1 **Self-Assembled Gelatin- κ -Carrageenan Encapsulation Structures for**
2 **Intestinal-Targeted Release Applications**

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9 **ABSTRACT**

10 In this work, natural biopolymeric encapsulation structures were developed through the self-
11 assembly of gelatin and ι-carrageenan in aqueous solutions. The interactions of this binary
12 system and of a ternary system containing a polyphenol-rich extract were deeply explored for the
13 development of intestinal delivery systems. The processing of the structures (extrusion vs.
14 freeze-drying) greatly influenced release properties, explained by the specific interactions
15 between gelatin and polyphenols, thus allowing for tuning the processing conditions depending
16 on the desired target application. Release was further controlled by incorporating a divalent salt,
17 giving raise to extract-loaded ι-carrageenan/gelatin capsules with adequate release profiles for
18 intestinal targeted delivery. These results demonstrate the potential of exploiting biopolymer
19 interactions for designing bioactive delivery systems using environmentally friendly processes
20 which do not involve the use of toxic or harsh solvents or cross-linkers.

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23 **KEYWORDS**

24 Coacervation; polyelectrolyte; complex; delivery; food

1. Introduction

Biopolymer mixtures are of great interest in the food area for the development of novel textures (Wu, Degner, & McClements, 2014), improved delivery systems (Timilsena, Wang, Adhikari, & Adhikari, 2017), for the stabilization of emulsions (Cho & McClements, 2009; Evans, Ratcliffe, & Williams, 2013) and, even, for providing novel functionalities (Turgeon & Laneuville, 2009). Understanding the interactions between them is of outmost importance to tailor design the systems for their intended application. Specifically, interactions between proteins and charged polysaccharides have been the focus of extensive research work, as the combination of electrostatic interactions between them leads to the formation of polyelectrolyte complexes (Devi, sarmah, Khatun, & Maji, 2017; Semenova, 2017). These complexes have a unique ability for changes in their phase state in response to variations in environmental factors (pH, ionic strength, temperature, etc.) and, thus, they are considered smart or intelligent polymer systems (Derkach, Zhabyko, Voron'ko, Maklakova, & Dyakina, 2015).

The phenomenon giving rise to polyelectrolyte complexes between two oppositely charged polymers is known as associative phase separation or complex coacervation (Timilsena et al., 2017). Amongst its many applications, coacervation is considered to be the first microencapsulation technique developed (Srivastava, Semwal, & Sharma, 2013), as the biopolymer complexes are able to entrap other molecules of interest, such as bioactive ingredients. In many cases, microencapsulation through complex coacervation can be achieved in mild conditions and without toxic solvents, therefore presenting obvious advantages for their application in food products (Đorđević et al., 2015). The food industry has an increasing commercial interest in the development of food-grade encapsulation strategies for the production of new functional foods enriched with bioactive ingredients (Ozen, Pons, & Tur, 2012), and

biopolymer mixtures giving rise to polyelectrolyte complexes could meet this demand. Moreover, these complexes are also of interest for many other applications, including the production of flexible electronic devices and electronic inks, as potential drug delivery systems and tissue regeneration scaffolds, or for the development of improved membrane separation technologies, among others (Zhao et al. 2011; Marciel et al. 2017; Facchi et al. 2017; Zhao et al. 2018).

Gelatins and carrageenans are two types of edible biopolymers which are able to form thermoreversible hydrogels through coil/helix conformational transitions (Michon, Vigouroux, Boulenguer, Cuvelier, & Launay, 2000). This property makes them ideal to be processed in aqueous media, while avoiding complete disruption of the obtained materials below their gel-sol transition temperature (Gómez-Mascaraque, Lagaron, & López-Rubio, 2015). Gelatin is a protein obtained from partial hydrolysis of collagen and its structure is based on repeating tripeptide sequences of glycine-aa₁-aa₂, where amino acids aa₁ and aa₂ are mainly proline and hydroxyproline (Gómez-Mascaraque, Soler, & López-Rubio, 2016). Gelatin has been largely employed for enhancing elasticity, stability and consistency of food products (Okutan, Terzi, & Altay, 2014). Moreover, it has been traditionally used by the pharmaceutical industry for the encapsulation of drugs (Roussanova et al., 2012), and has more recently attracted interest as a microencapsulation matrix for food ingredients (Gómez-Mascaraque et al., 2017). Particularly, it has been used to microencapsulate bioactive compounds through coacervation in combination with different polysaccharides (Devi et al., 2017).

Carrageenans are a family of linear, sulphated polysaccharides (galactans) extracted from marine red algae which have also been widely used as thickening, gelling agents, stabilizers and texture

enhancers in the food industry (Liu, Chan, & Li, 2015). The three most relevant commercial carrageenan types are *kappa* (κ), *iota* (ι) and *lambda* (λ), which have one, two and three sulphate ester groups, respectively (Daniel-da-Silva, Ferreira, Gil, & Trindade, 2011). Although the most extensively used has been κ -carrageenan, which forms harder and stronger gels (Azevedo, Torres, Sousa Pinto, & Hilliou, 2015), ι -carrageenan yields fragments of greater molecular weight upon hydrolysis in the gastrointestinal tract, and these are generally less harmful when ingested (Ekström, Kuivinen, & Johansson, 1983). Hence, ι -carrageenan was selected in this work as a more adequate candidate for food purposes, even though it has received relatively lower attention. The attractive electrostatic interactions between gelatin and ι -carrageenan in aqueous solution, at pHs below the isoelectric point of the protein, lead to the formation of polyelectrolyte complexes through complex coacervation (Michon et al., 2000).

The aim of this work was to study the interactions taking place between gelatin and ι -carrageenan in aqueous solutions, and further explore their interactions in the presence of a polyphenol-rich bioactive food extract, to optimise the protein/polysaccharide ratio for the design of encapsulation structures with tailored release properties. For this purpose, gelatin/ ι -carrageenan blends with different biopolymer ratios were prepared both in the absence and in the presence of the extract, and characterized in terms of pH, electrical conductivity, surface tension, turbidity and rheological behaviour. A grape juice extract (GJE) was selected as a model bioactive extract, since it is an important dietary source of health-promoting antioxidant molecules and has demonstrated to reduce the risk of various chronic diseases in epidemiological studies (Genova, Tosetti, & Tonutti, 2016). The blends were then freeze-dried in order to dry the structures and increase their shelf-stability. The resulting materials were further characterized through infrared spectroscopy, and their water uptake capacity and release properties were

94 studied to select the optimal composition for encapsulation of the bioactive extract. Finally, the
95 impact of the addition of a divalent salt, as well as the preparation protocol, on the release
96 properties of the materials was also investigated.

2. Experimental Section

2.1 Materials

Gelatin from porcine skin, with reported gel strength of 180 g Bloom, was supplied by Gelita AG (Eberbach, Germany). *ι*-Carrageenan, methylene blue reagent (MB) and calcium chloride dehydrate (purity >99%) were obtained from Sigma-Aldrich (Madrid, Spain). Citric acid/sodium hydroxide/hydrochloric acid and boric acid/potassium chloride/sodium hydroxide buffer solutions, of pH 2 and 8 respectively, were provided by Scharlab S.L. (Spain). A grape juice extract (GJE) with the European Economic Community (EEC) code E-163 was kindly donated by SECNA, S.L. (Valencia, Spain).

2.2 Preparation of biopolymer solutions and blends thereof

Gelatin and *ι*-carrageenan were separately dissolved in distilled water under magnetic stirring by mild heating at 40 °C. High biopolymer concentrations are usually desired for encapsulation purposes since they yield denser gel networks which provide greater barrier effects. However, processing of concentrated biopolymer solutions is frequently limited by their high viscosities or even gelling. Therefore, a concentration of 1% (w/v) was selected for both biopolymers, as it was low enough to avoid gelation of gelatin at room temperature (which occurred at concentrations above 1.5%) and too high viscosities for *ι*-carrageenan. Gelatin/*ι*-carrageenan blends with different protein to polysaccharide ratios, i.e. 0:100, 15:85, 30:70, 50:50, 70:30, 85:15 and 100:0, were prepared by adding the gelatin solution onto the corresponding amount of *ι*-carrageenan solution, followed by intense magnetic stirring for 2 h. For selected compositions, the impact of reversing the order of addition of the biopolymers (i.e. *ι*-carrageenan onto gelatin) was also explored (cf. Section 2.11). When the GJE extract was incorporated within the blends, it was

added in a proportion of 30% w/w with respect to the total mass of biopolymers, and the blends were thoroughly mixed by magnetic stirring for 2 h.

2.3 Characterization of the blends

The pH and electrical conductivity of the gelatin/*ι*-carrageenan blends were measured at room temperature using a pHmeter 50+ serie version 1.0 (LabProcess) and a XS Con6 conductivity meter (Labbox, Barcelona, Spain), respectively. The surface tension of the blends was measured at room temperature using the Wilhelmy plate method in an EasyDyne tensiometer (Krüss GmbH, Hamburg, Germany).

The turbidity of the blends was calculated at 600 nm using a VWR-1200 spectrophotometer (USA) according to Equation 1, where L is the optical path length (1 cm), I_0 the incident light intensity and I_t the transmitted light intensity (Cao et al., 2015).

$$\tau = \left(-\frac{1}{L}\right) * \ln\left(\frac{I_t}{I_0}\right) \quad (1)$$

The rheological behaviour of the blends was studied following the procedure described in Gómez-Mascaraque et al. (2015). Briefly, a rheometer model AR-G2 (TA Instruments, USA) with a parallel plate geometry was used. The stainless steel plate diameter was 60 mm and the gap was fixed to 0.5 mm. The tests were performed at a controlled temperature of $25 \text{ }^\circ\text{C} \pm 0.1 \text{ }^\circ\text{C}$. Continuous shear rate ramps were performed from 0.1 to 200 s^{-1} during 15 min after equilibrating the samples for 5 min, and the shear stress of the samples was registered. The obtained flow curves were adjusted to the Ostwald de Waele model following Equation 2, where σ was the shear stress, K was the flow consistency index, γ was the shear rate, and n was the flow behaviour index (López-Rubio et al., 2016).

$$\sigma = K \gamma^n \quad (2)$$

All measurements were made at least in triplicate.

2.4. Small-angle X-ray scattering (SAXS) experiments

The structure of the pure biopolymers and the blends at the nanoscale level was investigated by means of small angle X-ray scattering of the prepared solutions. SAXS experiments were carried out in the Non Crystalline Diffraction beamline, BL-11 at ALBA synchrotron light source (www.albasynchrotron.es), using the experimental settings previously described in Atay, et al. (2017). The data reduction was treated by pyFAI python code (ESRF) (Kieffer & Wright, 2013), modified by ALBA beamline staff. The intensity profiles were then represented as a function of q using the IRENA macro suite (Ilavsky & Jemian, 2009) within Igor procedures. A scattering background from a quartz capillary filled in with water was subtracted from all the samples. The experimental data were fitted using a two-level or three-level Beaucage model, depending on the sample and the amount of structural features apparent in the corresponding scattering patterns. This model considers that, for each individual level, the scattering intensity is the sum of a Guinier term and a power-law function (Beaucage, 1995; Beaucage, 1996):

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

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 i^{th} structural feature and the surrounding solvent), $R_{g,i}$ is the radius of gyration describing the average size of the i^{th} 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.

2.5. Methylene blue (MB) analysis

The interactions between gelatin and *ι*-carrageenan were studied using the methylene blue (MB) analysis described by Yang, et al. (2012). Firstly, a MB aqueous solution (0.0005% w/v) was prepared, and *ι*-carrageenan was subsequently dissolved in the MB solution at different concentrations in the range from 0.40% to 0.00625%. The absorbance of the MB-*ι*-carrageenan solutions was measured at 664 and 616 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), and the ratio A_{664}/A_{616} was calculated. The *ι*-carrageenan concentration above which all MB molecules were bound to *ι*-carrageenan molecules was determined as the concentration at which A_{664}/A_{616} reached a plateau value. Secondly, gelatin was dissolved in the MB-*ι*-carrageenan solution at the *ι*-carrageenan concentration determined above and at different gelatin concentrations ranging from 0.25% to 0.000244%. The ratio A_{664}/A_{616} was calculated for the different MB-*ι*-carrageenan-gelatin solutions to study the interactions between *ι*-carrageenan and gelatin. All measurements were performed in triplicate.

2.6. Drying of the blends

Gelatin/*ι*-carrageenan blends were freeze-dried using a Genesis 35-EL freeze-dryer (Virtis). The dry materials were then ground using a IKA A11 Basic grinder (Germany) after soaking in liquid nitrogen, to obtain powdery materials.

2.7. Fourier transform infrared (FT-IR) analysis of the materials

Freeze-dried, ground materials (*ca.* 1–2 mg) were dispersed in spectroscopic grade potassium bromide (*ca.* 130 mg) (KBr). A pellet was then formed by compressing the samples at 150 MPa. FT-IR spectra were collected in transmission mode using a Thermo Nicolet Nexus equipment. The acquisition time was 128 s at 4 cm⁻¹ resolution, and the average spectra are reported.

2.8. Water uptake capacity of the freeze-dried materials

The water-uptake capacity of the freeze-dried materials at high relative humidity (RH) was assessed following the method described in Costamagna et al. (2017). Briefly, the samples (*ca.* 100 mg) were previously stored in a desiccator at 0% RH for 48 h and the mass of dried capsules (m_0) was determined. The samples were then stored in another desiccator at 74.9% RH and weighted after different time intervals. The water uptake (WU) at 74.9% RH was calculated using Equation 4, where m_t is the mass of the capsules at time t after storage at 74.9% RH. Experiments were performed at least in triplicate.

$$WU_t (\%) = \frac{m_t - m_0}{m_t} \times 100 \quad (4)$$

2.9. Release of GJE from the freeze-dried materials

The release of the GJE from the different gelatin/*ι*-carrageenan freeze-dried blends was assessed following a method adapted from Atay et al. (2017). Briefly, the materials were suspended in 20 mL of distilled water at a theoretical concentration of GJE of 0.5 mg/mL and the absorbance of the supernatant at 523 nm was measured at different time intervals using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), prior calibration ($R^2_{H_2O} = 0.9999$). The samples were gently shaken to homogenize them and subsequently centrifuged in a centrifuge 5804R (Eppendorf AG, Hamburg, Germany) at 2400 rpm for 3 min at 20 °C before analysis of the supernatant. Experiments were performed in triplicates.

2.10. Ionic crosslinking of the materials

The optimal gelatin/*ι*-carrageenan blend selected from the results of the previous sections (85:15, cf. Section 3.6) was prepared in the presence of calcium chloride (CaCl_2) in order to assess the impact of ionic crosslinking on the GJE release profile from the blend materials. For this purpose, CaCl_2 was dissolved in the gelatin solution at different concentrations (i.e. 50mM, 100mM, 200mM) prior to its addition to the *ι*-carrageenan solution, and the procedure described above was followed to prepare GJE-containing blends, which were subsequently freeze-dried and ground as described in Section 2.6. The release profiles of GJE from the obtained powdery materials at two different pHs (i.e. pH=2 and pH=8, using citric acid/sodium hydroxide/hydrochloric acid and boric acid/potassium chloride/sodium hydroxide buffer solutions, respectively) were then studied following the protocol described above, prior calibration in both media ($R^2_{\text{pH}2} = 0.9998$; $R^2_{\text{pH}8} = 0.9999$).

2.11. Production of GJE-loaded *ι*-carrageenan-gelatin beads

The optimal gelatin/*ι*-carrageenan ratio previously selected (85:15, cf. Section 3.6) was also used to produce GJE-loaded biopolymeric beads (capsules) by extrusion. For this purpose, the order of addition of the biopolymers was reversed: the solution of *ι*-carrageenan containing the GJE was added drop-wise onto the corresponding amount of gelatin solution required to achieve a gelatin/*ι*-carrageenan ratio of 85:15, which contained different concentrations of CaCl_2 (i.e. 50mM, 100mM, 200mM). The as-prepared beads were then freeze-dried and their release profiles studied at pH=2 and pH=8 as described above.

2.12. Statistical analysis

IBM SPSS Statistics software (v.23) (IBM Corp., USA) was used to perform the statistical analysis of the data. The significance of the differences observed between samples was assessed through two-sided t-tests at $p < 0.05$. For multiple comparisons, the p-values were adjusted using the Bonferroni correction.

3. Results and Discussion

Initially, gelatin/*ι*-carrageenan blends with different protein/polysaccharide ratios (i.e. 100:0, 85:15, 70:30, 50:50, 30:70, 15:85 and 0:100) were prepared and characterized, both in the absence and in the presence of a polyphenol-rich food extract (i.e. grape juice extract, GJE), in order to find the optimal composition which maximized intermolecular interactions in the system.

3.1. Characterization of the blends

Figure 1 shows the pH, conductivity, surface tension and turbidity of gelatin/*ι*-carrageenan blends in the absence and in the presence of grape juice extract, as a function of the gelatin content. Their rheological profiles are depicted in Figure 2.

Figure 1. pH, conductivity, surface tension and turbidity of gelatin/*i*-carrageenan blends in the absence (solid lines) and in the presence (dotted lines) of grape juice extract, as a function of the gelatin content.

Figure 2. Rheological behaviour of gelatin/ ι -carrageenan blends in the absence (A) and in the presence (B) of grape juice extract.

Both the pH and the conductivity of the gelatin solutions were lower than that of the ι -carrageenan solutions. As a result, the pH and conductivity of the biopolymer blends in the absence of GJE decreased with increasing gelatin/ ι -carrageenan ratios, in a linear manner due to the weighted contribution of the individual components. The addition of the GJE caused a decrease in the pH (due to its acidic nature) and a slight increase in the conductivity of the blends. Remarkably, some deviations from the linearity were observed for the extract-containing

blends with the greatest protein contents, which suggested that interactions between the GJE and gelatin might take place, reducing the contribution of gelatin to the pH and conductivity of the blends. Indeed, proteins can strongly interact with polyphenol molecules via different mechanisms, including hydrogen bonding or hydrophobic interactions (Peña, de la Caba, Eceiza, Ruseckaite, & Mondragon, 2010).

Regarding the surface tension of the solutions, it was considerably lower for gelatin than for *ι*-carrageenan, given that proteins generally exhibit amphiphilic structures and, thus, effective surfactant properties (McClements, 2004). Again, the surface tension of the biopolymer blends in the absence of GJE decreased linearly with increasing gelatin/*ι*-carrageenan ratios, due to their individual contribution to the properties of the blend. However, when the extract was added, deviations from the linearity were observed for the blends with the greatest protein contents. For these samples, the surfactant properties of gelatin might have been altered, presumably due to its interaction with the polyphenol-rich extract.

The turbidity could only be estimated for the blends in the absence of the extract, as the colorant had a great contribution to the absorbance of the samples. This magnitude, which is frequently used to study electrostatic complexation between biopolymers (Cao et al., 2015), exhibited a significant increase for the gelatin/*ι*-carrageenan ratio 85:15 as compared to all the other blends. This suggested a greater extent of macroscopic complexation, due to intermolecular interactions between both biopolymers, for the blend containing the highest amount of protein.

The rheological behaviour of biopolymer systems depends on the characteristics of their molecular networks (Banerjee & Bhattacharya, 2012), so it reveals valuable information about the structure and intermolecular interactions within solutions and gels. The rheograms of the

gelatin/*ι*-carrageenan blends prepared in this work are shown in Figure 2. The obtained curves were fitted to the Ostwald de Waele model, since it is the most widely employed model for non-Newtonian liquids (Marcotte, Hoshahili, & Ramaswamy, 2001), and their flow consistency indexes (*K*) and flow behaviour indexes (*n*) are summarized in Table 1.

Table 1. Flow consistency indexes (*K*) and flow behaviour indexes (*n*) of the gelatin/*ι*-carrageenan blends according to the Ostwald de Waele model. Different superscripts (a–f) within the same column indicate significant differences at $p < 0.05$ among the samples.

Gelatin/ <i>ι</i> -carrageenan ratio	Blends without extract		Blends with extract	
	<i>n</i>	<i>K</i>	<i>n</i>	<i>K</i>
0:100	0.22±0.01	21.40±1.59 ^a	0.18±0.03	22.82±0.16 ^a
15:85	0.10±0.01	59.36±1.71 ^c	0.08±0.01	29.82±0.70 ^c
30:70	0.18±0.04	17.02±2.21 ^a	0.11±0.03	17.59±5.50 ^d
50:50	0.21±0.01	16.67±0.36 ^a	0.33±0.02	3.34±0.41 ^b
70:30	0.24±0.01	19.47±1.90 ^a	0.64±0.02	0.24±0.02 ^e
85:15	0.34±0.11	0.32±0.02 ^d	0.63±0.01	0.01±0.01 ^f
100:0	0.82±0.08	0.01±0.01 ^b	0.75±0.03	0.01±0.01 ^{b,f}

The rheological behaviour of gelatin was almost Newtonian, with a linear relationship between the shear stress and the shear rate in the whole range of study, and a flow behaviour index close to 1. Gelatin is known to behave as a Newtonian fluid in aqueous solutions under non-gelling conditions, even at high concentrations (Wulansari, Mitchell, Blanshard, & Paterson, 1998). On

the other hand, *ι*-carrageenan exhibited its typical shear-thinning behaviour (Marcotte et al., 2001), with a much higher flow consistency index than gelatin.

An intermediate rheological behaviour between that of gelatin and *ι*-carrageenan would be expected for the blends in the absence of intermolecular interactions. However, this was not the case for some gelatin/*ι*-carrageenan blends in the absence of GJE (Figure 2A). Specifically, the blend 15:85 showed the highest flow consistency index and, together with the blend 70:30, the most shear-thinning behaviour (lowest flow behaviour index). Moreover, these two blends exhibited a visco-plastic behaviour, i.e. there was a critical stress (yield stress) below which the fluids did not flow but deformed plastically like solids, and above which the fluids flowed like viscous materials. The existence of a yield stress is typical of gel-like materials (Coussot, Nguyen, Huynh, & Bonn, 2002), and has been previously observed in carrageenan solutions at higher concentrations (Marcotte et al., 2001). Michon et al. (2000) reported that gelatin chains were able to stabilize *ι*-carrageenan networks by connecting the double helices of the polysaccharide. The visco-plastic behaviour of the blends with ratio 15:85 and 70:30, thus, suggested that in these samples, the gelatin chains were able to reinforce the *ι*-carrageenan network in such a way that they behaved like weak gels. For a better understanding of the structural conformation giving rise to this behaviour, SAXS experiments were also conducted for the gelatin/*ι*-carrageenan blends (cf. Section 3.2).

The addition of GJE to the gelatin/*ι*-carrageenan blends substantially affected their rheological behaviour. For the highest protein ratios (50:50, 70:30 and 85:15), the flow behaviour index increased while the flow consistency index decreased, that is, their behaviour was closer to that of a Newtonian fluid and their viscosity decreased. Moreover, the yield stress of the blends with the lowest protein ratios (15:85, 30:70) significantly decreased, and the consistency index of the

sample 15:85 was lower than that of pure *ι*-carrageenan. Again, these results suggested that interactions between the polyphenol-rich extract and gelatin took place, so that they competed with the gelatin/*ι*-carrageenan interactions reducing the contribution of gelatin to the strengthening of the *ι*-carrageenan network.

3.2. Structural conformation at the nanoscale level

Several studies have reported on the complex nature of gelatin/*ι*-carrageenan systems, in which complex formation may be promoted by the existence of different interactions, i.e. electrostatic interactions, hydrophobic interactions and hydrogen bonding (Fang, Li, Inoue, Lundin, & Appelqvist, 2006; Michon et al., 2000; Voron'ko, Derkach, Vovk, & Tolstoy, 2017; Wang et al., 2015). The prevalence of each specific type of interaction gives rise to the formation of different structural features, such as coacervates and hydrogels. To investigate the structural conformation of gelatin and *ι*-carrageenan in the nanometric size range, the aqueous solutions of the pure biopolymers and their blends were characterised by means of SAXS. The scattering patterns of the different samples are shown in Figure 3.

Figure 3. SAXS patterns from pure ι -carrageenan and gelatin and gelatin/ ι -carrageenan blends (aqueous solutions). Markers correspond to the experimental data and solid lines correspond to the fits obtained using the Beaucage model.

The pure ι -carrageenan solution shows a similar pattern to that previously reported for both ι -carrageenan and κ -carrageenan in the semidilute regime (Mischenko, Deneff, Koch, & Reynaers, 1996), indicating the arrangement of ι -carrageenan chains in a random coil conformation. As observed in Figure 3, the scattering patterns from the pure gelatin and the gelatin/ ι -carrageenan blends were characterised by the appearance of shoulder-like features. The experimental data could be fitted by applying a two- or three-level Beaucage model, depending on the number of

scattering features visible in the patterns. The structural parameters obtained from the fits are gathered in Table 2. The pure gelatin showed a behavior very similar to that previously reported for 8 wt.-% gelatin solutions in acetic acid (Atay et al., 2017), with two characteristic structural features whose associated radii of gyration are $R_{g1}=50.7$ nm and $R_{g2}=2.4$ nm. The value of R_{g2} agrees with the correlation length reported in the literature for gelatin solutions (Mohanty, Aswal, Kohlbrecher, & Bohidar, 2006) and is characteristic of the distance between adjacent polymeric chains. Interestingly, the blends showed a very different behavior depending on the ratio of the two biopolymers. The 85:15, 50:50 and 30:70 blends present one broad shoulder-like feature, whereas the 15:85 and 70:30 blends display scattering patterns characteristic of weak gels, with two scattering features. For the latter ones, the power-law exponents in the low q region, which are close to -3, are indicative of the presence of branched polymeric networks, such as the ones present in gels. The associated radii of gyration, characteristic of the gel mesh size, are 83 nm and 77 nm for the 15:85 and 70:30 blends, respectively. This suggests that a more open gel network structure is attained when increasing the ι -carrageenan content. In contrast to this, the scattering patterns from the 50:50 and 30:70 blends are indicative of polymeric solutions. The power-law exponent in the intermediate q region is -1.69 for the 50:50 blend, suggesting the presence of linear polymer coils in a good solvent and -2.53 for the 30:70 blend, reflecting the existence of branched polymer chains in a theta solvent (Yang, et al., 2015). These results suggest that there must be an optimum gelatin/ ι -carrageenan ratio at which the blends behave like solutions. An excess of any of the biopolymers gives rise to the formation of gel-like network structures, due to the formation of intermolecular interactions such as hydrogen bonding. Surprisingly, the 85:15 blend presents a notably distinct behaviour. While the high q region is characterised by a very broad shoulder feature with an associated radius of gyration of

ca. 4 nm and a power-law exponent of -1.71, the scattering intensity in the low q region increases sharply, suggesting the presence of a scattering feature at lower q values, out of the experimental range. This behavior may be characteristic of large aggregates (larger than ca. 200 nm) which have an internal structure of swollen linear chains. This is in agreement with previous studies, which showed that the formation of gelatin/ ι -carrageenan complexes only takes place within a certain stoichiometric range. As a reference, the κ -carrageenan/gelatin stoichiometric range has been reported to be 0.03-0.75 (Voron'ko et al., 2017), but the presence of additional sulphate groups in ι -carrageenan is surely expected to affect the range at which complexes are formed.

Table 2. Parameters obtained from fits of the Beaucage model for the gelatin/ ι -carrageenan blends.

	0:100	15:85	30:70	50:50	70:30	85:15	100:0
G_1	---	$3.64 \cdot 10^{-6}$	---	---	$3.64 \cdot 10^{-6}$	---	
R_{g1}	---	83.47	---	---	77.38	---	50.70
B_1	---	$1.43 \cdot 10^{-3}$	---	---	$1.43 \cdot 10^{-3}$	---	---
P_1	---	3.15	---	---	2.91	---	---
G_2	---	11.80	21.84	28.33	5.06	43.68	12.56
R_{g2}	---	2.79	3.57	4.15	3.61	4.07	2.44
B_2	---	$1.81 \cdot 10^{-6}$	$1.31 \cdot 10^{-4}$	$6.25 \cdot 10^{-3}$	$1.02 \cdot 10^{-6}$	$3.66 \cdot 10^{-2}$	$1.36 \cdot 10^{-7}$
P_2	---	4.00	2.53	1.69	4.00	1.71	4.00

3.3. Methylene blue (MB) analysis

The electrostatic interactions between gelatin and ι -carrageenan were studied using the MB analysis. The MB reagent is known to interact with the sulphated groups of carrageenans, forming MB-carrageenan complexes. When gelatin, which interacts associatively with

carrageenan, is introduced in a methylene blue/carrageenan solution, a competition between the MB and the gelatin molecules for the sulphated groups of carrageenan takes place, so that MB molecules are released from the sulphated groups of carrageenan back to the solution in their free state (Michon, Konaté, Cuvelier, & Launay, 2002). These changes can be tracked using spectrophotometric techniques, since free MB in aqueous solutions show a maximum of absorbance at about 664 nm, while the soluble MB-polysaccharide complexes exhibit a maximum of absorbance at about 616 nm. When a low concentration of carrageenan is added to MB solutions, the peak at 664 nm decreases and a shoulder appears at 616 nm. Conversely, when gelatin is added to a MB-carrageenan solution, the peak at 664 nm increases and the shoulder at 616 nm decreases (Yang et al, 2012).

Figure 4. Ratio of absorbance at 664 and 616 nm (A_{664}/A_{616}) for aqueous solutions of 0.0005% methylene blue + *l*-carrageenan as a function of *l*-carrageenan concentration (dotted line) and for aqueous mixtures of 0.0005% methylene blue + 0.00625% *l*-carrageenan + gelatin as a function of gelatin concentration (solid line).

405

406 The results from the MB analysis are shown in Figure 4. The maximum *ι*-carrageenan
407 concentration capable of interacting with 0.0005% MB, determined as the concentration at which
408 the absorbance ratio A_{664}/A_{616} reached a plateau value, was found to be 0.00625% w/v. Using
409 this *ι*-carrageenan concentration, the maximum gelatin concentration capable of interacting with
410 *ι*-carrageenan in the presence of MB, again determined as the concentration at which the
411 absorbance ratio A_{664}/A_{616} reached a plateau, was found to be 0.03125% w/v. Hence, the
412 maximum amount of gelatin which could interact with *ι*-carrageenan was much higher than the
413 amount of *ι*-carrageenan present in the solution, specifically 5 times higher. Interestingly, this
414 gelatin/*ι*-carrageenan ratio is very close to the highest protein ratio used in the gelatin/*ι*-
415 carrageenan blends (i.e. 85:15), which also showed a distinct behaviour in the SAXS results and
416 the greatest extent of complexation in the turbidity tests (cf. Figure 1). These results were in
417 good agreement with those reported by Voron'ko et al. (2017) for κ -carrageenan, which
418 suggested that one κ -carrageenan macromolecule could interact with six gelatin macromolecules
419 in their system. Therefore, it was confirmed that the intermolecular interactions between both
420 biopolymers were maximal for the blends with a gelatin/*ι*-carrageenan ratio of 85:15.

421

422 **3.4. FT-IR analysis**

423 The freeze-dried blends, both in the absence and in the presence of GJE, as well as the pure
424 extract, were analysed by FT-IR and the spectra of representative samples are shown in Figure 5.
425 The characteristic bands of carrageenans were observed in the freeze-dried *ι*-carrageenan at 1260
426 cm^{-1} , attributed to the ester sulphate groups, 933 cm^{-1} , ascribed to the anhydride galactose group,
427 852 cm^{-1} , corresponding to the galactose-D-sulfate group and the hydroxyl band centered at 3460
428 cm^{-1} (Derkach, Ilyin, Maklakova, Kulichikhin, & Malkin, 2015). On the other hand, the spectrum

429 of the freeze-dried gelatin showed its four most characteristic bands centered at 3422 cm^{-1} ,
430 attributed to stretching vibrations of N-H bonds coupled with hydrogen bonding (Amide A),
431 1660 cm^{-1} , associated to the stretching mode of C=O coupled with C-N deformation (Amide I),
432 1548 cm^{-1} , representative of the bending vibration of N-H bonds in amino groups (Amide II) and
433 1244 cm^{-1} , attributed to the stretching vibrations of C-N groups (Amide III) (Gómez-Mascaraque
434 et al., 2015).

Figure 5. FT-IR spectra of representative samples. A) Individual biopolymers and their blend (ratio 50:50). B) ι -Carrageenan, the grape juice extract (GJE) and their blend (30% GJE). C) Gelatin, GJE and their blend (30% GJE).

The spectra of the gelatin-*ι*-carrageenan blends showed displacements in the amide I and amide II bands. Specifically, the amide I band was displaced towards lower wavenumbers (1650-1655 cm^{-1}) in the blends, while the amide II band shifted to higher wavenumbers (1554 cm^{-1} , irrespective of the protein content). These shifts indicate the interaction between both biopolymers, which are known to form polyelectrolyte complexes upon mixing. These complexes formation results in conformational changes in the protein as a consequence of the increase in order structures (Derkach et al., 2015). It is worth noting that the vibrational bands from the carrageenan sulfated groups also shifted towards lower wavenumbers, specifically 851-849 cm^{-1} in the case of the d-galactose-sulfate band and 1257-1255 cm^{-1} for the ester sulfate group band), thus confirming the interactions established between the two biopolymers.

When GJE was incorporated in the blends, shifts in its band attributed to hydroxyl groups (centered at 3390 cm^{-1} in the pure extract) were observed. Specifically, it experienced an important shift towards lower wavenumbers (3324 cm^{-1}) when it was incorporated within gelatin (Figure 5C). This shift, which was also observed in the gelatin/*ι*-carrageenan blends but not when the extract was incorporated within the pure *ι*-carrageenan, suggested the presence of intermolecular interactions between gelatin and the GJE via hydrogen bonding. Certainly, the ability of proteins to strongly interact with polyphenols is widely recognized, and one of the main proposed mechanisms of interaction is through hydrogen bonding (Jakobek, 2015). These results, together with the deviations from linearity observed in the solution properties of the GJE-containing blends at the highest protein concentrations (cf. Figure 1) suggest that the interactions of the extract with the biopolymer matrix take place preferentially with the protein.

3.5. Water uptake capacity of the freeze-dried materials

The water uptake (WU) capacity of encapsulation matrices is a feature of great relevance, since it is directly related to their ability to reduce the hygroscopicity of the formulated ingredients and, thus, to ensure their textural and storage stability. It can also be used as an indirect way of studying the biopolymer interactions and can provide information about the swelling ability of the materials, which in turns have an impact on their release properties. Hence, the water uptake of the GJE-containing freeze-dried blends was assessed in high RH conditions (i.e. 74.9%), and the results are shown in Figure 6A. All the dry blends exhibited a rapid water uptake during the first 4 h after storage at high RH, which reached a plateau after 24 h. This water uptake capacity at equilibrium was higher for *ι*-carrageenan than for gelatin, which could be attributed to both their differences in composition and the higher porosity of freeze-dried carrageenans as compared to freeze-dried gelatin (Varghese, Chellappa, & Fathima, 2014).

The blends containing higher amounts of *ι*-carrageenan exhibited an intermediate behaviour between that of the individual biopolymers, due to their individual contribution. However, blends containing higher amounts of gelatin had slightly lower water uptake capacities than the protein. This could be explained in the light of the greater extent of intermolecular interactions between both biopolymers at higher gelatin concentrations, as inferred in the previous sections. Indeed, these intermolecular interactions compete with water molecules for the available binding sites, so that a higher amount of functional groups participating in polymer-polymer interactions implies a lower water sorption. In all cases, the freeze-dried matrices were very effective in reducing the hygroscopicity of the GJE, which sorbed up to 32% water after 100 h at 74.9% RH (cf. Figure S1 of Supporting Information).

Figure 6. A) Water uptake capacity of GJE-containing freeze-dried powders with different gelatin/ ι -carrageenan ratios. B) Release of GJE from freeze-dried blends with different gelatin/ ι -carrageenan ratios.

3.6. Release of GJE from the freeze-dried materials

The release profile of the compounds of interest from their encapsulation matrices is another feature of outmost importance when designing delivery vehicles, since one of the aims of encapsulation is to deliver the bioactive molecules specifically to their target sites. Therefore, a preliminary release study was carried out for the different freeze-dried blends using distilled

water as an aqueous release medium, in order to select the optimal gelatin/*ι*-carrageenan composition. The results are shown in Figure 6B. The release of the selected blend was then further investigated as a function of the pH (see below).

The release profiles of the GJE from all the freeze-dried blends in water showed an initial burst release during the first 30 min, which is typical of hydrophilic matrices (Gómez-Mascaraque et al, 2015). This fast release was attributed to the presence of poorly bound GJE molecules on the surface of the freeze-dried materials which were rapidly released upon swelling of the matrices in the aqueous medium, facilitated by the high specific area of the porous, powdery freeze-dried materials. Interestingly, differences in the amount of extract released at equilibrium were observed among the samples depending on the blend composition. While the samples from the blends containing higher amounts of *ι*-carrageenan (including the pure *ι*-carrageenan) released more than 93% of the extract they contained, pure gelatin released only 85% of the GJE and the samples containing higher amounts of gelatin released about 60% of the extract. As previously discussed, the GJE interacts preferentially with the protein rather than the polysaccharide, so the more effective entrapment of the extract within gelatin than within blends containing high amounts of *ι*-carrageenan could be expected. However, the materials containing high amounts of gelatin but also some *ι*-carrageenan retained the extract much more efficiently than pure gelatin. Hence, the complexation of gelatin with *ι*-carrageenan, which was intensified at higher gelatin/*ι*-carrageenan ratios as discussed throughout the previous sections, was the main responsible of the enhanced entrapment of the GJE within the freeze-dried materials. Therefore, the blend with the highest gelatin content (i.e. the gelatin/*ι*-carrageenan ratio of 85:15) was selected for further experiments.

519

520 **3.7. Impact of pH and ionic crosslinking on the release of GJE**

521 Due to the polyelectrolyte nature of the biopolymers used in this work, and thus, their inherent
522 pH-responsiveness, the selected gelatin/*ι*-carrageenan matrices were expected to release the
523 extract in a different manner depending on the pH of the release media. For that reason, the
524 release of GJE from the selected blend was studied at two different pHs, i.e. pH=2 and pH=8,
525 which simulate the acidic and alkaline conditions of the gastric and intestinal environments,
526 respectively (Evans et al., 1988).

527 Moreover, ionic crosslinking of the selected blend was also attempted by adding different
528 concentrations of CaCl₂ (i.e. 50mM, 100mM, 200mM) to the gelatin solution prior to its mixing
529 with *ι*-carrageenan. Although monovalent cations induce faster gelation of *κ*-carrageenans than
530 divalent cations, the opposite trend has been reported for *ι*-carrageenans, and in particular
531 divalent salts such as CaCl₂ are effective in inducing gelation of *ι*-carrageenans (Michel,
532 Mestdagh, & Axelos, 1997). Crosslinking of biopolymer networks create additional bonds
533 between macromolecular chains which generally limit the swelling capacity of the structures
534 consequently constraining the diffusion of entrapped molecules and delaying their release
535 (Gómez-Mascaraque, Méndez, Fernández-Gutiérrez, Vázquez, & San Román, 2014). Hence, the
536 release of GJE from these crosslinked samples in acidic and alkaline media was also evaluated
537 and compared to that of the blend prepared in the absence of the salt. The results are shown in
538 Figure 7A.

539 Again, the release profiles of GJE from the freeze-dried samples prepared both in the absence
540 and the presence of CaCl₂ and in both media, showed a burst release during the first 30 min. The
541 more poorly bound extract molecules were rapidly released while the effectively entrapped

fraction of the GJE remained confined within the biopolymeric structures due to the intermolecular interactions taking place in the GJE-gelatin-*ι*-carrageenan ternary systems.

As expected, the release of the extract from the gelatin/*ι*-carrageenan matrices prepared without salts was pH-dependent, being higher at alkaline pH than at acidic pH. Hezaveh and Muhamad (2013) also found a similar pH-responsive behavior in κ -carrageenan/polyvinyl alcohol hydrogels, which exhibited increased β -carotene release at pH=7 as compared to pH=1.2. This finding reveals the great potential of gelatin/*ι*-carrageenan networks for the selective deliver of bioactive compounds to the intestine.

Remarkably, the amount of released extract decreased with increasing CaCl_2 concentrations, confirming that the divalent salt promoted the crosslinking of the biopolymeric matrices, strengthening the hydrogel network and therefore hindering the release of GJE to a greater extent as the concentration of CaCl_2 increased. Hence, crosslinking with CaCl_2 may be regarded as a suitable strategy to tailor the release of the extract from gelatin/*ι*-carrageenan networks. However, the pH-responsiveness of the biopolymeric matrix was lost upon addition of the salt, since the release from these samples was similar at both pHs. Given that crosslinking implies the formation of additional bonds in polymeric networks, these bonds could be hindering the relaxation of the polymer chains that presumably causes the increased release at pH=8 in the non-crosslinked samples (Hezaveh & Muhamad, 2013).

Nevertheless, the release of the GJE from all the freeze-dried blends studied in this section was considerably low (less than 15% in acidic medium and up to 20% in alkaline solution).

Figure 7. Release of GJE in aqueous media at two different pHs. A) Release from freeze-dried gelatin/ ι -carrageenan blends (ratio 85:15) crosslinked with different CaCl_2 concentrations. B) Release from freeze-dried ι -carrageenan/gelatin beads prepared with different CaCl_2 concentrations.

3.8. Release of GJE from ι -carrageenan-gelatin capsules

In order to produce ι -carrageenan/gelatin beads (capsules) containing GJE, the order of addition of the biopolymers was reversed. The relevance of adding gelatin onto the carrageenan solution or *vice versa* on the characteristics of the resulting materials had been observed in preliminary trials, in which the formation of quasi-spherical gel-like structures had been obtained by drop-

wise addition of *ι*-carrageenan onto the gelatin solution. The much higher viscosity of *ι*-carrageenan (cf. Figure 2, Table 1) favoured that its interaction with gelatin at the interface of the droplet was faster than the collapse or disintegration of the droplet. As a result, the *ι*-carrageenan droplet was surrounded by a layer of gelatin-*ι*-carrageenan network which hampered the diffusion of the carrageenan (plus extract) core of the developed capsules. Furthermore, when CaCl₂ was present in the gelatin solution, the additional crosslinking promoted by the salt resulted in less fragile capsules, presumably due to the strengthening of the biopolymeric network at the interface of the droplet and possibly to the diffusion of the small Ca²⁺ cations through the gelatin-*ι*-carrageenan complex layer inducing the gelation of the *ι*-carrageenan core. Consequently, while the beads prepared in the absence of salt were considerably fragile, those prepared in the presence of CaCl₂ could be easily handled. Thus, GJE-containing beads were prepared in the presence of different CaCl₂ concentrations, freeze-dried, and the release of the extract from these capsules was studied. Figure S2 of the Supporting Information shows representative images of the developed capsules and their release profiles are depicted in Figure 7B.

Interestingly, the release of the extract from the developed capsules was much more sustained than from the freeze-dried blends. Although an initial burst release occurred again in the first 30 min, a more gradual release was observed during the following 24 h. Moreover, despite the addition of the divalent salt, these encapsulation structures exhibited pH-responsiveness, which was more pronounced as the CaCl₂ concentration decreased. The notable differences with the freeze-dried blends despite having the same composition can be explained in the light of the physical organization of both materials.

Figure 8 illustrates a schematic representation of both systems. While in the freeze-dried blends both biopolymers were vigorously stirred to obtain a mixture as homogeneous as possible, where the extract was expected to be in contact with both the polysaccharide and the protein, in the capsular structures the gelatin was placed around a core of *ι*-carrageenan plus extract, so that only the small fraction of protein located at the interface was, in principle, available for interaction with the GJE. Since the interactions between the extract and the biopolymeric matrix were established mainly with the gelatin, the molecules of GJE located further from the interface were, presumably, not as strongly attached to the matrix, and could eventually be released from the carrageenan core through a diffusion mechanism.

Remarkably, the release profiles obtained for the *ι*-carrageenan/gelatin capsules prepared with 50 mM CaCl₂ were practically ideal for intestinal targeted delivery purposes, since less than a 30% of the extract was released in the acidic medium after 2 h, while almost complete release was achieved at alkaline pH. This emphasizes the great potential of the developed materials for the encapsulation and selective release of polyphenol-rich food extracts such as the GJE.

Figure 8. Schematic representation of the developed grape juice extract-containing structures. A) Freeze-dried gelatin/ ι -carrageenan blends. B) ι -Carrageenan/gelatin capsules.

4. Conclusions

Novel edible encapsulation structures based on gelatin and ι -carrageenan for intestinal-targeted delivery of functional ingredients were developed in this work, by exploiting the interactions between both biopolymers and a model polyphenol-rich extract. The extent of complexation between both biopolymers was dependent on the protein/polysaccharide ratio, being maximal for the highest ratio assessed (85:15). Although complexation in gelatin/ ι -carrageenan binary systems had been previously studied (Michon et al., 2000; Michon et al., 2002), the interactions taking place in a ternary system including a polyphenol-rich extract had not been explored yet, despite being crucial for the performance of these materials as delivery vehicles. The results suggested that the grape juice extract (GJE) used preferentially interacted with the protein. This finding, together with its greater extent of biopolymer complexation, resulted in the gelatin/ ι -carrageenan ratio 85:15 being the most efficient for the entrapment of the bioactive ingredient,

reducing the hygroscopicity of the formulated extract and hindering its dissolution in water to a greater extent.

Remarkably, the release of the extract from the selected gelatin/ ι -carrageenan matrices was pH-dependent, being higher at alkaline pH than at acidic pH. Although the addition of a divalent salt (CaCl_2) to the system favored crosslinking and, therefore, a more effective entrapment of the extract within the polymeric network, the pH-responsiveness of the system was lost, since the release in alkaline conditions was also limited. Nevertheless, the release properties could be tuned by tailoring the physical organization of the materials. This was shown by changing the preparation protocol, i.e. by reversing the order of addition of the biopolymers. Specifically, GJE-loaded ι -carrageenan/gelatin capsules were produced by extrusion of ι -carrageenan and GJE onto a gelatin bath. The capsules prepared in the presence of 50 mM CaCl_2 exhibited almost ideal release properties for intestinal targeted delivery purposes, suggesting the promising potential of these materials for the development of novel functional foods. Future work to assess their performance in real food systems should be conducted to further confirm their suitability as delivery vehicles for food ingredients.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge (PDF).

Figure S1. Water uptake capacity of the grape juice extract at 74.9% relative humidity.

Figure S2. ι -Carrageenan-gelatin beads containing grape juice extract.

ACKNOWLEDGMENT

L. G. Gómez-Mascaraque, M. Martínez-Sanz and M. J. Fabra are recipients of a predoctoral (BES-2013-065883), Juan de la Cierva (IJCI-2015-23389) and Ramón y Cajal (RYC-2014-158) contracts from the Spanish Ministry of Economy, Industry and Competitiveness (MINECO), respectively. The authors would like to thank the Spanish MINECO project AGL2015-63855-C2-1-R for financial support. Synchrotron experiments were performed at NCD beamline at ALBA Synchrotron with the collaboration of ALBA staff (2016021658 project).

ABBREVIATIONS

FT-IR, Fourier transform infrared spectroscopy; GJE, grape juice extract; MB, methylene blue; RH, relative humidity; SAXS, small-angle X-ray scattering; WU, water uptake.

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