Thermal gelation of mixed egg yolk/kappa-carrageenan dispersions

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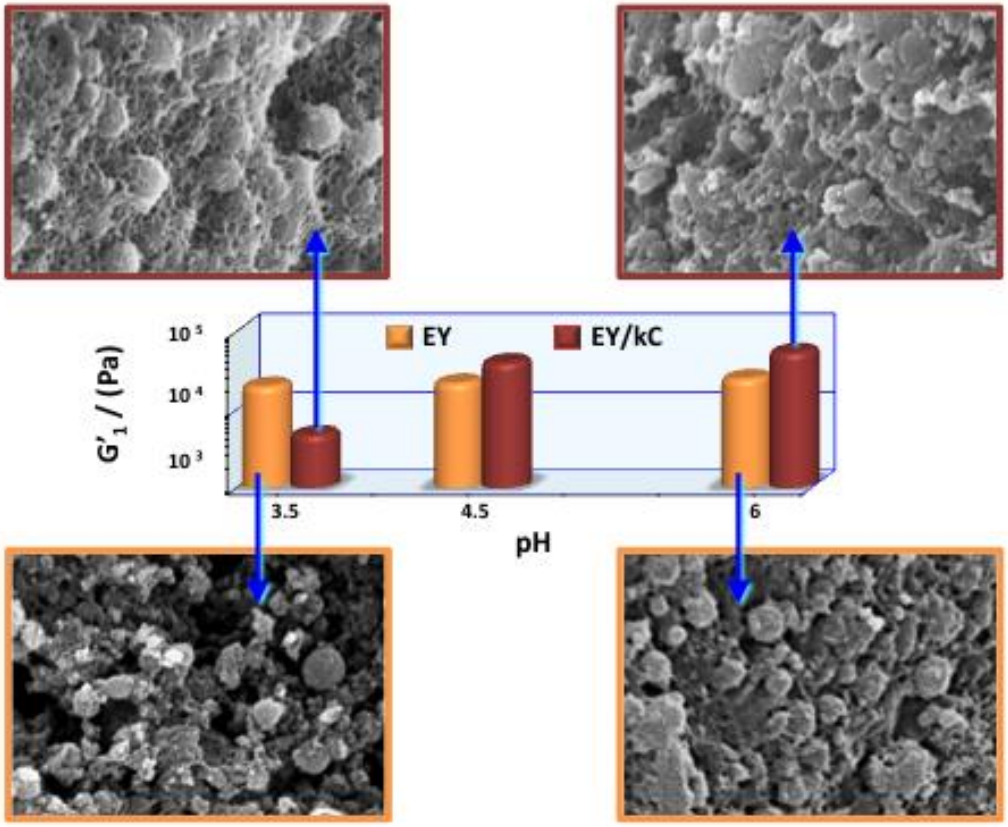
Graphical abstract



Highlights

- Heat induces dramatic changes in the microstructure and rheology of EY/κC systems
- Rheological properties of EY/κC gels can be tailored by κC content and pH
- The microstructure of EY/κC gels is generally dominated by the protein matrix
- At low pH, the gum dominates the gel microstructure of EY containing 0.3% κC
- At low pH, electrostatic attractions among EY aggregates and κC are favoured

Abstract

This study aims to evaluate the effect of gum content and pH on the thermal gelation of mixed egg yolk/κ-carrageenan (EY/κC) dispersions, monitored by linear viscoelastic measurements.

Heat processing induces dramatic changes in the microstructure and viscoelastic properties of EY/κC systems, which may be attributed to a multistage mechanism that yields an interparticle gel network. An increase in κC content generally induces an enhancement in viscoelasticity. A reduction in pH hinders this enhancement and causes an anticipation of the multistage process, which confirms the importance of the electrostatic interactions of EY/κC dispersions..

The viscoelastic properties of EY/κC gels generally fit a master mechanical spectrum, which suggests that the protein matrix generally dominates the microstructure of EY/κC gels. However, SEM images reveal formation of a κC network at low pH, at which some κC autohydrolysis may also play a role. Electrostatic attractions seem to favour interactions among EY aggregates and κC into the carrageenan network.

Keywords: *Egg yolk; κ-Carrageenan; Protein-Polysaccharide interactions; Thermal gelation; Small amplitude oscillatory shear; Scanning Electron Microscopy*

1. INTRODUCTION

Hen egg yolk is a multifunctional ingredient widely used in the manufacture of food products, since it provides an inexpensive and low calorie source of high-quality protein that possesses much appreciated organoleptic characteristics, distinctive colour and well-known functional properties (emulsifying, coagulating, and gelling properties).

In terms of composition, egg yolk is a complex association of lipids (33 wt%), proteins (17 wt%) and water (50 wt%) in which several types of solids are suspended in a protein solution or plasma (Pisuchpen, Chaim-ngoen, Intasanta, Supaphol, & Hoven, 2011) . Plasma (about 78% of yolk dry matter) is composed of 85% low-density lipoproteins (LDLs) and 15% livetins. More specifically, LDL consists of spherical particles (17-60 nm in diameter) with a liquid core formed by 61-66 % neutral lipids (triglycerides, cholesterol esters) surrounded by a layer of 21-23 % phospholipids and 11-17% apoprotein (Cook & Martin, 1969). It is generally accepted that the structure of LDL is fairly stable and basically remains unaltered even after protein gelation (V. Kiosseoglou, 2003). Granules (about 22% of yolk dry matter) are non-soluble protein aggregates suspended in the plasma which are mainly constituted by high-density lipoproteins (HDLs) and phosvitin (70% and 16%, respectively) linked by phosphocalcic bridges (Li, Guo, Wei, MacDiarmid, & Lelkes, 2006; Yu, Fridrikh, & Rutledge, 2006; Zhang, Reagan, & Kaplan, 2009).

Polysaccharides are also available natural macromolecules which are commonly used as functional ingredients, aiming for contributing to the structure, texture and stability of food systems through their thickening or gelling behaviour. Carrageenan is a sulphated anionic polysaccharide obtained from red seaweeds that consists of a galactose backbone with different proportions and locations of ester sulphate groups. The main carrageenan types -kappa, iota and lambda- exhibit a wide spectrum of rheological profiles and interactions with other biopolymers (Dzenis, 2004).

As is well known, κ-Carrageenan forms thermo-reversible gels in aqueous solution and

in presence of cations. Although the gelation mechanism is not fully understood, the model originally proposed by (Anderson, Dolan, & Rees, 1965) and subsequently modified by (E. R. Morris, Rees, & Robinson, 1980) is commonly accepted. At high temperature, κ-Carrageenan is presented as a random coil. On cooling, two-step gelation occurs and is characterised by a coil (disordered)-helix (ordered) transition followed by aggregation and network formation (Fernandez-Saiz, Lagaron, & Ocio, 2009). Conformational ordering of κ-carrageenan is promoted through lowering the electrostatic repulsion between the sulphate groups and by specific incorporation in the ordered structure of large Group I cations such as K^+, Rb^+, and Cs^+, in preference to both smaller (Li^+ and Na^+) ions (Luo et al., 2010). However, divalent cations such as Ca^{2+}, and Ba^{2+} can also lead to gelation (V. J. Morris, 1986), which suggests that gel formation depends to a great extent on the counterions size.

On the other hand, κ-Carrageenan solutions lose viscosity and gel strength when subjected to pH values below 5.5, but this effect is not significant for heat-set systems until pH lower than 4.3. This is caused by an autohydrolysis effect, occurring at low pH values as carrageenan in the acid form cleaves at the 3,6-anhydrogalactose linkage in the molecule (Hoffmann, Russell, & Gidley, 1996). The rate of autohydrolysis increases at elevated temperature and low cation levels. However, once the solution is cooled below the gelling temperature, potassium ions associate with the sulphate groups on the carrageenan, which has been found to prevent autohydrolysis (Imeson, 2009).

(Tolstoguzov, 2003) stressed out the importance of thermodynamic considerations in protein-polysaccharide interactions to design food formulations with novel or improved properties. (Ma, Kotaki, Yong, He, & Ramakrishna, 2005) reviewed the relative importance of non-covalent interactions, such as electrostatic interactions, steric exclusion, hydrophobic interactions and hydrogen bonding, between the particular molecules involved. A relatively strong electrostatic attraction between the two polymers could either result in soluble complexes evenly distributed throughout the

entire system or in a two-phase system with an enriched phase in both associated polymers, which may be a coacervate or a precipitate. In contrast, if the environmental conditions led to repulsive interactions between polymers another two scenarios could be possible. Thermodynamic incompatibility has been reported by some authors in mixed system consisting of κ-Carrageean and different proteins, such as pea protein (Nunes, Raymundo, & Sousa, 2006), soybean isolate (Molina Ortiz, Puppo, & Wagner, 2004), fish gelatine (Haug, Draget, & Smidsrod, 2004), or β-lactoglobulin (Dumay, Laligant, Zasypkin, & Cheftel, 2011; Roesch, Cox, Compton, Happek, & Corredig, 2004) . But, probably, the best known synergy is exhibited between carrageenan and positively charged amino acids of κ-casein micelles of milk proteins (Sambaer, Zatloukal, & Kimmer, 2012). On the other side, thermal stability of egg proteins have been investigated in presence of pectin or guar gum (Hernandez-Munoz, Kanavouras, Lagaron, & Gavara, 2005), modified starch (Grevellec, Marquie, Ferry, Crespy, & Vialettes, 2001), or different hydrocolloids gums including κ-carrageenan (Georgopoulos, Larsson, & Eliasson, 2004).

Furthermore, the effect of pH and protein-polysaccharide ratio on the linear viscoelastic behaviour of egg yolk/κ-carrageenan mixtures in aqueous solution and their heat-set gels was previously studied (Aguilar et al., 2011). And, recently, (Navidghasemizad, Temelli, & Wu, 2015) studied the types of interactions after mixing egg yolk components with some polysaccharides including ι-carrageenan. These authors evaluate their behaviour in selective phase separation of egg yolk proteins. Despite all the above developments, the complex interactions between egg yolk proteins and/or lipoproteins and polysaccharides have not been sufficiently studied, particularly when mixed systems are subjected to thermal processing.

The main objective of this work was the analysis of the effect of the progressive addition of kC on the behaviour of technical egg yolk over heat-induced gelation, at different pH values. To accomplish this objective, zeta-potential measurements of

EY/κC dispersions were previously determined, whereas the thermal gelation process were continuously monitored by using linear viscoelastic measurements. Likewise, linear viscoelastic properties and microstructure of the heat-set gels obtained were also evaluated.

2. EXPERIMENTAL

2.1 Materials

κ-Carrageenan (κC) was kindly provided by Degussa (France) under Satiagel AMP45 trademark, containing 94.86 wt% kappa-carrageenan and 5.14 wt% iota-carrageenan. Egg Yolk (EY) was obtained from fresh chicken eggs purchased in a local market according to grade A and type L (63-73 g) commercial specifications, being discarded all damaged or cracked eggs. Hydrochloric acid (analytical grade) was purchased from Merck (Germany).

2.2 Methods

Chicken eggs were hand broken and the white was carefully separated from the yolk using the preparation method proposed by (Harrison & Cunningham, 1986). Afterwards, EY solid content wt% was quantified by loss of mass after drying some aliquots at 105 °C (± 2 °C) in a temperature-controlled oven at atmospheric pressure for 24 hours (Miranda, Partal, Cordobes, & Guerrero, 2002).

Native EY pH (close to 6.0) was determined for each sample using a digit 501 pH-meter (Crison, Spain) and EY/ κC mixtures were prepared by dispersing different amounts of κC (0-0.5 wt%) in native EY containing 50.43 ± 0.8 wt % solids. Mixing was performed by using a mechanical stirring (300 rpm, 1h) at room temperature. The initial pH of the system was modified by using hydrochloric acid (1M and 2M solutions) as acidulant up to the desired pH value (i.e. 3.5, 4.5, 5 or 6), and adding later the amount of demineralised water required to raise a 45 wt% solids content. It is worth mentioning that κC is not fully hydrated under the experimental mixing conditions. In fact, given the

reduced amount of water required to prepare EY dispersions (from 50 to 45 wt%) it is not possible to use any pre-hydrated κC solution. Moreover, κC is added as late as possible, particularly at low pH, in order to minimize autohydrolysis.

Small-amplitude oscillatory shear (SAOS) measurements, including either stress sweep, frequency sweep or temperature ramp tests, were performed by means of controlled-stress rheometer Haake RS-300 (Thermo Scientific, Germany). A 20 mm plate–plate serrated geometry with 1 mm gap was selected for all measurements, except for kC solutions where a 60 mm hard anodized aluminium plate-plate geometry was used. All the samples were maintained in the sensor system for 20 min before running any rheological test, in order to reach temperature and structure equilibrium. To ensure a correct stress control, temperature ramp experiments, performed from 20 to 90 °C, were carried out into three zones using dynamic viscoelasticity monitoring (Cordobes, Partal, & Guerrero, 2004) at 1.5 °C/min heating rate and constant frequency (2π rad/s). A shear stress sweep test was previously performed at a frequency of 2π rad/s for each region and sample to keep measurements within the linear response regime. Similarly, all the dynamic viscoelasticity frequency sweep measurements were carried out using a shear stress clearly lower than the critical value for linear viscoelasticity. At least two replicates were performed on each rheological measurement.

Zeta potential measurements of all the systems under study were performed using a Zetasizer Nano ZS (Malvern Instruments, U.K.). Aliquots of 0.5 g were diluted in deionised water up to 100 ml of total volume, keeping adjusted the pH value of the dispersion with 0.1 N HCl. Prior to analysis, the samples were tempered at 20 ºC and stirred in order to keep the suspension homogeneous, avoiding segregation. The samples were measured in triplicate and each measurement was an average of twelve determinations. During the experiments, no flocculation was observed and each measurement was conducted at 20 ºC. Zeta potential values were calculated from the

electrophoretic mobility using the Henry equation and the Smoluchowski approximation (Aydt, Weller, & Testin, 1991)

Scanning Electron Microscopy (SEM) was used in collaboration with the Microscopy Service (CITIUS, Universidad de Sevilla), to evaluate the microstructure of the gels formed, following the same procedure used in a previous work for heat-set egg yolk gels (Cordobes et al., 2004). Gel samples were immersed in 3% glutaraldehyde for 72 h and washed several times with distilled water and then post-fixed in 1% osmium tetroxide at 4 °C. SEM samples were rinsed for 1 h in distilled water before being dehydrated in a grade of ethanol series, 50, 70, 90 and 3×100 vol. % and dried at the critical point. Each dried sample was mounted on a bronze stub and coated with gold, the specimens being observed with a 6460LV Microscope (JEOL, USA).

3. RESULTS

3.1. Thermal gelation of EY/κC dispersions

3.1.1. Evolution of SAOS properties over thermal gelation of κC-free EY

Figure 1 shows the evolution of the complex modulus (G*) over a thermal cycle for native EY (pH 6 and 45 wt% solids) and for an aqueous solution containing 0.3 wt% κC (pH 6).

The behaviour of EY may be described using four different stages: (i) The first region, which is previous to any protein denaturation, displays low values for G* and a slight decrease with increasing temperature, due to a thermal-induced increase in mobility. (ii) The second region is characterised by a dramatic increase in G*, reflecting a remarkable reinforcement of the gel network, which has been explained in terms of a mechanism of sequential stages: thermal induced protein denaturation; aggregation of partially denatured protein molecules and random association of the aggregates to form a protein gel network (2001). This mechanism has been successfully applied to thermal gelation of egg yolk (Cordobes et al., 2004). (iii) In the third region, which corresponds to the isothermal stage, a tendency to a plateau value in G* takes place.

(iv) In the cooling stage, a noticeable reinforcement of the gel network structures comes about.

Consequently, the final EY gel can be envisaged as an interparticle network, where the particles consist of neutral triglycerides buried into the particle cores, which are surrounded and stabilized by protein. The network itself is formed by gelation of the protein-dominated particle surfaces according the above-mentioned mechanism (V. Kiosseoglou, 2003).

As may be also observed in Figure 1, the behaviour of □C solution over the thermal cycle is completely different, displaying much lower viscoelastic properties for the whole test (e.g. G*). As mentioned above, κC is basically unsolvated below 40ºC. A slight decrease in G* takes initially place at region (i) and the beginning of region (ii) as a consequence of thermal agitation. A fluid-like behaviour takes place in these first regions, where tan δ show values much higher than unity. As temperature is raised above 75ºC a moderate increase in G* occurs. It is known that hydration of carrageenan initially results in an increase in viscosity as swollen particles offer more resistance to flow (Stanley, 1990). A plateau may be observed at the end of region (iii), at which κC should be fully hydrated. This region is well above the so-called coil-helix transition for κC, which is known to depend on the type of cation used (Rochas & Rinaudo, 1980). However, the most important effect takes place on cooling, at region (iv), where a significant increase in viscoelastic properties can be clearly observed. Kappa-carrageenan forms a gel in this region as temperature is reduced below the coil-helix transition, showing tan δ values in the order of 0.4.

It should be pointed out that the values obtained at region (i) for EY and regions (i), (ii) and the beginning of (iii) for κC must be regarded with caution since they can show some accuracy problems. Thus, the sensor geometry used in this test for EY was selected to obtain accurate values for the subsequent regions (ii, iii and iv). In fact, using a larger surface geometry (e.g. 60 mm cone-plate) egg yolk always yields

viscous-like behaviour at room temperature (Aguilar et al., 2011). Thus, although region (i) should generally reflect a fluid-like behaviour, EY samples in this region may either show apparent values for tanδ higher or lower than unity.

Stress-sweep tests were previously carried out at regions (i) and (iii), in order to confirm that the system remains in the linear viscoelastic region over the whole thermal cycle. Different critical stresses were selected for each region to ensure permanence within the linear viscoelastic range.

3.1.2. Influence of pH on SAOS properties over gelation

The isoelectric point (IEP) of EY is around 5.6 (V. D. Kiosseoglou & Sherman, 1983). As is well known, any depart of pH from the IEP in the aqueous media may lead to marked changes in the net charge of the protein surfaces, considerably affecting to the balance of forces involved in the early gelation process. The presence of an anionic polysaccharide will also contribute to the net balance of charges (e.g. anionic polysaccharide may reduce the pH at which net neutrality can be achieved).

Measurement of z-potential values is regarded as a suitable technique for determining the occurrence of electrostatic interactions in the protein-polysaccharide dispersions before gelation, thus providing useful information to explain the effect of pH on the evolution of linear viscoelastic properties over gelation.

Table 1 shows the z-potential values obtained for EY/κC dispersions at different values of pH and κC content. As may be observed, these dispersions display a strong dependence on pH, shifting from positive to negative values, regardless of the κC content.

The isoelectric point of EY is around 5.6 (V. D. Kiosseoglou & Sherman, 1983) such that protein surfaces show moderately negative charges at pH 6 and clearly positive charges at pH 3.5. These results are rather coincident with those shown by

(Navidghasemizad et al., 2015) who reported zeta-potential values for fresh EY and iota-carrageenaan as a function of pH.

Even though kappa-carrageenan is basically swelled, rather than being solvated, an increase in its concentration shows an apparent effect on the z-potential, which becomes more pronounced by increasing pH. This effect indicates that some protein-polysaccharide electrostatic interactions occur. Thus, as may be observed in Table 1, positive charges, obtained at pH 3.5, are slightly balanced by the subsequent addition of κC, whereas negative charges, obtained at pH 6, are significantly enhanced as κC concentration increases. Moreover, at pH 3.5 and 6 the z-potential decreases 5.8 mV and 13.6 mV when κC content is raised from 0 to 0.5 wt%. According to (Grizzuti & Perlmann, 1973) some egg proteins such as phosvitin show a poorer capacity of binding Ca^{+2} and Mg^{2+} ions at low pH, which may explain the above-described uneven behaviour since positively charged protein surfaces may compete with bivalent cations to interact with κC anions. Similar behaviour has been also found for other proteins such as β-casein of milk (Baumy & Brule, 1988). This is also consistent with previously results that showed a reduction in the pH at which net neutrality (zero z-potential value) is reached in the presence of κC (Aguilar et al., 2011). (Navidghasemizad et al., 2015) also found a reduction in the net neutrality by adding ι-carrageenan or xanthan gum to egg yolk. Other authors have also reported a similar effect for whey protein isolate (Stone & Nickerson, 2012) or canola protein isolate (Stone, Cheung, Chang, & Nickerson, 2013) in combination with κ–, ι–, and λ–type carrageenan. It is worth mentioning that in the current study this net neutral value is shifted to a pH of ca. 4.5 by adding 0.3% κC, which is similar to that shifting reported by (Navidghasemizad et al., 2015).

Figure 2 shows the evolution of G* and tanδ as a function of temperature for EY containing 0.3 wt% κC at different pH values. It should be noted that the x-axis is

divided into three different sections: the first one represents the increasing temperature from 20ºC to 90ºC; the second section represents the evolution over time at constant temperature (90ºC) and the last section plots the evolution with decreasing temperature from 90ºC to 20ºC. It is worth pointing out that κC would probably remain basically unsolvated over the upward section of the thermal cycle applied. However, it is expected than solvation of κC should take place at the isothermal section, such that κC would be eventually able to interact with egg yolk proteins as a fully solvated polysaccharide. In fact, as may be observed, a reduction in the pH value leads to an anticipation of the upward gelation profile, which is consistent with previous results (Guerrero, Carmona, Martinez, Cordobes, & Partal, 2004). Thus, region (ii), which corresponds to the increase in G*, and region (iii), corresponding to the plateau zone, take place at lower temperature as pH departs from the native value. However, only slight differences can be observed between pH 4.5 and 3.5. Moreover, it should be pointed out that both the initial and final G* values of the upward profile remain practically unaffected by pH.

The loss tangent, tanδ, which always shows a peak followed by a pronounced decrease towards a plateau value, also illustrates the regions described above for each gelation profile. The reduction in pH leads to an anticipation of the first section of the profile. This anticipation affects to the end of region (i) and to the whole temperature range for region (ii), where several specific events can be found. Thus, a clear increase in tanδ takes place at temperature T_i, which is followed by a peak, occurring at T_m, and a crossover point (tanδ=1), taking place at T_x. The end of region (ii) is reached at T_f, which corresponds to the plateau temperature. A linear decrease of all these specific temperatures can be observed in the insert of Fig. 2 as pH is reduced from the EY IEP. As may denoted, the separation between these temperatures becomes larger as the pH decreases, reflecting a progressive broadening of tanδ peak and, as a result, much

slower gelation kinetics. This anticipation may be attributed to an increase in electrostatic interactions among charged protein residues, as pH departs from the EY IEP, which may lead to changes in the aggregation mechanism and consequently affecting to the gelation kinetics.

In any case, from the beginning of region (iii) the values for the tanδ remained basically independent on pH. Region (iv) does not involve any remarkable dependence on pH. These results suggest that the thermal process applied tend to develop the same type of gel structure. Thus, all the gels eventually display similar properties, in spite of the apparent acidic-induced anticipation of the multistage gelation mechanism that takes place.

3.1.3. Influence of κC concentration on SAOS properties over gelation

Figure 3 shows G' and G'' profiles obtained at 2π rad/s and pH 6, as a function of time for different κC concentrations. This figure also shows the thermal cycle applied. It is worth pointing out that, although the contribution of the swelled carrageenan powder grains at low temperature is clearly shown for 0.5%, all the viscoelasticity functions display similar thermal profiles as described for Fig. 1, for the single EY system. This means that the mixed EY/κC system is dominated by the heat-induced gelation of EY protein with no apparent contribution of κC. However, SAOS moduli generally show a significant increase with κC concentration as previously reported for EY/κC dispersions and gels (Aguilar et al., 2011). Moreover, the extent of this evolution may also show a strong dependence on the region of the thermal cycle considered. As observed in Fig. 3, the effect of κC becomes more remarkable at the cooling region. On cooling, when κC is present, the increase in viscoelastic properties becomes steeper below 60ºC. This change, which is particularly apparent for EY containing 0.5 wt% κC, coincides with the increase in G* appreciated for κC solution in Figure 1 and may be

attributed to the coil-helix transition reported for carrageenans at 50-40ºC (Stanley, 1990). This effect has been also found at pH 3.5.

Application of the Winter-Chambon criterion (Winter & Chambon, 1986) is the most generally accepted method to define the gel point and thus, it has been previously used for EY gelation induced either by heat (Cordobes et al., 2004) or pressure (Aguilar, Cordobes, Jerez, & Guerrero, 2007). However, some authors have used the crossover temperature, at which G' and G'' become equal (i.e. G_x), as an estimation of the gel point (Te Nijenhuis, 1981; Sanchez & Burgos, 1997). Although the crossover point shows a certain dependence on frequency, it may be used as a first approximation of the gel point because of its easier determination. In any case, the modulus obtained for both points are closely related. Figure 4 shows the values obtained for the crossover modulus in region (ii), G_x, as a function of κC concentration at pH 3.5 and 6. As may be seen, G_x exhibits a strong dependence on κC concentration that may be represented by an exponential growth for both pH values. In any case, the values of G_x are always higher at pH 3.5 for the whole range of polysaccharide concentration. Thus, T_x undergoes a reduction from 76ºC to almost 65ºC as the pH value decreases from 6 to 3.5. Interestingly, both temperature values remain hardly affected by κC content (data not shown). In addition to the modulation effect of κC on the interactions among protein segments, it is worth mentioning that some solvation and autohydrolysis (at low pH) may take place in region (ii). However the larger values of G_x (and shorter values of T_x) found at pH 3.5 suggest that neither of these effects exert any noticeable contribution and protein aggregation dominates in this region, confirming the behaviour observed in Figure 1.

3.2. Heat-set EY/κC gels

3.2.1. Linear viscoelastic behaviour of gels

Figure 5A shows the master curves for the mechanical spectra of EY/κC gels obtained at different pH values and κC concentrations. To obtain this mater curves, the y-axis has been normalized using parameter G'_1, which corresponds to the storage modulus obtained at 1 rad/s for each gel sample.

All the gels included in Figure 5A show mechanical spectra that can be reasonably normalized onto a single master mechanical spectrum. Only a slight deviation in G''/G'_1 can be noticed at the highest κC content. In any case, the slope for the elastic modulus is ca. 0.063 ± 0.008 for these EY/κC gels. The normalized spectra were fitted to a Generalized Maxwell (GM) model for linear viscoelasticity, using 7 Maxwell elements.

The expressions for the normalized moduli according to this model are as follows:

$$\frac{G'(\omega)}{G'_1} = \sum_{k=1}^{7} \frac{G_k}{G'_1}\left(\frac{\omega^2\lambda_k^2}{1+\omega^2\lambda_k^2}\right) \quad (1)$$

$$\frac{G''(\omega)}{G'_1} = \sum_{k=1}^{7} \frac{G_k}{G'_1}\left(\frac{\omega\lambda_k}{1+\omega^2\lambda_k^2}\right) \quad (2)$$

where G_k and λ_k are the relaxation strength and relaxation time corresponding to each Maxwell element. Figure 5B shows the discrete master relaxation spectrum obtained by plotting the values for the normalized relaxation strength vs. the relaxation time from the GM model. It may be pointed out that the shape of the master relaxation spectrum specifically corresponds to the so-called plateau region.

The plateau region has been extensively described in polymer rheology in terms of an entanglement network among polymer chains in which the motion of molecules is constrained by the neighbouring molecules (Ferry, 1980). This behaviour is also typical of other polymeric systems such as protein-stabilised emulsions (Carlos Bengoechea, Cordobes, & Guerrero, 2006; Carlos Bengoechea, Romero, Manuel Aguilar, Cordobes, & Guerrero, 2010; C. Bengoechea et al., 2008; Calero, Munoz, Cox, Heuer, & Guerrero, 2013; Alberto Romero, Cordobes, & Guerrero, 2009; A. Romero, Cordobes, Puppo, Guerrero, & Bengoechea, 2008).

A straightforward consequence derived from the superposition of the normalized mechanical spectra into a master curve is the usefulness of the normalization parameter (G'_1), which completely represents the effect of the concentration of κC and pH on the linear viscoelastic properties of EY/κC gels.

Parameter G'_1 is plotted against κC concentration (Fig.6A) and pH (Fig. 6B) for the different heat-induced gels studied. These values are also compared with their corresponding unprocessed dispersions taken from a previous study (Aguilar et al., 2011). As may be observed in Fig. 6A, G'_1 shows higher values for the heat-induced gels at pH 6 than at pH 3.5, even though the opposite behaviour was found for unprocessed dispersions.

This change in gel behaviour may be explained in terms of protein surface charges. At pH 3.5 electrostatic repulsive interactions favour the increase in viscoelastic properties of EY/κC dispersions, but also interfere in the development of the gel network. In addition, although the exposition time at low pH was minimised, some hydrolysis of κC is expected to occur at pH 3.5, which might help the solvation of some κC chains leading to a dispersion with higher elasticity. With shorter chains, the interactions may be different which may change its influence on the formation of the protein.

In contrast, dispersions show lower viscoelastic properties at pH 6, leading to proper gel network development due to the lack of electrostatic interactions. Moreover, an increase in κC content at pH 6 leads to an enhancement of the gel since a slight, but significant, increase in G'_1 may be noticed. In the same way, the increase in κC concentration induces an evolution towards higher values in G'_1 for EY/κC dispersions at the same pH.

Addition of κC at pH 3.5 yields a similar evolution in viscoelastic parameters of EY/κC gels, but showing significantly lower values of G'_1. These results indicate that electrostatic repulsions, taking place at pH far from the isoelectric point of EY protein,

tend to inhibit protein aggregation (at least partially). This inhibition effect may be discussed in terms of a partial compensation of protein surface charge, taking place as κC content is being increased, which might hinder protein-protein aggregation and, as a consequence, gel formation. Another alternative might be to explain this change in behaviour in terms of a different scenario relying on the dominant role of polysaccharide molecules on the formation of a 3D network. However, not enough evidences can support this scenario at this point.

Fig 6B shows how unprocessed EY dispersions containing 0.3 % κC passes through a minimum value in G'_1 at pH around 5. This behaviour reinforces the assumption related to the above-mentioned partial compensation of protein surface charges by κC and the displacement of the net neutrality towards a pH lower than the EY IEP. This effect is also supported by the results shown in Table 1.

As may be seen in Fig. 6B, parameter G'_1 for heat-induced EY gels undergoes an increase with pH. This increase is slightly more remarkable at low pH at which the unprocessed dispersion shows a positive z-potential value. Therefore, the predominance of positive charges at protein surfaces seems to interfere the thermal gelation process leading to lower gel strength. A reduction in protein surface charges would favour formation of a heterogeneous gel. The presence of κC may contribute to a partial compensation of positive charges but, as mentioned above, this contribution does not seem to be enough to prevent their effect on protein gelation. The above-mentioned hydrolysis at low pH may also contribute to the lower gel strength. In any case, the highest gel strength found corresponds to pH 6, which is the closer value to the IEP of EY proteins.

3.2.2. Microstructure of EY/κC gels

Figure 7 shows the SEM images of κC-free and κC-containing (0.3 wt%) EY gels at different pH values. In absence of κC, SEM images (Fig. 7A, 7B and 7C) illustrate the dependence of EY gels on pH. At low pH the predominance of positive charges on protein surfaces seems to lead to smaller protein aggregates (Fig. 7A). In contrast, fairly heterogeneous gels coming from larger aggregates may be observed when the pH value is closer to the IEP (i.e. at pH 4.5 and 6, corresponding to Fig. 7B and 7C, respectively). This moderate microstructural change, driven by the increase in pH from 3.5, is consistent with the slight (but significant) increase observed for the linear viscoelastic properties of the gel (Fig. 6B).

Likewise, fairly developed gel networks may be also noticed in those SEM images corresponding to EY/κC mixed gels at pH 4.5 and 6 (Fig. 7E and 7F, respectively). In both cases the presence of κC is hardly perceptible. Interestingly, the image corresponding to the mixed gel containing 0.3 % κC at pH 3.5 (Fig. 7D) reveals occurrence of a rather different microstructure. In fact, EY has shown generally poorer gel formation ability at pH 3.5, as a consequence of both electrostatic repulsions among protein segments and some hydrolysis effect of κC, leading to a lower gel strength. This moderate gel ability of EY protein under such conditions seems to facilitate the above mentioned scenario based on the development of the polysaccharide network, which becomes apparent in the microstructure of this particular mixed gel, as illustrated in the SEM image (Fig. 7D).

This image also suggests a relevant contribution of interactions between positively charged protein surfaces and negatively charged polysaccharide macromolecules. These electrostatic attractive interactions seem to greatly contribute to the inhibition of association between protein aggregates, as well as to the entrapment of protein aggregates into the polysaccharide network, as illustrated in Fig. 7D. In fact, this SEM

image suggests formation of protein-polysaccharide complexes at pH < IEP, as reported by (Stone et al., 2013) for canola protein isolate and carrageenan.

4. CONCLUSIONS

Heat processing induces dramatic changes in the microstructure and rheological properties of EY/κC systems, which has been previously explained in terms of a multistage mechanism that leads to formation of an interparticle EY gel network. A reduction in pH causes an anticipation of the multistage thermal gelation process but does not seem to impart any remarkable change in viscoelastic properties at the end of the cooling region.

An increase in κC concentration generally leads to an enhancement of viscoelastic properties of the thermal cycle applied, which is particularly apparent on the cooling stage and near the IEP. This effect lessens as pH is reduced, thus confirming the importance of the electrostatic interactions of EY/κC dispersions. Autohydrolysis may also play a role at low pH.

All the viscoelastic properties of EY/κC gels obtained at different values of pH and κC concentration may be represented by a master mechanical spectrum. Generally, a moderate enhancement of viscoelastic properties, represented by the normalization parameter, G'_1, takes place with increasing κC content and when pH is approaching to the IEP.

According to the results of this study, it may be concluded that the gel network formed upon heating is generally dominated by protein, which is particularly apparent at pH close to the IEP. However, at low pH, electrostatic repulsions between protein surfaces interfere on protein gelation. As a result, formation of the carrageenan network becomes apparent.

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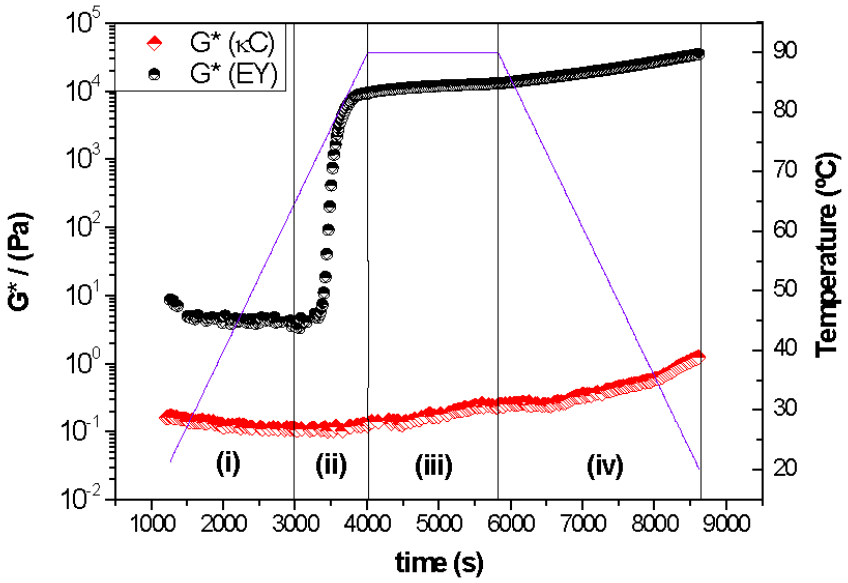

Figure 1. Evolution of the storage (G') and loss (G'') moduli at 2π rad/s upon application of a thermal cycle (continuous line) for native EY (pH 6 and 45 wt% solids).

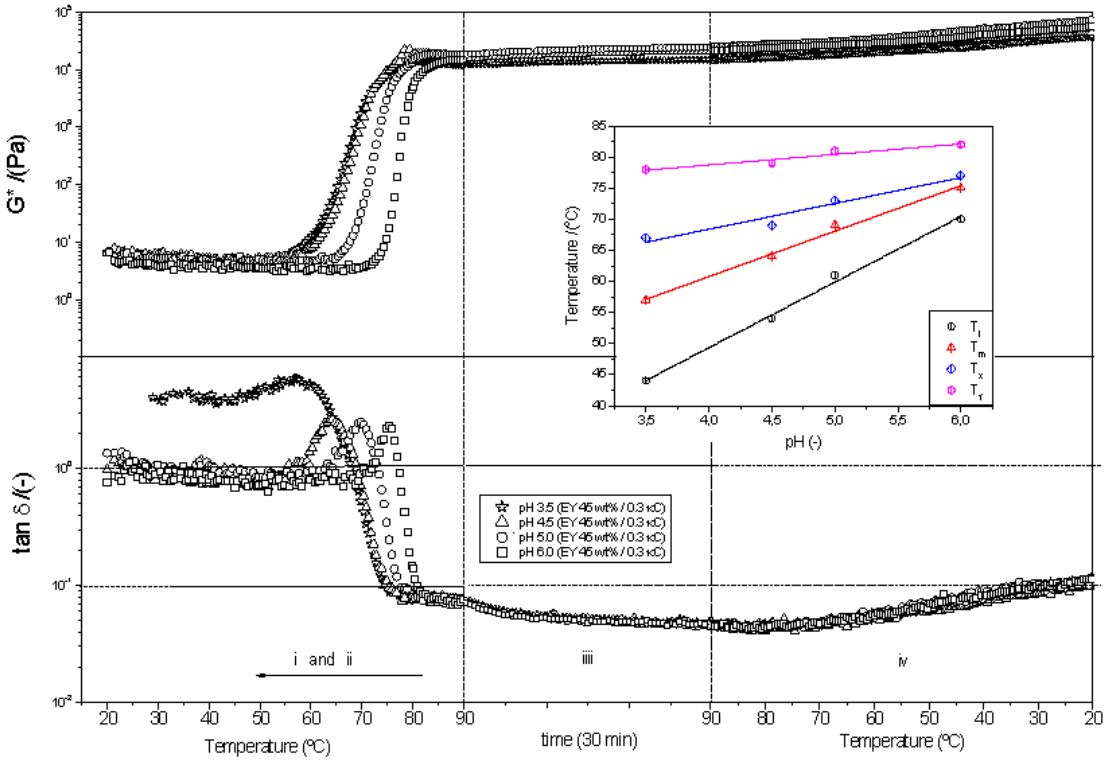

Figure 2. Evolution of the storage (G') and the loss tangent (tan δ) at 2π rad/s, upon application of the thermal cycle, for EY/κC systems containing 45 wt% solids and 0.3 wt% κC at different pH values. The insert graph plots the evolution of the temperature at specific locations of tan δ peak (at the initial growth, T_i; at the peak value, T_m; at tan δ=1; at the end of the peak, T_f) as a function of pH.

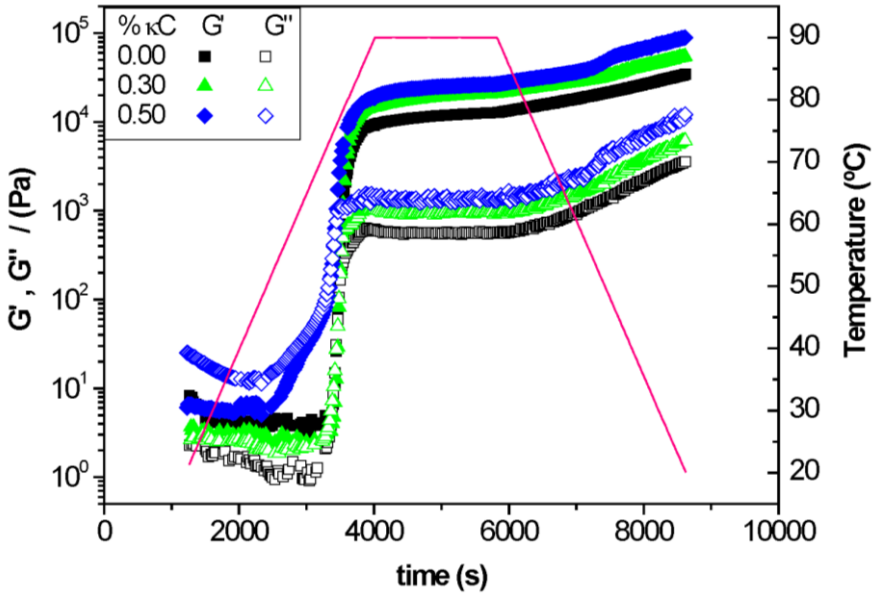

Figure 3. Influence of κC content on the gelation profiles (G' and G'' at 2π rad/s) of EY/κC systems containing 45 wt% solids at pH 6.

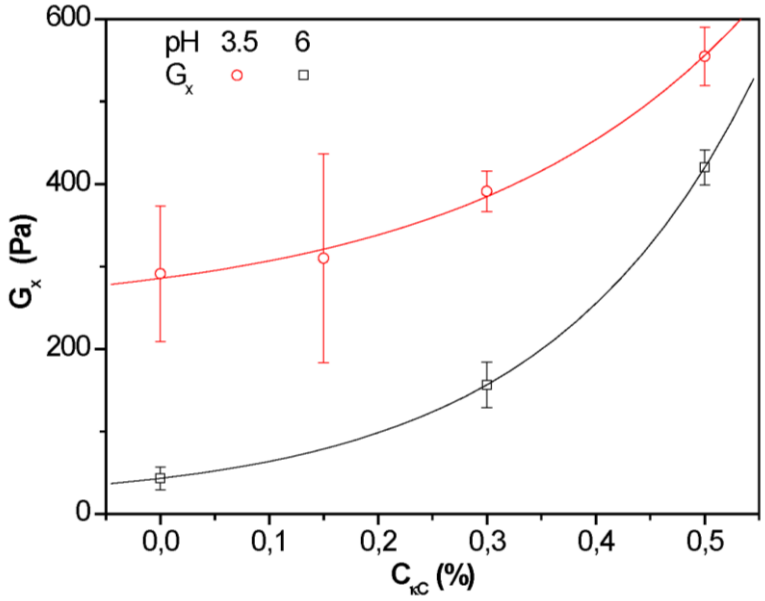

Figure 4. Evolution of the crossover modulus, G_x obtained from temperature ramp measurements as a function of κC for EY/κC systems containing 45 wt% solids at pH 3.5 and 6.

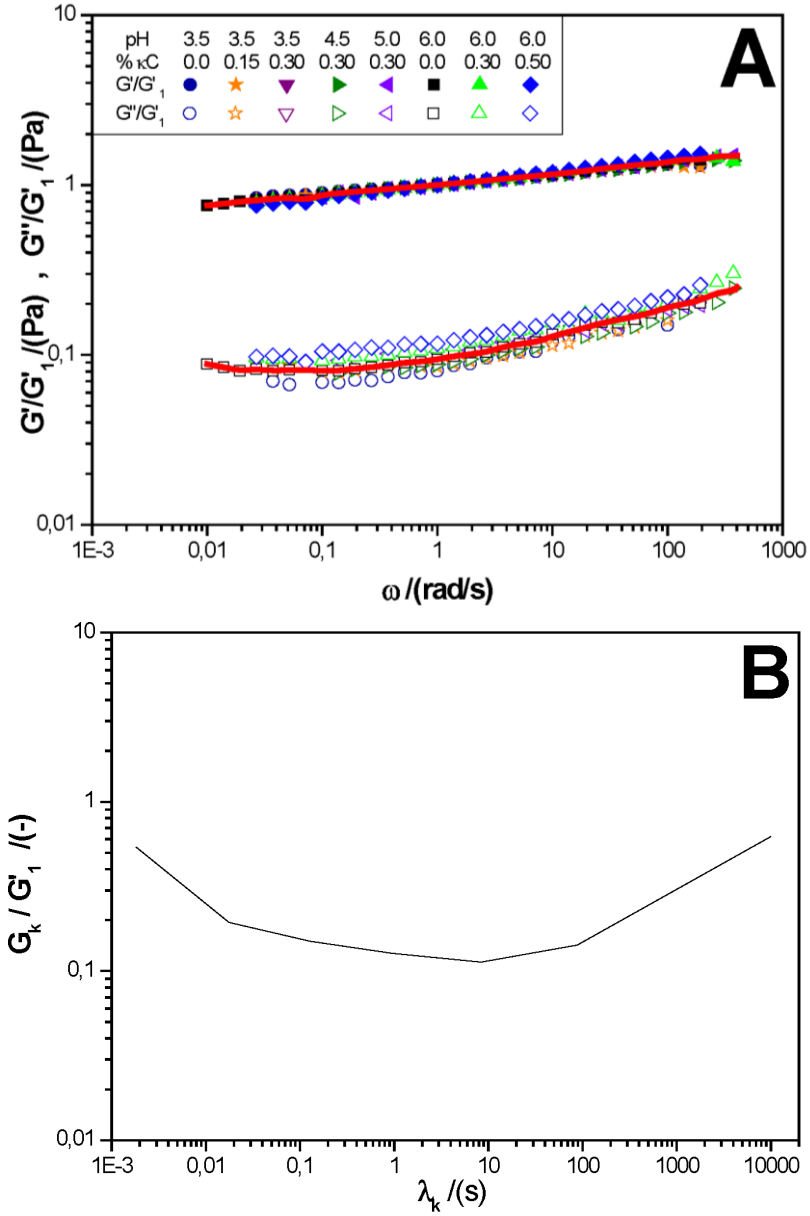

Figure 5. Normalization with G'_1 for EY/κC gels containing 45 wt% solids for different κC concentrations and pH values. (A) Master mechanical spectrum, where the continuous lines represent the viscoelastic moduli recalculated from the Generalized Maxwell Model; (B) Normalized discrete relaxation spectrum

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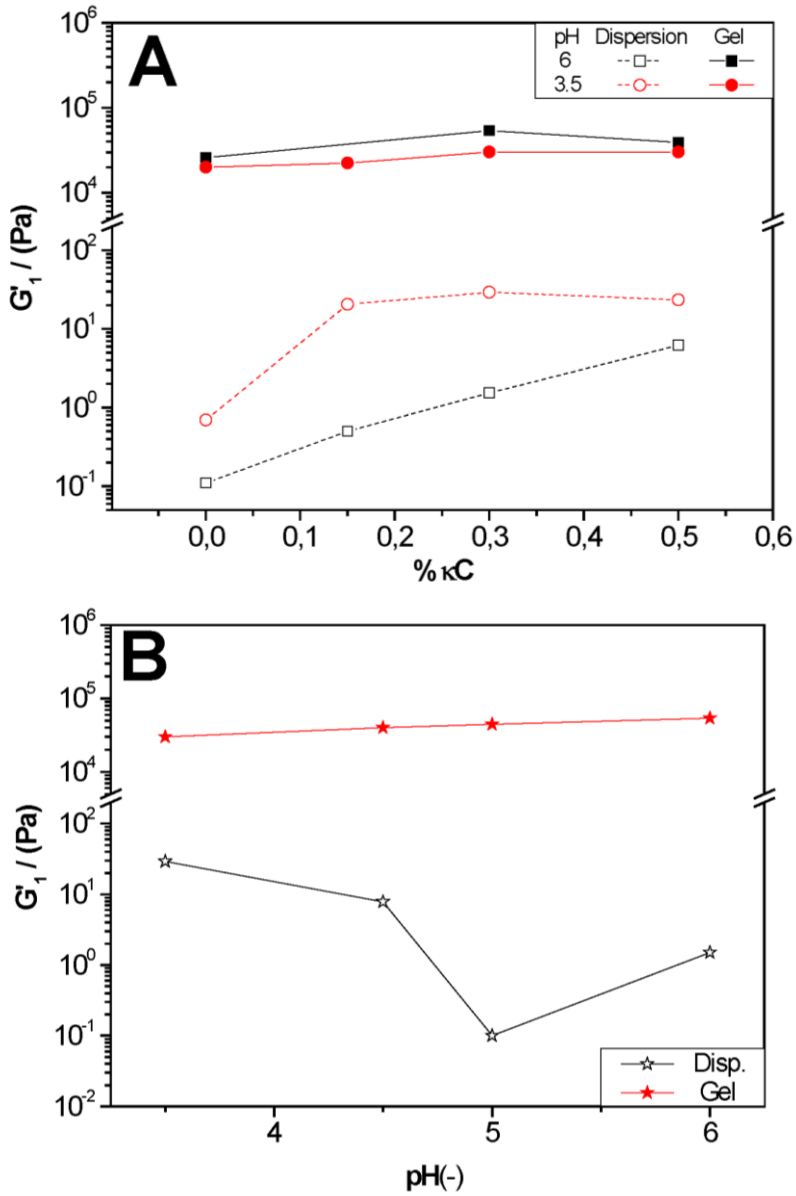

Figure 6. Normalization parameter (G'_1) for EY/κC dispersions and gels containing 45 wt% solids: (A) G'_1 as a function of κC concentration at pH 3.5 and 6; (B) G'_1 as a function of pH at 0.3% κC. The relative standard deviation is always lower than 7% for gels and 10% for dispersions.

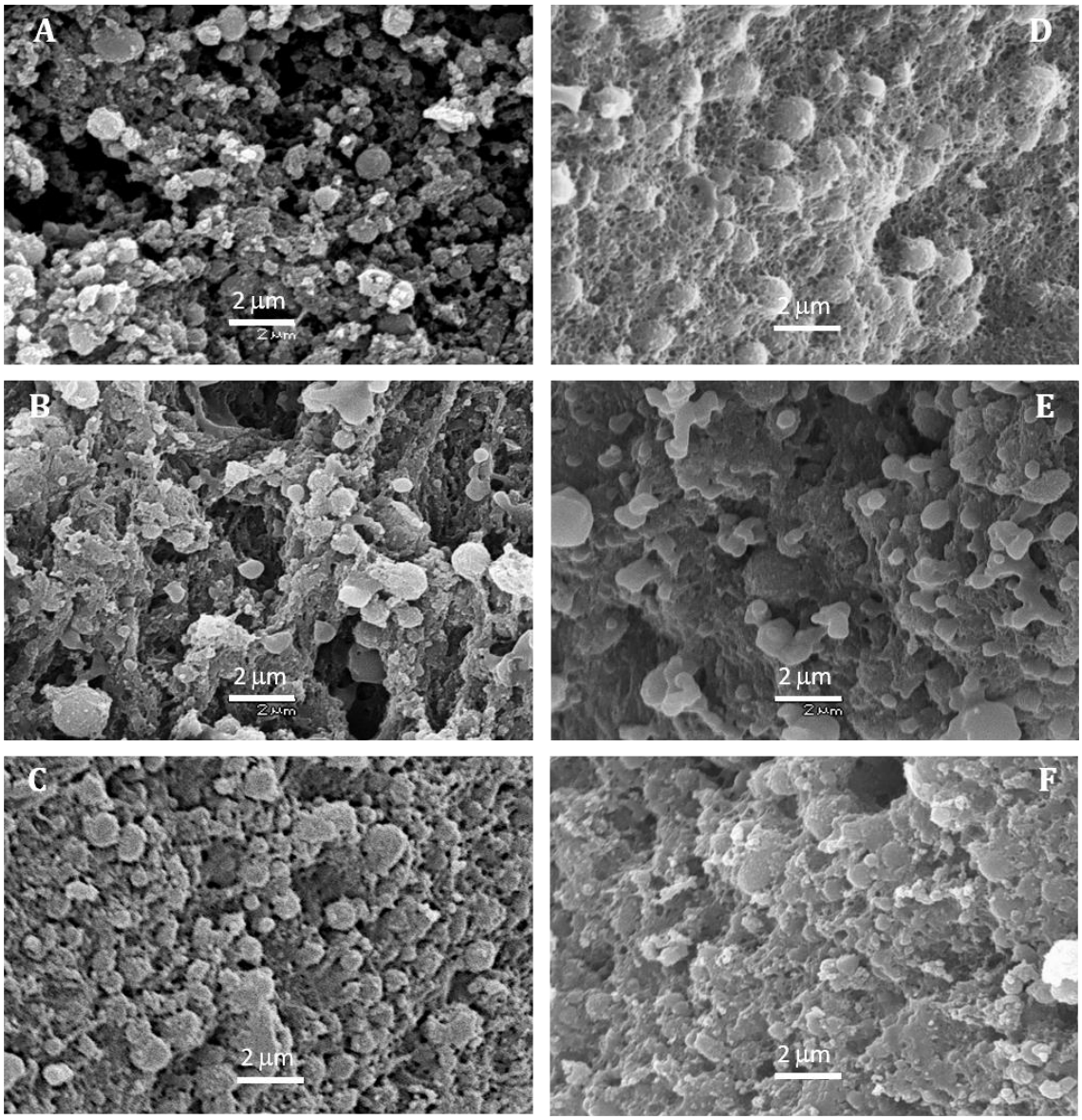

Figure 7. SEM images for EY and EY/κC gels containing 45 wt% solids at different pH values: EY gels at (A) pH 3.5; (B) pH 4.5; (C) pH 6; EY/κC gels with 0.3 wt% κC at (D) pH 3.5; (E) pH 4.5; (F) pH 6.

Table 1. Zeta potential values for egg yolk dispersions with different κC content

pH values (-)	Zeta potential (mV)		
	0 wt% κC	0.3 wt% κC	0.5 wt% κC
3.5	40.0 ± 0.6	38.9 ± 0.6	34.2 ± 0.8
4.5	4.9 ± 0.8	-0.2 ± 0.7	-3.7 ± 0.2
6.0	-13.3 ± 1.0	-20.0 ± 0.6	-26.9 ± 1.4