**Development and characterization of chitosan/gelatin electrosprayed
microparticles as food grade delivery vehicles for anthocyanin extracts**

**Elif Atay¹, Maria Jose Fabra², Marta Martínez-Sanz², Laura G. Gomez-
Mascaraque², Aylin Altan¹, Amparo Lopez-Rubio^{2*}**

¹ Department of Food Engineering, Mersin University, Ciftlikkoy, Mersin 33343,
Turkey

²Food Safety and Preservation Department, IATA-CSIC, Avda. Agustin Escardino 7,
46980 Paterna (Valencia), Spain

*Corresponding author: Tel.: +34 963900022; fax: +34 963636301

E-mail address: amparo.lopez@iata.csic.es (A. López-Rubio)

ABSTRACT

This work reports on the development of electrosprayed chitosan/gelatin microparticles for encapsulation purposes. Initially, the effects of the chitosan molecular weight (Mw), biopolymers and solvent composition on solution properties and capsule formation were investigated. Results demonstrated that the sprayability of the blend solutions was mainly determined by a critical range of solids content, which was needed for chain entanglement and subsequent capsule formation, and by their rheological properties. SAXS experiments from selected electrospraying solutions showed that clustered networks between both biopolymers were formed due to the electrostatic interactions between the positively charged amino groups in chitosan and negatively charged amino acid residues in gelatin. As a model bioactive extract, an anthocyanin-rich black carrot extract (BCE) was loaded into selected chitosan/gelatin electrosprayed structures and the encapsulation efficiency and release into two food simulants (ethanol 10 % and acetic acid 3%) was evaluated. Faster and greater release was observed in the acetic acid medium as expected and the results showed that the main factor affecting the bioactive release was the blend composition, i.e. increasing the chitosan content of the formulations limited BCE release.

KEYWORDS: chitosan; molecular weight; food simulants; anthocyanins extract; electrospraying.

1. INTRODUCTION

Microencapsulation technologies are gaining interest in the food area for the preservation of different bioactive compounds (Dias, Ferreira, & Barreiro, 2015) during food processing and storage, preventing undesirable interactions with the food matrix and ensuring their targeted delivery. In recent years, several technologies have been developed and successfully applied to generate micro- and nanoencapsulated materials (spray-drying, extrusion, coacervation, etc.) being the selection of the micro- or nanoencapsulation process mainly governed by the physicochemical properties of both the core and shell materials, and the intended final application. As an alternative to conventional microencapsulation techniques, the electrohydrodynamic process has been recently postulated as a simple and advantageous method for generating micro-, submicro- and nanoencapsulation structures for a variety of bioactive molecules (Pérez-Masiá, Lagaron et al. 2014, Gómez-Mascaraque and López-Rubio 2016, Gómez-Mascaraque, Morfin et al. 2016)), being specially interesting for the encapsulation and protection of thermosensitive molecules, as the drying of the structures is based on the application of an external electrical voltage and, thus, no temperature is required. As a result, compared with the conventional spray-drying technique, electrospraying can achieve higher microencapsulation efficiencies for thermosensitive bioactive compounds (Pérez-Masiá, Lagaron et al. 2015, Gómez-Mascaraque and López-Rubio 2016). The morphology of the obtained structures is greatly affected by the solution properties and process parameters and, for certain materials, reduced-size capsules can be attained when adjusting both.

Different substances may be used as shell materials for encapsulating solids, liquids or gases of different types and properties. However, it should be taken into account that regulations for food additives are stricter than for other applications (*i.e.* pharma). In

this regard, the materials used as protective shells have to be food-grade, biodegradable and able to form a barrier between the internal phase and its surroundings.

Natural biopolymer blends are attracting increased attention as encapsulation matrices, because by changing the composition of the blend or fostering biopolymer interactions, it is possible to tune the release properties of the structures obtained. Specifically, chitosan and gelatin blends are particularly interesting as both materials are bio-based, renewable, biocompatible and biodegradable natural polymers which, moreover, are able to form stimulus-responsive hydrogels and are known to interact through hydrogen bonding and electrostatic interactions, thus forming compatible blends. Chitosan is a cationic polysaccharide obtained from the deacetylation of chitin and it is considered a GRAS food additive by the FDA (Luo & Wang, 2013) while gelatin is a polyelectrolyte protein obtained by controlled hydrolysis of collagen and contains repeating sequences of glycine aa1-aa2, where amino acids aa1 and aa2 are mainly proline and hydroxyproline (Lai, 2013). Although encapsulation structures from chitosan/gelatin blends have been already developed using different techniques (Hussain & Maji, 2008; Morelli, Holdich, & Dragosavac, 2017; Prata & Grosso, 2015), to the best of our knowledge, the development of electrosprayed capsules has not been yet explored.

Unlike electrospinning, electrospraying results from the interaction of bulk and surface electrohydrodynamic forces breaking the jet into droplets, which yield nano- or microparticles upon drying. The electrospraying processing of chitosan is mainly affected by its polycationic nature in solution, rigid chemical structure and specific inter- and intra-molecular interactions which makes the chitosan solutions highly viscous either at acidic pH or at room temperature (Homayoni, Ravandi, & Valizadeh, 2009). Given that the molecular weight of chitosan is one of the key factors which can affect not only its processability but also its performance as a delivery matrix, the focus

of the first part of this work was to study the influence of the chitosan molecular weight and the chitosan/gelatin ratio on the sprayability. Furthermore, two different acetic acid concentrations (20 and 80 %v/v) for chitosan solubilization were used as solvent properties are also known to greatly influence electrohydrodynamic processes (Chalco-Sandoval, Fabra, López-Rubio, & Lagaron, 2015).

In order to evaluate the release properties from the developed structures, an anthocyanin-rich black carrot extract (BCE) was incorporated within the electrosprayed biopolymer blends. This extract was selected because apart from the well-known health promoting effects of anthocyanins (Alasalvar et al., 2005) and their inherent low chemical stability (thus requiring some protection for functional food development), it could be easily quantified through spectroscopic techniques as the anthocyanins present in the extract are hydrophilic pigments (Delgado-Vargas, Jiménez, & Paredes-López, 2000). Therefore, in the second part of this work the encapsulation efficiency and anthocyanins release in two food simulants (3% acetic acid and 10% ethanol media) from selected electrosprayed hydrogel microparticles were evaluated.

2. METHODS AND MATERIALS

2.1. Materials

Chitosans with reported degree of deacetylation of $85 \pm 2.5\%$ and different average molecular weights 25, 350 and 600 kDa (commercial grades 85/5, 85/500 and 85/3000, respectively), were purchased from Heppe Medical Chitosan GmbH. Type A gelatin from porcine skin (Gel), with reported gel strength of 180 g Bloom was obtained from Gelita (Germany). 96% (v/v) acetic acid was supplied by Scharlab. 96% (v/v) ethanol was purchased from Panreac (Spain). Black carrot extract (BCE) was kindly donated by SECNA, S.L. (Valencia, Spain).

2.2. Preparation of chitosan and gelatin dispersions

8% (w/w) gelatin solutions were prepared following the method described in Gómez-Mascaraque, Lagaron, & López-Rubio (2015). Briefly, the protein was dissolved in acetic acid 20% (v/v) under magnetic agitation and mild heating, and were cooled down to room temperature before processing. Similarly, solutions from different molecular weight Mw chitosans (low Mw - 25 kDa; intermediate Mw - 350 kDa; high Mw -600 kDa) were prepared by dissolving the biopolymer in acetic acid either at 80% or 20% (v/v) under magnetic agitation and room temperature before processing. Different contents of chitosan were incorporated depending on the chitosan Mw, as there was a viscosity limitation for handling of the solutions and subsequent electrospraying.

Gelatin/chitosan blends were prepared by mixing the individual protein and polysaccharide solutions at different volume ratios 20:80, 50:50, 60:40 and 80:20, resulting in the different gelatin/chitosan molar percentages shown in Table 1, where all the prepared blends are summarized. For the second part of the work, a theoretical BCE content of 17% w/w with respect to the total mass of solids was incorporated within the selected gelatin/chitosan blends before processing.

2.3. Characterization of the solutions

The electrical conductivity of the solutions was measured using a conductivity meter XS Con6 (Labbox, Barcelona, Spain) at room temperature. The rheological behavior of the solutions was studied using a rheometer model AR-G2 (TA Instruments, USA), with a parallel plate geometry following the method described in Gómez-Mascaraque, Lagaron, & López-Rubio (2015). Briefly, the shear stress (σ) was measured as a

function of the shear rate ($\dot{\gamma}$) from 0 to 200 s⁻¹ during 15 min at 25 ± 0.1 °C using a stainless steel plate with a diameter of 60 mm and a gap of 0.5 mm. The power law model Eq. (1) was applied to determine consistency index (k) and flow behavior index (n). Apparent viscosities were determined at 100 s⁻¹. All measurements were made at least in triplicate.

$$\sigma = k \cdot \dot{\gamma}^n \quad (1)$$

Pure gelatin and chitosan solutions and selected blends were characterized by means of small angle X-ray scattering (SAXS). SAXS experiments were carried out in the Non Crystalline Diffraction beamline, BL-11 at ALBA synchrotron light source (www.albasynchrotron.es), using the experimental settings previously described in (Fabra et al. 2017). The data reduction was treated by pyFAI python code (ESRF) (Kieffer and 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. The experimental data were fitted using a two-level Beaucage model. 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, 1996):

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

where $G_i = c_i V_i \Delta SLD_i^2$ (cm⁻¹·sr⁻¹) 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), $R_{g,i}$ (Å) is the radius of gyration describing the average size of the i^{th} level structural feature, B_i (cm⁻¹·sr⁻¹·Å^{-P_i}) is a q -independent prefactor specific to the type of power-law scattering with power-law exponent, P_i , and bkg (cm⁻¹) is the background.

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159 **2.4. Electro-hydrodynamic processing of the solutions**

160 Microparticles were obtained using the electrospraying technique, following a method
161 adapted from Gómez-Mascaraque, Lagaron, & López-Rubio (2015). The
162 electrospinning apparatus, equipped with a variable high-voltage 0–30 kV power
163 supply, was supplied by Spraybase® (Dublin, Ireland). The solutions were
164 electrosprayed under a steady flow-rate using a stainless-steel needle situated toward a
165 metallic plate used as collector. The needle was connected through a
166 polytetrafluoroethylene wire to the polymer solution kept in a 10 mL plastic syringe
167 which was disposed vertically lying on a digitally controlled syringe pump. The
168 electrospraying conditions were the following: 0.3 mL/h flow-rate, 13 kV voltage and
169 10 cm tip-to-collector distance for all the solutions, as selected from preliminary trials.

170

171 **2.5. Morphological characterization of the particles**

172 The morphology of the electrosprayed capsules was analyzed using scanning electron
173 microscope (Hitachi S-4800), following the method described in Gómez-Mascaraque,
174 Sanchez, & López-Rubio (2016). An accelerating voltage of 10 kV and a working
175 distance of 7–10 mm were used. The samples were sputter-coated with a gold-palladium
176 mixture under vacuum prior to examination.

177

178 **2.6. Fourier transform infrared (FT-IR) analysis of the particles**

179 FT-IR spectra were collected following the method described in Gómez-Mascaraque,
180 Sanchez, & López-Rubio (2016). Samples (*ca.* 1–2 mg) of selected chitosan/gelatin
181 capsules, were ground and dispersed in about 130 mg of spectroscopic grade potassium
182 bromide (KBr). A pellet was then formed by compressing the samples at *ca.* 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.

2.7 Microencapsulation efficiency (MEE)

The microencapsulation efficiency (MEE) of the BCE-loaded samples was calculated after dissolution of the structures in acetic acid and centrifugation, taking aliquots of the supernatant for UV-vis absorbance measurements. A calibration curve ($R^2=0.9993$) was obtained for the BCE in acetic acid (3% v/v) at a wavelength of 526 nm (cf. Figure S1 in the Supplementary Material). The BCE content in the electrosprayed capsules was interpolated from the obtained linear calibration equation. The MEE of the extract-loaded capsules was then calculated using Eq. (3) (Gómez-Mascaraque, Casagrande Sipoli et al. 2017):

$$MEE = \frac{\text{Actual BCE content in the capsules}}{\text{Theoretical BCE content in the capsules}} \times 100 \quad (\text{Eq. 3})$$

2.8 *In vitro* release assays

The *in vitro* release assays were carried out for selected microcapsules in two different media, i.e. ethanol 10 % and acetic acid 3%, which are considered as food simulants for aqueous and acidic food products, respectively, according to the Commission Regulation 10/2011 EU (10/2011/EC). Acetic acid 3 % was selected as the worst-case scenario in which the release was expected to be faster due to the quick swelling or even dissolution of the polymeric matrices in acidic conditions. Ethanol 10% was selected as a hydrophilic food simulant, compatible with the shell material and also representing a wide variety of foodstuffs. A method adapted from Costamagna, Gómez-Mascaraque et

al. (2017) was used to build the release profiles. Briefly, 17 mg of microcapsules were incorporated in 10 mL of the food simulants at 23 °C. At appropriate time intervals, the concentration of BCE in the release media was estimated by measuring the absorbance of the supernatant at a wavelength of 526 nm using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA). The obtained data were used to determine the total amount of the BCE released from the microcapsules at each time point. Three independent replicates of each sample were analyzed.

In order to gain a better understanding of the factors influencing the release from the microcapsules, and given that some of the gelatin/chitosan blends did not have the adequate solution properties to yield electrosprayed capsules, films were prepared by casting of selected gelatin/chitosan blends containing 17% BCE with respect to the total mass of solids. For this purpose, blends prepared as explained in Section 2.2 were placed on Teflon molds and left at room temperature until complete evaporation of the solvent. Pieces of the obtained films (ca. 45 mg/mL) were used to conduct *in-vitro* release assays following the same procedure as for the electrosprayed microparticles, in both release media.

2.9 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, the solids content and acetic acid concentration of each biopolymeric solution were adjusted so as to have suitable starting materials (especially in terms of viscosity) for their subsequent blending. In the case of gelatin, an 8 wt.% solids content and 20% (v/v) acetic acid concentration were selected based on the adequacy of this solution composition for capsule development through electrospraying (Gómez-Mascaraque et al., 2015). In the case of chitosan, depending on the molecular weight, the maximum amounts of carbohydrate that could be incorporated in solution were 5, 2 and 1.25 wt.% for the low, intermediate and high Mw, respectively. Greater amounts gave rise to too viscous solutions difficult to work with. It has been previously observed that both the acetic acid concentration and biopolymer concentration have an important effect on chitosan solution properties and, thus, on the subsequent morphology of the electrohydrodynamic processed materials (Gómez-Mascaraque, Sanchez et al. 2016). Consequently, two different carbohydrate concentrations (5 and 2 wt.% for low Mw chitosan, 2 and 1 wt.% for intermediate and 1.25 and 0.5 wt.% for the high Mw chitosan) and two acetic acid concentrations were tested (20 and 80% v/v).

3.1. Impact of the chitosan molecular weight, gelatin/chitosan ratio and acetic acid concentration on solution properties

Figure 1 shows the pH and conductivity values of the different solutions prepared. As it can be observed from the graphs, the pH and conductivity values for the various blends were intermediate between those of the pure biopolymeric solutions. For the same acetic acid concentration in solution, decreasing the chitosan concentration led to an increase in both the conductivity and pH, i.e., more protonated groups were present in the solution. On the other hand, for the same chitosan concentration, decreasing the acetic

acid concentration also led to greater pH and conductivity values, which could be ascribed to the higher degree of dissociation of the acid at low concentrations.

It is important to highlight that most of the solutions became unstable and phase separation took place, ascribed to the electrostatic interactions and polyelectrolyte complex (PEC) formation between gelatin and chitosan. In the case of low Mw chitosan, the compositions that remained stable and did not phase separate were the ones with greater chitosan contents, having a solids content in solution ranging between 3.2 and 6.5 g/100 mL and between ~30 and 80 wt.% of gelatin of the solids composition. For the high Mw chitosan, only the blends with the greatest chitosan percentage were the ones stable. In the case of the intermediate Mw chitosan, only the samples prepared with the low acid concentration chitosan solution were stable. PEC formation was not an issue for electrospraying, as it will be detailed below, but the total solids content, which determined the formation of chain entanglements and the solutions properties were the main factors affecting capsule development.

3.2. Electrosprayability of the blends

The electrosprayability of the gelatin/chitosan blends was dependent on the total solids content of the solutions and on the solution composition which, at the same time, determined solution properties. Figure 2 shows selected SEM images of the electrosprayed materials as a function of the total solids content and composition. Although processing through electrospraying was possible using solutions containing from 2 to 8 wt.% solids content, encapsulation structures with suitable morphologies were only attained in a relatively narrow range of total solids content. In the case of the blends with low and high Mw chitosan, solids content between 6.5 and 6.8 wt.% were needed to achieve the required biopolymer chain entanglements leading to capsule

development. In fact, the formation of chain entanglements has been highlighted as the main factor determining the different morphologies attained by means of the electrohydrodynamic process (Shenoy, Bates, Frisch, & Wnek, 2005). Increasing or decreasing the content of solids in solution resulted in collapsed structures, either due to excessive viscosity, thus hindering proper solvent evaporation, or insufficient chain entanglements, respectively. For pure chitosan solutions, lower acetic acid concentrations have been observed to result in particle aggregates and solution dripping, attributed to a slight decrease in the viscosity of the solutions together with a sharp increase in their electrical conductivity at 20% acetic acid (Gómez-Mascaraque et al, 2016; Zhang & Kawakami). In contrast, when blending chitosan with gelatin, the interplay between composition and solids content determines capsule formation as further explained below.

Figure 2A, corresponding to the electrosprayed structures from the blends with low Mw chitosan shows that the compositions with 60 and 50% in volume of the gelatin solutions within this range of solids content gave raise to smooth spherical capsules with sizes ranging from ~100 nm to ~1.5 μ m. In contrast, the blend compositions with 80% in volume of gelatin solution which were also in the solids content range suitable for capsule production (cf. Figure 2A), resulted in collapsed capsules (in the case of the blend with low chitosan content and high acid concentration) or smaller capsule clusters connected with fibers (in the case of the blend with low chitosan content and low acid concentration). Having similar pH and conductivity values than the previous ones, the differences in morphology could be ascribed to their viscosity as it will be shown below. Solution rheology has been shown to play a key role in the processing of materials using electrospinning (Gómez-Mascaraque et al., 2016) and it is further confirmed in this work for capsule development.

Figures 2B and 2C show the SEM images obtained from the blends with intermediate and high Mw chitosan, respectively. Although literature reports that capsules are preferentially formed for lower molecular weight and that the chitosan molecular weight from which nanofibers have been obtained varies from 85 kDa (Pakravan et al., 2012) up to 570 kDa (Min et al., 2015, Sangsanoh et al., 2006), micro and nanospheres could be obtained for all the molecular weights used in the chitosan/gelatin mixtures. Similarly, Gómez-Mascaraque et al., 2016 have recently reported the formation of electrosprayed capsules not only for low molecular weight chitosan but also for intermediate and high molecular weight chitosan. However, as the amount of chitosan that could be incorporated into the blends decreased with the chitosan Mw, the electrosprayability of the solutions in this case was mainly governed by the gelatin fraction. Again, there was a range of solids contents which favored capsule formation, being this range greater for the blends with intermediate Mw chitosan (from 4.5 to 6.8 wt. %) than for the blends with low or high Mw chitosans. Moreover, while for these latter blends conductivity values greater than 1800 $\mu\text{S}/\text{cm}$ were needed for proper solvent removal, thus avoiding the formation of fused structures, spherical capsules were obtained for the 60/40 (v/v) gelatin/chitosan blend obtained with the intermediate chitosan Mw (at low chitosan concentration and high acetic acid concentration), having a conductivity value of $\sim 1400 \mu\text{S}/\text{cm}$. This result can also be explained taking into account the viscosity of the obtained solutions as it will be explained below.

3.3 Molecular conformation in pure solutions and selected blends

Considering the better morphologies attained for the different blend compositions, the molecular conformation of the electrospraying solutions which provided better capsules was also evaluated and compared with selected counterparts' solutions which did not

form capsule morphologies. Therefore, SAXS experiments were carried out to investigate the structural conformation of pure gelatin, chitosan solutions and selected gelatin/chitosan blends in the nanometre scale. The scattering patterns of the samples are shown in Figure 3. As observed, the spectrum for the pure gelatin is characterized by the appearance of two shoulder-like features, one located at ca. 0.003-0.008 $\text{\AA}^{-1}$ and a broader shoulder at ca. 0.03-0.22 $\text{\AA}^{-1}$. The pure chitosan solutions also show a more prominent shoulder feature within the range of ca. 0.02-0.22 $\text{\AA}^{-1}$ and a weak feature within the range of ca. 0.004-0.008 $\text{\AA}^{-1}$. While most of the gelatin/chitosan blends displayed patterns similar to that of the pure chitosan solutions, some particular formulations led to the appearance of structural features characteristic of the development of strong molecular interactions between gelatin and chitosan.

As shown in Figure 3, the experimental data could be properly described by a two-level Beaucage model and the most relevant structural parameters obtained from the fits are summarized in Table 2. The radius of gyration (R_g) is related to the dimensions of the structural feature originating the shoulders in the scattering patterns. The pure gelatin solution can be described by two structural levels whose associated radii of gyration present values of $R_{g1} = 43.4$ nm and $R_{g2} = 1.5$ nm. The correlation length values (characteristic of the distance between adjacent polymeric chains) reported in the literature for gelatin solutions and gels are around 2.6 nm, while gelatin coacervates have been reported to present values of 1.2 nm, which is very similar to R_{g2} (Mohanty, Aswal, Kohlbrecher & Bohidar, 2006). Additionally, the power-law exponent in the q region comprised between the two shoulder features presents a value of 2.4, which is characteristic of fractal features or aggregates. This is in contrast to the exponents of 1.6 obtained for polymeric chains in good solvents and 2.0 for Gaussian polymeric chains

in θ solvents. This suggests that in the presence of acetic acid, the gelatin chains may form aggregates due to intra and intermolecular electrostatic interactions. At the next structural level, Rg_1 is indicative of the size of the aggregates or coacervates formed.

Chitosan, similarly to other polysaccharides, has been reported to form aggregates under certain conditions, such as acidic pH (Anthonsen, Vårm, Hermansson, Smidsrød, & Brant, 1994; Dogsa, Kriechbaum, Stopar, & Laggner, 2005; Matsumoto, Kawai, & Masuda, 1991), although this aggregation phenomenon is still not fully understood. Thus, it may be reasonable to hypothesize that the origin of the features observed for the chitosan solutions arises from the presence of aggregates in acidic pH conditions. Considering the size ranges associated to the scattering features, it seems reasonable to assume that whereas Rg_1 is dependent on the size of the aggregates, Rg_2 is related to the correlation length, i.e. the distance between the polymeric chains forming the aggregates. The Rg_2 values, ranging from 0.6 to 1.4 nm, seem to increase slightly when comparing the low molecular weight chitosan solutions with the intermediate and high molecular weight chitosan solutions. The power-law exponents decrease with the chitosan molecular weight, presenting values close to 3 for the low molecular weight, close to 2 for the intermediate molecular weight and close to 1 for the high molecular weight chitosan. This shift in the power-law exponent is indicative of the presence of clustered polymeric chains in the low and intermediate molecular weight solutions and extended polymeric chains in the high molecular weight solutions. This could be explained by the lower chitosan concentration utilized to prepare the latter ones, which may result in an increased distance between polymeric chains at the corresponding size range. The Rg_1 values, ranging from 11.1 nm to 44.5 nm, also tend to increase for the high molecular weight chitosan. The same effect of increased Rg_1 is observed for the

intermediate and high molecular weight chitosan when the acetic acid concentration increases from 20% to 80%. This suggests that the chitosan polymeric chains are less compacted in the less concentrated systems and when stronger electrostatic repulsions are induced by the presence of acetic acid.

Regarding the selected gelatin/chitosan blends, although the shape of most of the scattering patterns is similar to that from the pure chitosan solutions, the power-law exponents and the R_{g1} values are significantly modified with regards to the pure chitosan solutions. It seems that clustered networks, similar to those occurring in the pure chitosan solutions, are formed in the blends. However, due to the electrostatic interactions between the positively charged amino groups in chitosan and negatively charged amino acid residues in gelatin, more compacted structures with reduced aggregate size are formed. Interestingly, unlike the rest of the samples, the H1 80:20 blend presented a well-defined shoulder within the range of 0.008-0.04 $\text{\AA}^{-1}$, whose corresponding R_{g1} value was 8.8 nm. The appearance of this scattering feature may be indicative of the formation of nanoscale complexed structures due to stronger interactions between gelatin and chitosan for this particular formulation. Indeed, it has been reported that in acidic conditions and at certain gelatin concentrations, gelatin/chitosan soluble polyelectrolyte complexes (PECs) are formed (Voron'ko, Derkach, Kuchina & Sokolan, 2016). Thus, according to the SAXS results, it seems that, from the characterized blends, only this formulation presented the pH and gelatin concentration conditions required for the formation of nanoscale soluble polyelectrolyte complexes, although as commented before, macroscopic PECs were also formed for other gelatin/chitosan blends.

3.4. Rheological properties of selected blends

Apart from the molecular conformation, the rheological behavior of the electro spraying solutions which provided better capsules was also evaluated and they were also compared with the selected counterparts' solutions which did not form capsule morphologies, in order to understand the role of solution rheology on the electro sprayability of the samples.

Complete flow curves presented in Figure 4 show that the pure gelatin, low molecular weight chitosan and the most diluted chitosan solutions in the blend samples behave as Newtonian liquids, with a linear relationship between the shear-stress (σ) and the shear rate ($\dot{\gamma}$), regardless of the molecular weight of the chitosan. However, a shear thinning behavior (pseudoplastic) was observed for pure intermediate (350kDa) and high (600kDa) molecular weight chitosan solutions and the blend solutions with greater volumes of the chitosan solution, which was accentuated as the chitosan/gelatin ratio increased. The rheological data of pseudoplastic solutions were fitted to the Ostwald de Waale model (Eq (1)). Table 3 gathers the viscosity values for the solutions with Newtonian behavior and, the flow and consistency indexes, together with the apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1} for the pseudoplastic solutions. The values of the correlation coefficient were in all cases around 0.99. Rheological parameters and the viscosity or apparent viscosity at 100 s^{-1} for pure gelatin or chitosan are in the order of those found by Wulansari et al. (1998) and No et al. (2006) for these biopolymers. The first clear observation was that the polymer concentration of the intermediate and high molecular weight chitosan solutions (without gelatin) promoted slight but significant changes in the rheological pattern: the apparent viscosity and consistency index (κ) increased whereas the flow index (n) decreased. This result indicated that high chitosan concentrations made the fluid solutions more viscous and

more shear thinning. In contrast, the acetic acid concentration did not significantly modify the rheological parameters of the neat chitosan matrices.

The formation of chain entanglements, needed for structure development, is highly affected by the concentration and molecular weight of the biopolymers, being directly linked to the viscosity of the solutions. In fact, stable electro spraying and capsule formation was only achieved when the viscosity of the solutions was high enough to produce the necessary polymer entanglements. For instance, as observed in Table 3, when comparing L1 60:40 with L3 50:50, both having similar conductivity and pH values, the higher content of solids of the former, which gave rise to a considerable higher viscosity, was determinant for capsule formation. However, in the case of the blends with intermediate molecular weight chitosan, as the viscosity of the starting chitosan solution was much higher than that of gelatin, the viscosity of the blend was more influenced by the ratio between the two biopolymers than by the total solids content. Specifically, when comparing I2 60:40 with I3 80:20, even though the latter had higher solids content (6.6 vs. 5.2 g/100 mL), its lower viscosity made this solution unsuitable for capsule formation. Further increasing the amount of chitosan in the blends containing the intermediate Mw chitosan, resulted in shear thinning solutions with higher apparent viscosity which did not form capsular structures, probably due to the very low content of solids (3.2 and 2.4 g/100 mL) which hampered the required entanglement (see I1 20:80 and I2 20:80 samples in Figure 3 and Table 3). The results for the blends with high Mw chitosan further confirmed the previous observations, i.e. that a minimum solids content and viscosity were required for capsule formation. From the results of Table 3 it seems that viscosities greater than 0.0134 Pa.s, together with solids content greater than 4.5 g/100 mL were required for capsule formation.

3.5. Characterization of the loaded samples

Based on the results obtained in the first part of this study, three different compositions were selected for the production of electrosprayed capsules as delivery systems of a black carrot extract (BCE) with a theoretical extract content of 17% w/w with respect to the total mass of solids. The amount of extract was selected based on some preliminary studies related to the extract solubility and trying to maximize the capsule loading. The compositions selected for this part of the study were based on the best morphologies attained in the first part of the present work and considering the three different chitosan molecular weights. Specifically, the blends L1 60:40, I2 60:40 and H1 80:20 were developed with the BCE and the release properties from the capsules developed were studied.

3.5.1. Encapsulation efficiency

Prior to release studies, the encapsulation efficiency of the BCE in the chitosan/gelatin microcapsules was previously calculated and the results are gathered in Table 4. Given the hydrophilic nature of the BCE, the encapsulation efficiency of the BCE loaded electrosprayed capsules was relatively high (greater in all cases than 75%). Although higher encapsulation efficiencies have been previously obtained for the encapsulation of water-soluble polyphenols within electrosprayed gelatin microcapsules (Gómez-Mascaraque, Lagarón et al. 2015)), similar values to those obtained in this work have been previously reported for the encapsulation of polyphenols within electrosprayed chitosan microstructures (Gómez-Mascaraque, Sanchez et al. 2016). Moreover, it should be highlighted that in this case, a number of different anthocyanin molecules together with probably some carbohydrates, as observed through FTIR (see as an example Figure S2 corresponding to the blend with high Mw chitosan in the

Supplementary material) were present, which may have an impact on the encapsulation efficiency.

3.5.2. Release assays

The effect of the matrix composition in the BCE release profile of the electrosprayed microcapsules in two food simulants is shown in Figure 5. The release of the BCE from the developed structures, both in 10% ethanol and 3% acid acetic media as aqueous and acidic food simulants, respectively, agrees with the typical behavior of active compound release from hydrophilic matrices (De Nobile et al., 2008). There was an initial burst release which occurred during the first 20 minutes, which is normally related to the presence of bioactive molecules on the surface of the capsules or close to it that are quickly released when the matrix swells upon contact with the release medium, followed by a sustained release (lag time) where the active compounds diffuse through the mesh of the biopolymeric network. However, slight differences were observed between samples and, the release of the BCE was affected by the properties of the food simulant and the composition of the shell matrix.

As observed in Fig. 5, the release was greater in the acidic food simulant, which can be attributed to the greater solubility of both chitosan and gelatin in acidic conditions. In both simulants, the release was lower for the microcapsules prepared with low molecular weight chitosan. Whereas in 10% ethanol, an increase in the percentage of BCE released was observed when increasing the chitosan molecular weight in the blends, no significant differences between intermediate and high molecular weight chitosan were observed in the acetic acid media. These results can be explained on the basis of the matrix composition, as the blend L1 60:40, i.e. the one with the low Mw chitosan, had the greatest chitosan content (see Table 1) which is less soluble than

gelatin in this specific release media. From the results, it appears that in both food simulants, an increase in the chitosan content resulted in a lower anthocyanin-rich BCE release. Therefore, the lower release attained for microcapsules containing low molecular weight chitosan can be explained by the higher chitosan concentration in the polymer matrix which might have better retained the BCE in the polymer network due to the lower solubility of chitosan as compared to gelatin in the release media.

To confirm these results with a wider range of matrix compositions, and given that not all the biopolymer blends yielded electrosprayed microcapsules, gelatin/chitosan films containing BCE were prepared and the release of the extract from these materials in both media was similarly evaluated. The release profiles from selected films are shown in Figure 6. Again, the blends containing higher chitosan contents exhibited the lowest BCE release from the films regardless of the Mw of the polysaccharide, confirming that the gelatin/chitosan ratio in the matrix is the main factor affecting the BCE release extent in these systems.

4. CONCLUSIONS

Electrosprayed chitosan/gelatin microparticles have been developed and the effects of the chitosan molecular weight (low, intermediate and high Mw), biopolymers ratio and solvent composition on solution properties and capsule formation were investigated. Even though electrosprayed structures were obtained using the three different chitosan Mw, it was found that there was a critical range of solids content, which was needed for chain entanglement and subsequent capsule formation. Moreover, the intrinsic viscosity

of the chitosan solutions (especially the ones from the intermediate and high Mw chitosans) limited its content in the blend compositions, as it greatly affected the rheological properties of the solutions, having an impact on their sprayability. Interactions from both biopolymers in the electrospraying solutions were deduced from the SAXS experiments which showed typical patterns from clustered networks. Loaded electrosprayed structures containing 17 wt.% of an anthocyanin-rich black carrot extract (BCE) were developed with the optimal chitosan/gelatin compositions and the encapsulation efficiency and release into two food simulants (ethanol 10 % and acetic acid 3%) was evaluated. The electrosprayed microhydrogels displayed a faster and greater release in the acetic acid medium, given the greater swelling of the structures in this medium and it was found that the main factor affecting the bioactive release was the blend composition, i.e. increasing the chitosan content of the formulations limited BCE release.

ACKNOWLEDGEMENTS

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

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List of Figures

Figure 1. Conductivity (dot lines) and pH values (solid lines) of the different gelatin:chitosan solutions prepared with: (A) low, (B) intermediate and (C) high Mw chitosan. The gelatin solutions had an 8wt. % solids content and 20% (v/v) acetic acid, while different chitosan solutions were used: (■) with high acid content (80%) and high chitosan concentration (5, 2 and 1.25wt. % for the low, intermediate and high Mw, respectively); (▲) with high acid content (80%) and low chitosan concentration (2.5, 1 and 0.5 wt.% for the low, intermediate and high Mw, respectively); (●) with low acid content (20%) and low chitosan concentration.

Figure 2. Solids content (g/100 mL) of the different solutions as a function of the volume concentration of gelatin for the blends with: (A) low, (B) intermediate and (C) high Mw chitosan, together with selected SEM images of the electrosprayed materials obtained.

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Figure 6. BCE release profiles from gelatin/chitosan films in two food simulants: acetic acid 3% (A) and ethanol 10 % (B) at 25 °C.

Table 1. Description of the different gelatin/chitosan blends assayed

Chitosan solution			Gelatin/chitosan blend		Solids content (g/100mL)	Sample identification (*)
Mw (kDa)	[Chitosan] (w/w %)	[Acetic acid] (v/v %)	Gel:Ch volume ratio	Chitosan percentage (mol/mol %)		
25	5	80	20:80	80.0	5.6	L1 20:80
	2			61.5	3.2	L2 20:80
		20		61.5	3.2	L3 20:80
	5	80	50:50	50.0	6.5	L1 50:50
	2			28.6	5	L2 50:50
		20		28.6	5	L3 50:50
	5	80	60:40	40.0	6.8	L1 60:40
	2			21.1	5.6	L2 60:40
		20		21.1	5.6	L3 60:40
	5	80	80:20	20.0	7.4	L1 80:20
	2			9.1	6.8	L2 80:20
		20		9.1	6.8	L3 80:20
350	2	80	20:80	10.3	3.2	I1 20:80
	1			5.4	2.4	I2 20:80
		20		5.4	2.4	I3 20:80
	2	80	50:50	2.8	5	I1 50:50
	1			1.4	4.5	I2 50:50
		20		1.4	4.5	I3 50:50
	2	80	60:40	1.9	5.6	I1 60:40
	1			0.9	5.2	I2 60:40
		20		0.9	5.2	I3 60:40
	2	80	80:20	0.7	6.8	I1 80:20
	1			0.35	6.6	I2 80:20
		20		0.35	6.6	I3 80:20
600	1.25	80	20:80	4.0	2.6	H1 20:80
	0.5			1.6	2	H2 20:80
		20		1.6	2	H3 20:80
	1.25	80	50:50	1.0	4.6	H1 50:50
	0.5			0.4	4.3	H2 50:50
		20		0.4	4.3	H3 50:50
	1.25	80	60:40	0.7	5.3	H1 60:40
	0.5			0.3	5	H2 60:40
		20		0.3	5	H3 60:40
	1.25	80	80:20	0.3	6.65	H1 80:20
	0.5			0.1	6.5	H2 80:20
		20		0.1	6.5	H3 80:20

710 (*) The sample identification is based on the following code: a letter indicating the Mw of the chitosan
711 used, followed by a number referring to the chitosan and acetic acid concentrations in the chitosan
712 solutions, and finally a ratio indicating the gelatin:chitosan volume ratio in the final blend. Accordingly,
713 letter L stands for low Mw, I for intermediate Mw and H for high Mw. Number 1 stands for the highest

714 chitosan and acid concentrations assayed, 2 for the lowest chitosan concentration and highest acetic acid
715 concentration, and 3 for the lowest chitosan and acetic acid concentrations.

Table 2. Radii of gyration (R_{g1} and R_{g2}) and power-law exponents (PL_1) extracted from the SAXS fitting results.

	Gelatin	L1	L2	L3	L1 60:40	L3 50:50	I1	I2	I3	I1 20:80	I2 20:80	I2 60:40	H1	H2	H3	H1 80:20	H3 60:40
R_{g1} (nm)	43.4	22.1	21.5	22.4	10.7	6.2	24.5	20.4	11.1	7.2	10.0	12.2	41.7	44.5	34.5	8.8	7.1
PL_1	2.4	2.8	2.9	3.8	3.4	2.3	2.1	1.9	2.4	1.0	1.2	1.0	1.0	1.0	1.7	1.7	1.1
R_{g2} (nm)	1.5	0.6	0.9	1.1	1.0	1.3	1.0	1.2	1.4	0.9	1.2	1.1	1.1	1.2	---	1.3	1.2

Table 3. Rheological parameters of the electrospinning solutions: Ostwald de Waale model parameters and apparent viscosity (η_{ap}) at 100 s^{-1} , at 25°C . Mean values and standard deviation.

SAMPLE NAME(*)	electrospinning solutions				rheological behaviour	$0 \leq \gamma \leq 200^{-1}$				
	MwCh	%mol Ch	%mol Gel	% acetic acid		$\mu \text{ (Pa}\cdot\text{s)}$	n	$\kappa \text{ (Pa}\cdot\text{s})^n$	r^2	$\eta_{ap} \text{ (Pa}\cdot\text{s)}$ ($\gamma = 100^{-1}$)
Gelatin			100		Newtonian	0.027 (0.007) ^a			0.9999	
L3 (100% Chitosan)		100		20	Newtonian	0.017 (0.001) ^a				
L2 (100% Chitosan)		100		80	Newtonian	0.030 (0.001) ^a				
L1 (100% Chitosan)	25 kDa	100		80	Newtonian	0.195 (0.0001) ^b				
L3 50:50		28.6	71.4	20	Newtonian	0.0134 (0.001) ^a			0.9998	
L1 60:40		40	60	80	Newtonian	0.037 (0.001) ^a			0.9999	
I3 (100% Chitosan)		100		20	Pseudoplastic		0.931 (0.004)	0.508 (0.005)	0.9987	0.370 (0.004) ^c
I2 (100% Chitosan)		100		80	Pseudoplastic		0.898 (0.005)	0.484 (0.013)	0.9995	0.302 (0.003) ^c
I1 (100% Chitosan)		100		80	Pseudoplastic		0.713 (0.018)	7.211(0.716)	0.9974	1.916 (0.077) ^d
I3 80:20	350 kDa	0.35	99.65	20	Newtonian	0.0134 (0.001) ^a			0.9998	
I2 60:40		0.95	99.05	80	Newtonian	0.0244 (0.001) ^a			0.9966	
I2 20:80		5.4	94.6	80	Pseudoplastic		0.925 (0.010)	0.222(0.015)	0.9999	0.157 (0.005) ^{b,e}
I1 20:80		10.2	89.8	80	Pseudoplastic		0.686 (0.011)	4.792(0.414)	0.9994	1.128 (0.041) ^f
H3 (100% Chitosan)		100		20	Pseudoplastic		0.943 (0.021)	0.190 (0.015)	0.9994	0.146 (0.002) ^{b,e}
H2 (100% Chitosan)		100		80	Pseudoplastic		0.957 (0.006)	0.143 (0.004)	0.9997	0.117 (0.0001) ^e
H1 (100% Chitosan)	600 kDa	100		80	Pseudoplastic		0.774 (0.090)	2.279 (0.320)	0.9982	1.059 (0.079) ^g
H3 60:40		0.3	99.7	20	Newtonian	0.008 (0.0001) ^a			0.9969	
H1 80:20		0.3	99.7	80	Newtonian	0.016 (0.001) ^a			0.9973	

Different letters in superscripts (a-g) indicate significant differences among the samples

(*) The sample identification is based on the following code: the first letter indicates the chitosan Mw (L for low, I for intermediate and H for high), the following number indicates the amount of chitosan and acid concentration in the starting solution (1 for high amount of chitosan and high acid concentration; 2 for low amount of chitosan and high acid concentration; and 3 for low amount of chitosan and low acid concentration) and the following ratio indicates the volume ratio of gelatin:chitosan.

Table 4. Microencapsulation efficiency (MEE) of the selected electrosprayed microcapsules

Sample identification	Absorbance at 526 nm	mg BCE	Theoretical content (mg)	MEE (%)
L1 60:40	0.047	0.86	1.19	75.6 ± 3.2
	0.052	0.94	1.19	
	0.049	0.89	1.19	
I2 60:40	0.05	0.91	1.19	76.5 ± 1.6
	0.051	0.93	1.19	
	0.049	0.90	1.19	
H1 80:20	0.048	0.88	1.19	76.9 ± 2.6
	0.052	0.94	1.19	
	0.051	0.93	1.19

Figure 1. Conductivity (dot lines) and pH values (solid lines) of the different gelatin:chitosan solutions prepared with the: (A) low, (B) intermediate and (C) high Mw chitosans. The gelatin solutions had an 8wt. % solids content and 20% (v/v) acetic acid, while different chitosan solutions were used according to the sample identification names given in Table 1: (■) L1, I1 and H1 samples; (▲) L2, I2 and H2 samples; (●) L3, I3 and H3 samples.

Figure 2. Solids content (g/100 mL) of the different solutions as a function of the volume concentration of gelatin for the blends with: (A) low, (B) intermediate and (C) high Mw chitosan, together with selected SEM images of the electrospayed materials obtained.

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Supplementary material

Figure S1. Calibration curve obtained for different amounts of black carrot extract (BCE) in acetic acid at a wavelength of 526 nm

Figure S2. FTIR spectra of the electrospayed blend H1 80:20 control (solid line) and with 17% black carrot extract (dotted line).