± 0.1
	CaCl$_2$	2.0	1.1	0.34	0.37 ± 0.02

$$HS_{KCl} = -2.9 + 2.01 * C + 2.37 * S - 0.38 * C^2 - 0.67 * S^2 - 0.17 * CS \quad (10)$$

$$HS_{CaCl_2} = 1.6 - 1.3 * C + 0.05 * S + 0.26 * C^2 - 0.06 * S^2 + 0.07 * CS \quad (11)$$

$$HS_{KCl} = -0.10 + 0.35 * C + 0.12 * S - 0.27 * C^2 - 0.72 * S^2 + 1.12 * CS \quad (12)$$

$$HS_{CaCl_2} = -0.23 + 0.04 * C + 0.38 * S + 0.09 * C^2 - 0.09 * S^2 - 0.12 * CS \quad (13)$$

The formulations that would theoretically produce the highest hydrogel strength values for λ↓-C would be 2.2% λ↓-C + 1.5% KCl (1.1 N) and 3.0% λ↓-C + 2.3% CaCl$_2$ (0.5 N). On the other hand, the optimal formulations in terms of hydrogel strength for λ↑-C would correspond to 2.0% λ↑-C + 1.6% KCl (1.4 N) and 2.0% λ↑-C + 1.1% CaCl$_2$ (0.4 N). In all the cases there was a very good agreement between the model-predicted and the experimental strength values.

3.3. Rheological characterization of selected hydrogels

The effect of the presence of cations and the valency of these in the rheological behaviour of the different carrageenan grades studied were investigated by performing oscillatory rheological measurements on selected hydrogel formulations (using the same carrageenan and salt concentrations for all of them). The elastic modulus (G′) and storage modulus (G″) were measured as a function of temperature, at a fixed frequency (1 Hz) and 1% strain. The gelling temperatures (T$_{gel}$), which were determined as the cross-over point between G′ and G″, as well as G′, G″ and tan δ values of the hydrogels at 20 °C were determined and the results are compiled in Table 3. It should be mentioned that, as opposed to other gelling polysaccharides such as agar [50] and agarose [51], the crossover point between G′ and G″ was clearly observed in all the samples, showing all of them a behaviour characteristic of solutions

Table 3

Rheological properties of carrageenan hydrogels. Gelation temperature (T$_{gel}$), elastic modulus (G′$_{20 °C}$), viscous modulus (G″$_{20 °C}$) and tan δ at 20 °C.

	T$_{gel}$ (°C)	G′$_{20 °C}$ (Pa)	G″$_{20 °C}$ (Pa)	Tan δ
2.0%κ-C	44	3273	640	0.2
2.0%κ-C + 1.5%KCl	79	11,364	6950	0.6
2.0%κ-C + 1.5%CaCl$_2$	52	134,000	2300	0.02
2.0%ι-C	41	300	30	0.1
2.0%ι + 1.5%KCl	74	500	20	0.04
2.0%ι + 1.5%CaCl$_2$	87	310	10	0.04
2.0%λ↓-C	26	40	30	0.9
2.0%λ↓ + 1.5%KCl	78	3600	300	0.08
2.0%λ↓ + 1.5% CaCl$_2$	64	170	40	0.2
2.0%λ↑-C	66	80	50	0.6
2.0%λ↑ + 1.5%KCl	80	2600	170	0.07
2.0%λ↑ + 1.5% CaCl$_2$	82	600	100	0.2

at high temperatures. As a general observation, the addition of salts increased the gelation temperature in the four carrageenan grades, suggesting that the presence of cations facilitated the association of carrageenan molecular chains to originate the network structures responsible for hydrogel formation. Although most of the hydrogels were not fully cured at 20 °C, in general, the incorporation of salts produced hydrogels with higher strength (as suggested by the higher G′ values) and increased their elastic character (as indicated by the lower tan δ values).

Fig. 4A shows the temperature sweep plots from the κ-C samples. As observed, the formulations showed a notably different behaviour depending on the nature of the cations added. In particular, the formulation containing KCl was seen to form a hydrogel at much higher temperatures (T$_{gel}$ = 79 °C) than the formulation containing CaCl$_2$ (T$_{gel}$ = 52 °C) and the plain carrageenan (T$_{gel}$ = 44 °C). This is consistent with the hypothesis of K$^+$ being able to promote the association of κ-carrageenan chains by neutralizing the negative charges from sulphate groups, thus facilitating the sol-gel transition. Wang et al. [48] reported the same behaviour, suggesting that K$^+$ decreases the amount of negative charges in κ-carrageenan, facilitating intermolecular association of carrageenan double helices by hydrogen bonding; on the other hand, divalent cations form ionic cross-links between the sulphate groups of κ-carrageenan. As observed, the plain κ-C and especially the formulation containing KCl showed a bump in G′ after the sol-gel transition, i.e. G′ showed a sharp decrease after reaching a maximum value. This phenomenon has been previously reported to take place in κ-carrageenan systems [52,53]. Despite some works have attributed this bump to syneresis effects, a more in-depth study reported the appearance of this feature as a result of a transient state formed during the gelation process. This structure is reported to consist of a fine network where the junction zones may be double helices. Since it is an unstable structure, when the temperature is further decreased an aggregation process is initiated, forming more tightly packed structures ([23]). To discard the appearance of this bump as a result of slippage effects associated to water syneresis, additional experiments were carried out by running the κ-C formulation with added KCl with a rough plate geometry. As shown in Fig. S2A, the bump was still clearly observed under these conditions even though no significant amount of water was expelled from the hydrogel and no apparent slippage took place. Furthermore, to discard nonlinear viscoelastic effects, the sample was also run at lower strains of 0.05% and 0.1% (cf. Fig. S2B and C), obtaining very similar results regardless of the parameters selected for the cooling run. Although the hydrogels were not fully cured at 20 °C, the G′ and G″ values for the κ-C hydrogels at that temperature were in general much higher than those of the other carrageenan grades, indicating that stronger networks (i.e. stronger interactions between carrageenan chains) were formed in the case of κ-C.

As observed in Fig. 4B, the ι-C formulations presented a very different behaviour to that of κ-C. The absolute values of the moduli were lower in comparison to those of κ-C after the sol-gel transition. Contrarily to κ-C, the sol-gel transition in ι-C was more strongly promoted by the addition of the divalent salt. Whereas the plain ι-C showed a gelation temperature of ca. 41 °C, higher gelation temperatures of 74 °C and 87 °C were observed with the addition of KCl and CaCl$_2$, respectively. Interestingly, and in agreement with the hydrogel strength values compiled in Table 2, there were no great differences between the G′ values of the hydrogels at 20 °C, suggesting that, even though the presence of Ca^{2+} facilitated the association of carrageenan double helices upon cooling to a greater extent than K$^+$, similar supramolecular structures may have been formed with both cations, which is also confirmed by the same tan δ values for both salts. This is in agreement with previous studies which showed the low cation selectivity of ι-carrageenan in terms of rheological properties [17].

With regards to the λ-C samples, Fig. 4C shows the results for λ↓-C, whereas the data from λ↑-C are shown in Fig. 4D. As deduced from the results listed in Table 3, the hydrogel structure formed at 20 °C (not
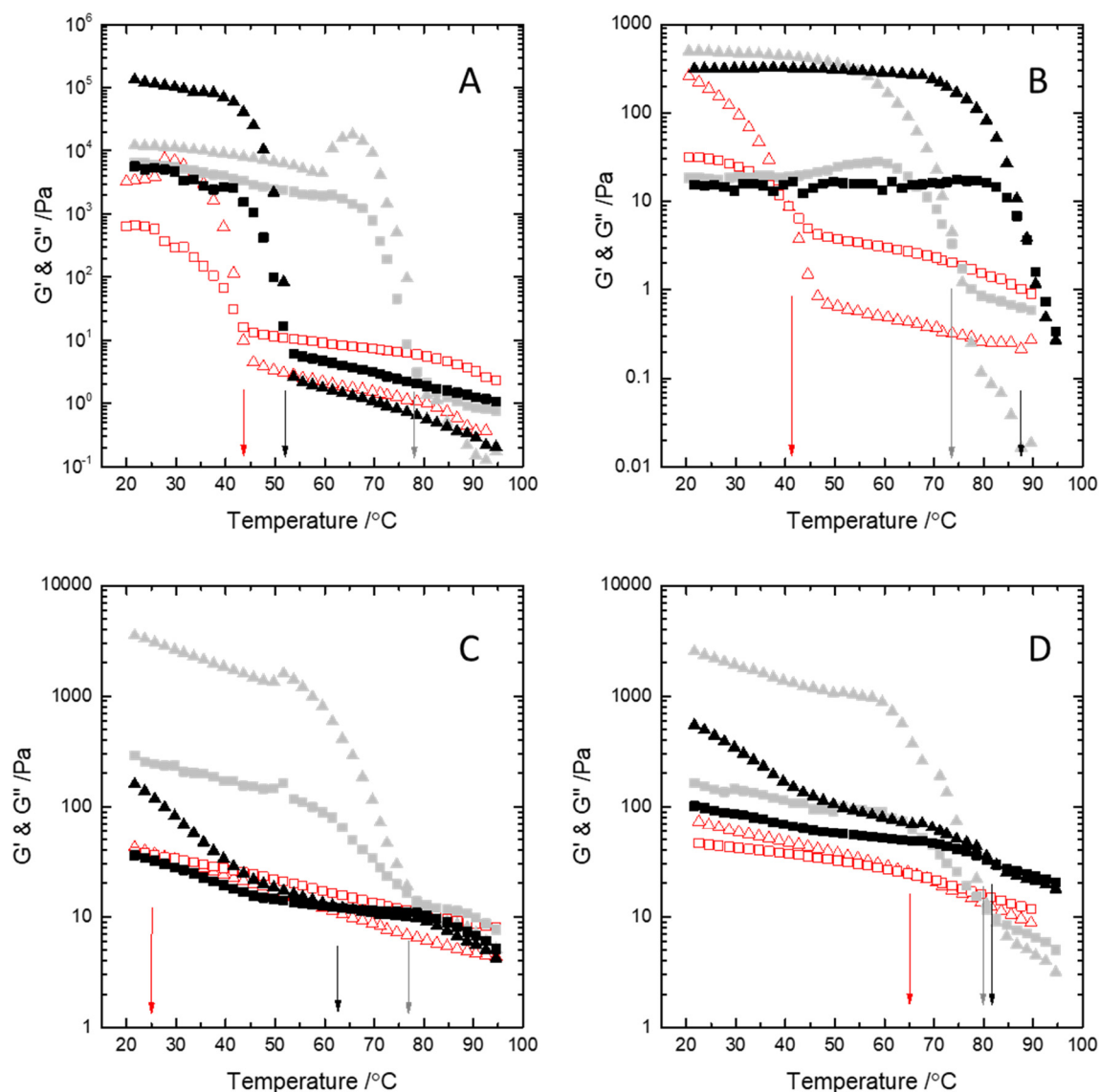
Fig. 4. Temperature dependence of G′ (triangles) and G″ (squares) moduli of carrageenan solutions upon cooling. (A) κ-C, (B) ι-C, (C) λ↓-C and (D) λ↑-C. 2% carrageenan (red markers), 2% carrageenan + 1.5% KCl (grey markers), 2% carrageenan + 1.5% CaCl₂ (black markers). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fully cured) showed a greater strength for λ↑-C (G′₂₀ = 80 Pa) than λ↓-C (G′₂₀ = 40 Pa), which may be attributed to the higher molecular weight of the former. The addition of salts had a strong impact in the gelation behaviour of λ↓-C, increasing the gelation temperature from 26 °C to 78 °C and 64 °C with the addition of KCl and CaCl₂, respectively. Furthermore, stronger hydrogel networks (not fully cured) were obtained at 20 °C with salt addition, especially with KCl, as indicated by the higher G′ values. The addition of salts also had a strong impact on the gelation temperature of λ↑-C, (although to a lower extent than in the case of λ↓-C), which increased from 66 °C to 80 °C and 82 °C with the addition of KCl and CaCl₂, respectively. On the other hand, although the systems were still not completely settled at 20 °C, the type of salt strongly affected the rheological properties of the hydrogels, as deduced from the G′ and tan δ values. In agreement with the measured hydrogels strength values (cf. Table 2), KCl was able to promote the formation of stronger λ↑-C hydrogels with higher degree of elastic contribution than CaCl₂. These results would suggest that the presence of monovalent cations is more favourable to generate load-bearing cross-links between the carrageenan chains in both λ-C grades. Since the λ-C grades used in this work were actually λ-, κ-, μ- and θ-

carrageenan hybrids, explaining their gelation behaviour is not straightforward. In principle, one may think that for the same concentration of added salt, Ca²⁺ should be able to neutralize the negative charges from sulphate groups more efficiently than K⁺. However, it seems that the nature of the intermolecular interactions established was distinct depending on the added salt, with K⁺ promoting stronger interactions than Ca²⁺. This might be also related to the fact that κ-carrageenan was one of the major components in both λ-C grades and therefore, their gelling behaviour showed some similarities with that from the κ-C grade.

3.4. Nanostructural characterization of selected hydrogels

To investigate the structural differences between the various hydrogels at the nanoscale level, SAXS experiments were carried out on selected formulations. In particular, hydrogels with 2% of carrageenan and 1.5% of salt were chosen since these concentrations seemed to provide hydrogel strength values relatively close to the optimum for all the carrageenan and salt combinations. Fig. 5 shows the scattering patterns for the different hydrogels, which as observed, presented
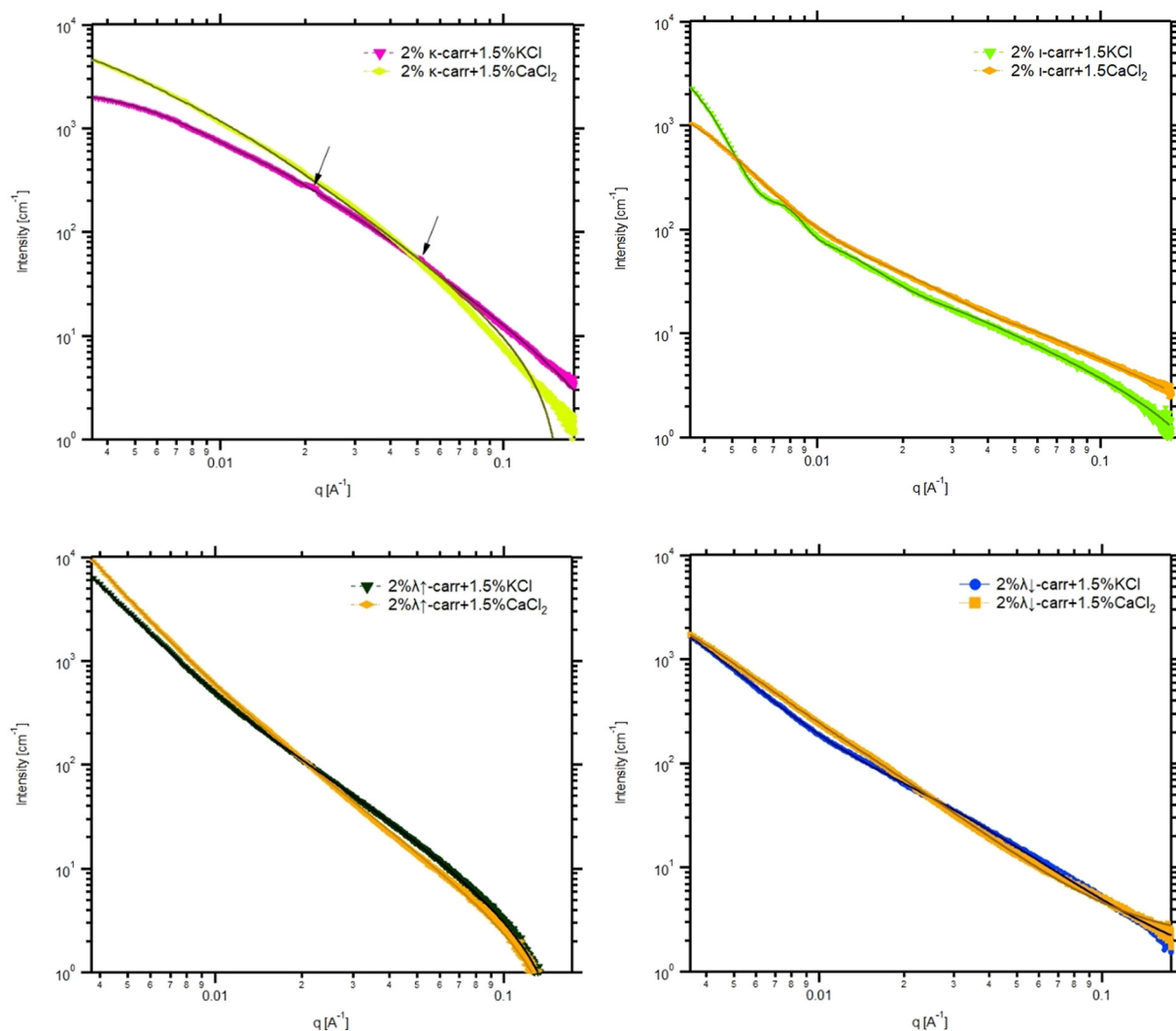
Fig. 5. SAXS patterns from the different carrageenan hydrogels. Markers represent the experimental data and solid lines show the fits obtained using theoretical models.

marked differences depending on the type of carrageenan used. Consequently, different models had to be used to fit the experimental data and extract structural information from the scattering results. The SAXS patterns from the κ-C hydrogels could be properly described by means of a Gauss-Lorentz gel model, which to date has been the only model proposed in the literature to fit the small angle scattering data from carrageenan hydrogels over a relatively broad q range [29]. The obtained fitting parameters are summarized in Table 4a. The Gaussian parameter (Ξ) is the characteristic mean size of the static heterogeneities and can be affected by the introduction of cross-links into the system, whereas the Lorentzian parameter (ξ) is the correlation length for the polymer chains and is related to the interactions between the fluctuating chains of polymer. While values of $\Xi \sim$ 8–10 nm and $\xi \sim$ 3–4 nm have been previously reported for pure 1% κ-carrageenan hydrogels [29], the addition of salts had a strong effect and both parameters presented significantly larger values. Although κ-carrageenan chains are able to associate into double helices to form gel-like structures, the addition of salts is known to promote aggregation of carrageenan helices through neutralization of the electrostatic repulsions originated by the presence of sulphate groups [22]. Thus, the increased values for the structural parameters seem to be indicative of a greater degree of cross-linking taking place in the hydrogels produced in the presence of salts. The correlation length values, which are in agreement with the value of 8.5 nm previously reported for κ-carrageenan

hydrogels with KCl [54], can be associated to the size of the carrageenan double helices. Similarly to carrageenan, agarose is known to associate into double helices, whose cross-section size has been reported to be within the range of 5–10 nm [55], which then further aggregate into bundles, responsible for the formation of strong hydrogels. As observed in Table 4a, the hydrogel prepared in the presence of KCl seemed to present a more compact structure than the hydrogel with CaCl₂, as suggested by the smaller values of Ξ and ξ. It should be noted that the hydrogel prepared in the presence of KCl showed two small peaks located at 0.021 Å^{-1} and 0.050 Å^{-1}, indicative of the existence of well-ordered structures with real distances of ca. 29 nm and 12 nm. As observed, these values are similar to the structural parameters Ξ and ξ, which may indicate that the association of carrageenan chains into double helices and bundles led to the formation of well-ordered structures arranged with a certain periodicity. This suggests that K^+ is able to promote the formation of more ordered and densely packed κ-C hydrogel networks, which may be the consequence of stronger intermolecular interactions being developed. This denser network could explain the higher strength observed for the κ-C hydrogels obtained with the K^+ as compared with Ca^{2+} (cf. Table S7 for the measured hydrogel strength values).

Although the Gauss-Lorentz model provided satisfactory fits for the ι-C hydrogels within the low q range, the data within the region $q > 0.01 \text{ Å}^{-1}$ was not properly described by the model. This confirms

Table 4
Parameters obtained from the fits corresponding to the κ-C (a), ι-C (b) and the λ-C (c) hydrogels. Standard deviations (i.e. errors associated to the fitting results) on the last digit are shown in parentheses.

(a)

	2% κ-C + 1.5% KCl	2% κ-C + 1.5% CaCl$_2$
I$_G$	1236 (33)	3091 (168)
Ξ (nm)	25.1 (4)	41 (1)
I$_L$	1314 (33)	4888 (110)
ξ (nm)	9.5 (1)	17.7 (2)

(b)

	2% ι-C + 1.5% KCl	2% ι-C + 1.5% CaCl$_2$
G$_1$	11,068 (200)	2461 (86)
Rg$_1$ (nm)	61.8 (3)	47.6 (9)
B$_1$	–	$3.6 \cdot 10^{-3}$ (1)
P$_1$	–	3.14 (8)
G$_2$	132 (13)	57 (4)
Rg$_2$ (nm)	14.4 (9)	7.3 (5)
B$_2$	0.4 (1)	0.34 (3)
P$_2$	1.09 (8)	1.22 (4)
I$_0$ (cm^{-1})	68 (6)	–
d (nm)	84.9	–

(c)

	2% λ↑-C + 1.5% KCl	2% λ↑-C + 1.5% CaCl$_2$	2% λ↓-C + 1.5% KCl	2% λ↓-C + 1.5% CaCl$_2$
A	$1.4 \cdot 10^{-3}$ (1)	$2.7 \cdot 10^{-4}$ (6)	$4.8 \cdot 10^{-3}$ (1)	–
n	2.75 (1)	3.10 (4)	2.25 (3)	–
C	77 (6)	350 (61)	44 (6)	–
m	2.2 (1)	2.09 (8)	2.10 (1)	–
ξ_L (nm)	4.0 (4)	10 (2)	3.5 (3)	–

that a different type of network structure was originated in the κ-C and ι-C hydrogels. As an alternative, a two-level unified model was suitable to fit the data through the whole q range (cf. Fig. 5). In the particular case of the ι-C hydrogel with KCl, an additional term was added to the fitting function to account for an additional shoulder-like feature centred at ca. 0.007 Å^{-1}. As deduced from the fitting results (cf. Table 4b), the radius of gyration corresponding to the high q range (Rg$_2$) could be assigned to the size of the carrageenan double helices, while the radius of gyration calculated from the data within the low q range (Rg$_1$) could be ascribed to the aggregates of these double helices, giving rise to the gel network structure. Although the gelation of ι-carrageenan has been proposed to take place exclusively as a result of double helix formation [56], the addition of salts has been shown to induce further helix aggregation through the establishment of ionic interactions [57]. From the scattering results it is clear that both salts induced the association of carrageenan double helices to form larger structures. The size of the ι-C double helices in the presence of both salts was within the range of values determined for the κ-C hydrogels. On the other hand, larger aggregates were formed as compared to κ-C, as suggested by the *Rg$_1$* values. Comparing the two different salts, it seems that more densely packed network structures were attained with the addition of CaCl$_2$, which could be related to the greater capacity of Ca^{2+} to neutralize the repulsive forces provided by the sulphate groups in ι-carrageenan. The real distance corresponding to the shoulder-like feature observed in the ι-C hydrogel with KCl (ca. 85 nm), has not been previously attributed to any particular structural feature in ι-C, but it might be associated to the formation of ordered domains in the longitudinal axis of the carrageenan bundles. The differences encountered between the κ-C and ι-C hydrogels are related to the different specific role of the cations on the gelation mechanism of the κ- and ι-

carrageenans present in these commercial grades [21]. It has been proposed that K$^+$ forms an ionic bond with the sulphate group of the galactose residues, inducing intermolecular association of κ-carrageenan, subsequently forming an electrostatic bond with the anhydrobridge oxygen atom of the adjacent galactose residue upon cooling and, at the same time, favouring the association of carrageenan double helices by strong hydrogen bonds. On the other hand, due to its divalent character, Ca^{2+} can form intra-molecular bridges between the sulphate groups of adjacent anhydrogalactose and D-galactose residues of ι-carrageenan. Upon cooling, further aggregation occurs due to intermolecular Ca^{2+} bridging. According to this, and considering the SAXS results, it may be reasonable to hypothesise that in the presence of salts, κ-carrageenan and ι-carrageenan can form similar structures of double helices and bundles, although the nature of the bridges holding together the bundles is different depending on the type of carrageenan and valency of the salt added. K$^+$ binds more efficiently to κ-carrageenan chains and is able to form strong interactions (most likely hydrogen bonds) between helices to form the bundles. On the other hand, Ca^{2+} is more efficient in creating intermolecular bonds between ι-carrageenan chains and induces the formation of larger aggregates, but weaker ionic cross-links are originated in this case.

The application of the Gauss-Lorentz gel or the unified models for the λ-C hydrogels gave rise to large uncertainties in the errors associated to the obtained fitting parameters. This is not unexpected given the absence of marked scattering features in the data from these heterogeneous samples. Instead, a simpler correlation length model, which has been previously applied to describe the scattering data from different gelling systems such as physically and chemically cross-linked gelatin networks [58], was applied to fit the data (except for the λ↓-C hydrogel with the addition of CaCl$_2$, where the fitting parameters still presented very large uncertainty values) and the obtained parameters are listed in Table 4c. The obtained power-law exponents (n) were indicative of mass fractal structures, i.e. branched network structures which, in the particular case of 2% λ↓-C + 1.5% KCl, were characteristic of randomly branched Gaussian chains. The greater exponents obtained for the high molecular weight λ-C hydrogels suggested the presence of more branched structures, which may be a direct consequence of their higher molecular weight. The Lorentz exponent (m) may be related to the local chain conformation and lower values are typically attained for more expanded polymer chains. Very similar values close to 2, which are indicative of randomly branched Gaussian polymer chains, were obtained for the different λ-C hydrogels. All these results point out towards the fact that λ-C gelation took place by means of a very distinct mechanism to that of κ-C and ι-C, forming less ordered structures. This is reasonable considering the fact that both λ-C grades were actually hybrid carrageenans. The heterogeneity in the molecular structures composing these materials would then lead to the appearance of less defined scattering features.

Based on the characterization of the carrageenan hydrogels used in this study and according to models described in the literature for the gelation of pure carrageenans, a hypothesis for the distinct gelation mechanism of the studied commercial carrageenan grades has been developed (see Fig. 6 for a graphical representation of the proposed gelation mechanisms). The κ-carrageenan found in the κ-C grade contains one sulphate group per disaccharide repeating unit, located at the C4 from the 3–linked galactose unit. The amount of sulphate groups in this type of carrageenan is low enough not to impede the association of carrageenan double helices by hydrogen bonding to form bundles, thus generating hydrogel structures. The addition of K$^+$ promotes a further aggregation of double helices by neutralizing the negative charges of the sulphate groups. In contrast, Ca^{2+} seems to induce weaker ionic cross-links between the sulphate groups from adjacent κ-carrageenan double helices, hence generating softer hydrogels. On the other hand, the ι-carrageenan present in the ι-C commercial grade possesses two sulphate groups per disaccharide repeating unit (one located at the C4 from the 3–linked galactose unit and another at the C-2 from the 4-
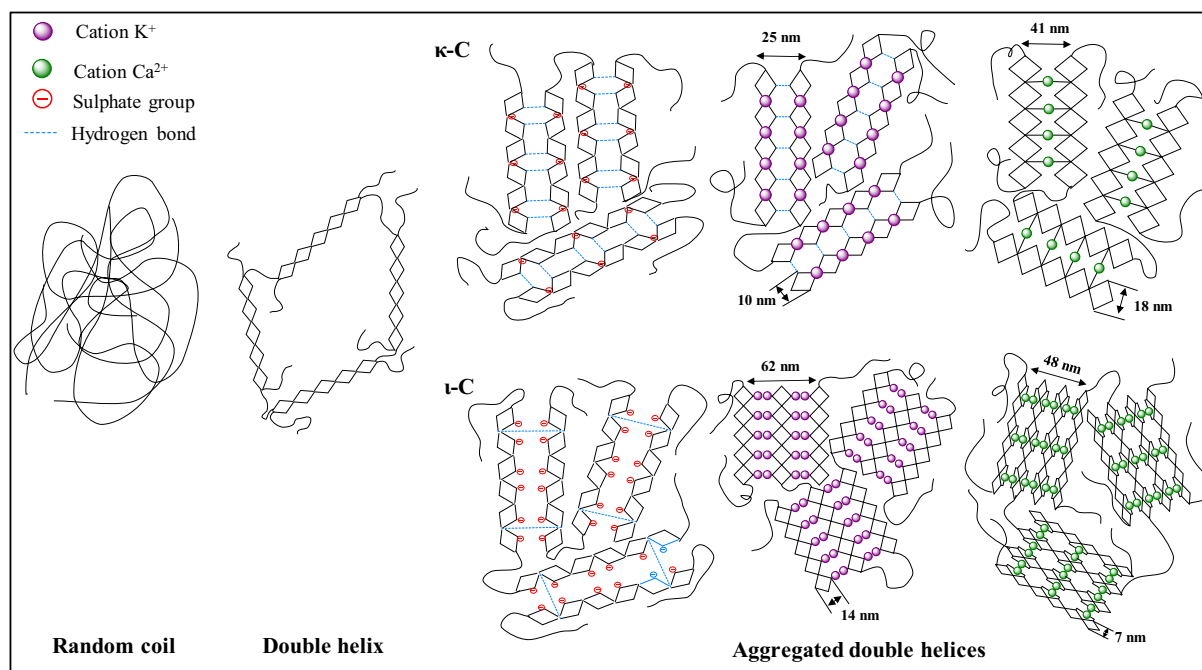
Fig. 6. Proposed gelation mechanism for the κ-C and ι-C commercial grades and the effect of added salts.

linked, 3–6 anhydrogalactose). Therefore, although the ι-carrageenan chains are able to associate to a certain extent to form weak hydrogels without salt addition, less elastic and stable networks are formed (as seen from the tan δ values in Table 3) and lower temperatures are required for hydrogel formation. As opposed to κ-C, the addition of salts in the case of ι-C did not seem to mediate hydrogel formation via hydrogen bonding of carrageenan double helices, but it rather promoted gelation through ionic cross-linking and as a result, much softer hydrogels were formed. The Ca^{2+} ions are thought to form ionic cross-links between the sulphate groups of adjacent anhydrogalactose and galactose residues, whereas the K^+ ions are believed to form an ionic bond with the sulphate group from galactose residues. Subsequently, intermolecular association may take place through electrostatic forces of attraction between the sulphate groups and the cations from adjacent carrageenan double helices. Thus, Ca^{2+} ions are more efficient in promoting ι-C gelation (lower concentrations are required to neutralize the same amount of sulphate groups) but a very similar network of ionic-bonded carrageenan double helices is formed with Ca^{2+} and K^+ (producing soft hydrogels with similar strength values). In the case of the λ-C grades, the results indicated that they were composed of the λ-, κ-, θ- and μ-C forms, thus forming very heterogeneous network structures. Due to the greater amount of sulphate groups in the λ-, θ- and μ-carrageenan grades (two or three per disaccharide unit), the association of carrageenan chains via hydrogen bonding is hindered and the addition of salts is necessary to form strong hydrogels. According to our results, the presence of K^+ is more favourable to generate stronger cross-links between the carrageenan chains in the λ-C grades (as suggested by the greater hydrogel strength values), suggesting that the gelation mechanism was mostly governed by the κ-carrageenan component in these commercial grades.

4. Conclusions

Four different commercial carrageenan grades (κ-C, ι-C, λ↓-C and λ↑-C) have been used to produce hydrogels with and without the addition of salts (KCl and CaCl₂). The effect of carrageenan and salt concentration on the hydrogel strength were evaluated through a response surface design and the rheological behaviour and nanostructure of selected hydrogels were characterized. The results evidenced that the

composition, which conditioned the amount and location of sulphate groups in the different carrageenan grades studied, had a strong impact on their gelation mechanism and on the properties of the formed hydrogels.

The lower amount of sulphate substitution in κ-carrageenan (the major component in κ-C) enabled strong association of double helices, leading to the formation of stronger hydrogel networks than the other types of carrageenan, and could be properly modelled as Gauss-Lorentz gels. The addition of K^+ resulted in the neutralization of the negative charges from sulphate groups and, thus, promoted further aggregation of the κ-carrageenan double helices into more ordered and densely packed networks, hence increasing the hydrogel strength. In contrast, the presence of Ca^{2+} induced the formation of weaker ionic cross-links between the sulphate groups from adjacent κ-carrageenan double helices. The greater amount of sulphate groups in ι-carrageenan (the major component in ι-C) and in the hybrid λ-C grades hindered the proper association of double helices due to repulsive forces, hence making necessary the addition of salts to form hydrogels with sufficient mechanical integrity. The presence of cations produced the gelation of ι-C by the establishment of ionic cross-links between the carrageenan double helices, leading to much softer hydrogels than those from κ-C. Due to the greater ability of Ca^{2+} to form ionic cross-links with sulphate groups, these were more efficient in promoting ι-C gelation (i.e. lower concentrations were required and gelation temperatures were higher) than K^+, although similar network structures (consisting of larger but weaker aggregates than those formed in the case of κ-C) were formed in both cases, producing hydrogels with similar strength values. On the other hand, the heterogeneous composition of λ-C, which was seen to consist of λ-, κ-, θ- and μ-carrageenan hybrids, resulted in the formation of hydrogels with irregular branched networks. In presence of K^+, stronger intermolecular cross-links were attained, as suggested by the greater hydrogel strength values. On the other hand, although lower carrageenan and salt concentrations were required to form hydrogels from λ↑-C as compared with λ↓-C, the molecular weight did not have a dramatic impact on the hydrogel strength.

These results evidence that the combination of spectroscopy, rheology and small angle scattering techniques is an efficient approach to characterize the composition and structure of commercial carrageenan grades. Understanding the distinct gelation mechanism of

carrageenan hydrogels and their implications in the nanostructure of the formed hydrogels will open up the possibility of designing hydrogels with specific properties for targeted applications within the food industry and any other relevant fields.

Author contributions

MMS, CFC and ALR conceived the research and designed experiments; CFC and LGGM performed the experiments; CFC and AS analysed the data; CFC and MMS wrote the manuscript; all the authors critically revised and edited the manuscript; funding was obtained by ALR; all authors read and approved the final manuscript. MMS (mmartinez@iata.csic.es) and ALR (amparo.lopez@iata.csic.es) take responsibility for the integrity of the work as a whole, from inception to finished article.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

CRediT authorship contribution statement

Cynthia Fontes-Candia: Investigation, Formal analysis, Methodology, Writing - original draft. **Anna Ström:** Investigation, Formal analysis, Writing - review & editing, Supervision. **Laura G. Gómez-Mascaraque:** Investigation, Formal analysis, Writing - review & editing. **Amparo López-Rubio:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Funding acquisition, Supervision. **Marta Martínez-Sanz:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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